Biochimica et Biophysica Acta, 445 (1976) 486—499

BBA 67891

PURIFICATION AND KINETIC STUDY OF GLYOXALASE-I FROM RAT LIVER, ERYTHROCYTES, BRAIN AND KIDNEY

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(Received March 2nd, 1976)

Summary

Glyoxalase-I (*S*-lactoyl-glutathione methylglyoxal-lyase (isomerizing), EC 4.4.1.5) was purified from rat liver, erythrocytes, brain and kidney using two different purification procedures. The similarities of the purification profiles, electrophoretic mobilities and kinetics suggest that a single major form of the enzyme exists in these tissues. The highest purification (9300-fold) of the erythrocyte enzyme gave nearly homogeneous protein, molecular weight 50 000, specific activity 2410 μmol/min per mg. Kinetic studies of the rat glyoxalase-I-catalyzed disproportionation of the hemimercaptals of GSH and aromatic or aliphatic α-ketoaldehydes revealed broad substrate specificity with V and K_m values quite insensitive to the nature of the α-ketoaldehydes. Use of deuterated analogs of the α-ketoaldehydes methylglyoxal and phenylglyoxal showed that the intramolecular hydride migration is the rate-determining step.

Introduction

The disproportionation of α-ketoaldehydes into the corresponding α-hydroxycarboxylic acids, catalyzed by the ubiquitous glyoxalase system, has been recognized for many years [1,2] although the importance of the reaction remains unclear. A number of roles have been suggested for the glyoxalase system such as protection against α-ketoaldehyde toxicity [3] and regulation of cellular growth [4—6]. More recently the glyoxalase system has been shown to affect cell-free microtubule assembly [7].

Two enzymic reactions take place: the first, catalyzed by glyoxalase-I (*S*-lactoyl-glutathione methylglyoxal-lyase (isomerizing), EC 4.4.1.5), requires glutathione for the conversion of an α-ketoaldehyde into the thiol ester of GSH and the corresponding α-hydroxycarboxylic acid; the second, catalyzed by gly-

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oxalase-II (*S*-2-Hydroxyacylglutathione hydrolase, EC 3.1.2.6), involves the hydrolysis of the thiol ester to regenerate GSH and liberate a free α-hydroxycarboxylic acid. There is considerable interest in the mechanism of glyoxalase-I esespecially with respect to the question of a one- or two-substrate pathway [8,9]. Owing to the rapid, reversible addition of GSH to α-ketoaldehydes to form hemimercaptals in a non-enzymic reaction, the determination of whether the hemimercaptal is the substrate for glyoxalase-I or whether the α-ketoaldehyde and GSH are the substrates has not been a simple problem [10—14]. Most mechanism studies in the past have concentrated on the yeast enzyme, although more recently data have been reported on mammalian sources of the enzyme [10,15,16]. Relatively little has been reported on any procaryotic source of glyoxalase-I [17].

We report here some results from studies on rat glyoxalase-I isolated from various tissues. We wished to determine the tissue distribution of glyoxalase-I with respect to the possible existence of tissue-specific isozymes. Recently, it was reported that tumor-bearing animals manifest an altered liver form of glyoxalase-I [18]. The functional significance of this change is not understood, nor is it known whether the altered liver enzyme represents the expression of a gene product commonly expressed in other tissue. No data on the tissue distribution of glyoxalase-I have been reported other than that there is glyoxalase-I activity in all tissues. We also wished to examine the kinetic properties of a mammalian source of glyoxalase-I, using some of the mechanism probes employed in our earlier studies of yeast glyoxalase-I [12—14]. These include an evaluation of the substrate specificity of the enzyme and an evaluation of the kinetic isotope effects in the disproportionation reaction.

Materials and Methods

Chemicals. Commercial methylglyoxal, 40% aqueous solution (Aldrich), was purified by distillation [9,12]. Kethoxal (β-ethoxy-α-ketobutyraldehyde) was a gift from the Upjohn Co., Kalamazoo, Mich., U.S.A. The series of meta- or para-substituted phenylglyoxals, 2,4-dimethylphenylglyoxal and 2,4,6-trimethylphenylglyoxal were prepared as described previously [14,19]. The synthesis of the deuterated derivatives of methylglyoxal and phenylglyoxal have been described [13]. Glutathione (Sigma) was more than 99% pure by sulfhydryl titration with *N*-ethylmaleimide [20].

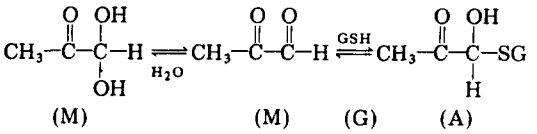
Column materials. CM-Sephadex C-50, DEAE-Sephadex A-50, Sephadex G-100 (Pharmacia) and hydroxyapatite (Bio Gel HPT) were prepared according to the manufacturers' instructions. Blue dextran affinity material was prepared according to the procedure of Ryan and Vestling [21] except that commercial CNBr-activated Sepharose 4B (Pharmacia) was used.

Enzyme kinetics. The dissociation constants of the hemimercaptals of glutathione and the α-ketoaldehydes are defined as:

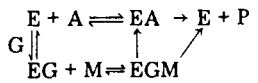
$$K_{diss} = \frac{[\text{total } \alpha\text{-ketoaldehyde}]\ [\text{glutathione}]}{[\text{hemimercaptal}]}$$

where total α-ketoaldehyde is essentially equal to the concentration of the hydrated form, owing to the high degree of hydration of these very reactive alde-

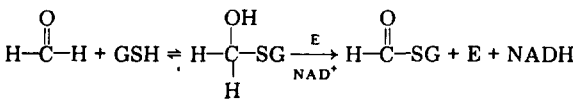
Glyoxalase-I



$$K_{diss} = \frac{[\text{total M}]\,[G]}{[A]}$$



Formaldehyde Dehydrogenase



Scheme 1

hydes (Scheme 1). K_{diss} values and pertinent spectral data have been reported [12,14]. Initial rate kinetic studies for glyoxalase-I were carried out as before [12] by following thiol ester formation at 240 nm for the alipathic α-ketoaldehydes and by following loss of reactant at the apparent isosbestic point between α-ketoaldehyde and hemimercaptal for the aromatic α-ketoaldehydes. Kinetic constants (Table IV) were determined under conditions where the initial rates are proportional to the enzyme concentration. Enzyme assays for glyoxalase-I activity in the various purification procedures were carried out using methylglyoxal (3 mM) and glutathione (3 mM) in pH 7 phosphate buffer (I = 0.2) with 10 mM $MgCl_2$, 25° C, and allowing 10 min for hemimercaptal formation to reach equilibrium. Enzyme solution was added in quantities which gave initial rates (ΔA/min) not exceeding 0.5. Under these conditions, the amount of substrate present is sufficient to saturate glyoxalase-I. Both the kinetic studies and the enzyme assays employed a Gilford modified Beckman DU, temperature controlled with a circulating water bath.

Protein determinations. The procedure of Lowry et al. [22] was used generally for determination of protein concentrations except for very dilute solutions where the procedure of Waddell [23] was used. Both methods were standardized with bovine serum albumin. The protein content of the column effluents was monitored at 280 nm. Specific activities are expressed in μmol/min per mg (I.U.).

Results

Purification of rat liver glyoxalase-I

Step 1. Livers from 36 male Sprague-Dawley rats, 250—300 g, which had

been sacrificed by decapitation, were cut into small pieces and were homogenized in a glass tissue homogenizer with a close-fitting teflon pestle in pH 7 phosphate buffer (I = 0.2) with 10 mM $MgCl_2$, 4°C, in a total volume of 500 ml. The homogenate was centrifuged at 27 000 × g for 20 min, and the pellet was washed and centrifuged again giving 600 ml of combined supernatant fractions (Table I).

Step 2. Solid $(NH_4)_2SO_4$, 146 g, was added slowly at 0°C to the 600 ml combined supernatant fractions, with addition of small volumes of NaOH solution to keep the pH above 6. The precipitate from the resulting 40% saturated solution was centrifuged at 27 000 × g for 20 min. The supernatant was treated with 170 g $(NH_4)_2SO_4$ to give an 80% saturated solution, and the precipitate was centrifuged at 27 000 × *g* for 20 min. Most of the glyoxalase-I activity was in the 40—80% pellet which was dissolved in pH 7 phosphate buffer giving 188 ml of solution (Table I).

Step 3. To the above solution at 0°C, 94 ml of 3 : 5 (v/v) mixture of chloroform/ethanol was added dropwise with stirring. After 30 min, the mixture was centrifuged in glass bottles at 10 000 × g for 15 min, and the supernatant fraction, 200 ml, was collected and concentrated to 5 ml by ultrafiltration with a Diaflo PM 30 membrane (Table I).

Step 4. The sample from Step 3 was placed on a CM-Sephadex C-50 column (2.5 × 45 cm) equilibrated at 4°C with pH 7 phosphate buffer (I = 0.02) containing 10 mM $MgCl_2$. Protein was eluted using the same buffer. Glyoxalase-I activity appeared as a single peak of activity immediately after the void volume (Fig. 1) and was collected in a volume of 110 ml which was concentrated by ultrafiltration to 5 ml (Table I).

Step 5. The sample from Step 4 was placed on a Sephadex G-100 column (2.5 × 100 cm) equilibrated with pH 7 phosphate buffer, as in Step 4. Glyoxalase-I was eluted with this buffer and appeared as a single peak of activity (Fig. 2) which was collected in a volume of 75 ml and concentrated to 9.5 ml (Table I).

Step 6. One half of the sample from Step 5 was placed on a DEAE-Sephadex A-50 column (2.5 × 30 cm) equilibrated with pH 6 phosphate buffer containing 0.07 M NaCl and 10 mM $MgCl_2$, total I = 0.12. The column was developed with

TABLE I

PURIFICATION SCHEME FOR RAT LIVER GLYOXALASE-I

Step	Volume (ml)	Protein (mg/ml)	Specific activity (I.U.)	Yield (step) (%)	Overall purification
1 27 000 × *g* supernatant fraction	600	41	0.83	100	1
2 $(NH_4)_2SO_4$ fractionation 40—80%	188	70	1.46	94	1.7
3 Chloroform/ethanol fractionation	200	7	11.2	89	13.4
4 CM-Sephadex C-50 (pH 7)	110	4	25.1	65	30
5 Sephadex G-100 (pH 7)	75	2.5	54.3	93	64.7
6 DEAE-Sephadex A-50 (pH 6)	7	0.18	983	45	1170

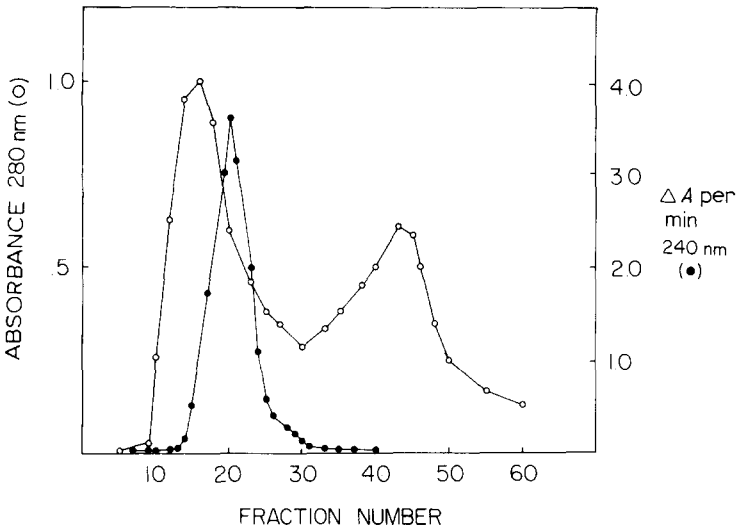


Fig. 1. Purification of rat liver glyoxalase-I on CM-Sephadex C-50, pH 7. ○, protein profile 280 nm; •, activity profile; 5 ml/fraction. The activity profile was obtained from 20-μl aliquots added to the standard assay buffer. Glyoxalase-I activity in fractions 14—32 was pooled and concentrated.

a linear NaCl gradient which increased 0.01 M per 300 ml eluant. Glyoxalase-I activity appeared as a single peak of activity (Fig. 3) which was collected in a volume of 150 ml and concentrated to 7 ml (Table I). This purification scheme for rat liver glyoxalase-I, summarized in Table I, gave a single form of the en-

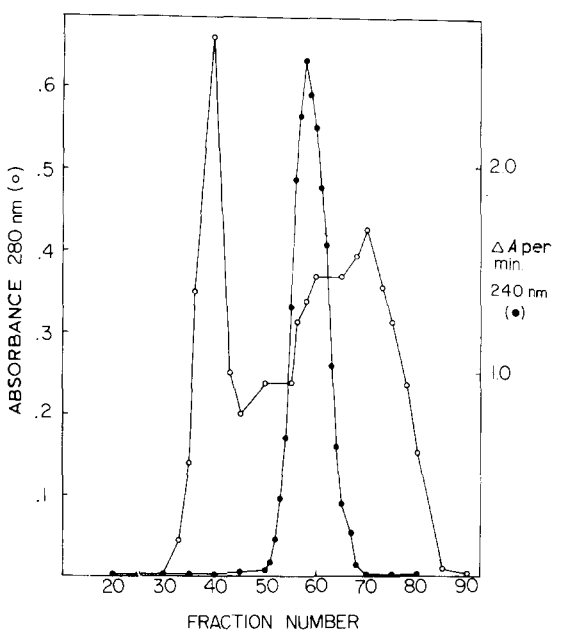


Fig. 2. Purification of rat liver glyoxalase-I on Sephadex G-100, pH 7. ○, protein profile 280 nm; •, activity profile; 5 ml/fraction. Activity was measured using 10-μl aliquots. Glyoxalase-I activity in fraction 52—67 was pooled and concentrated.

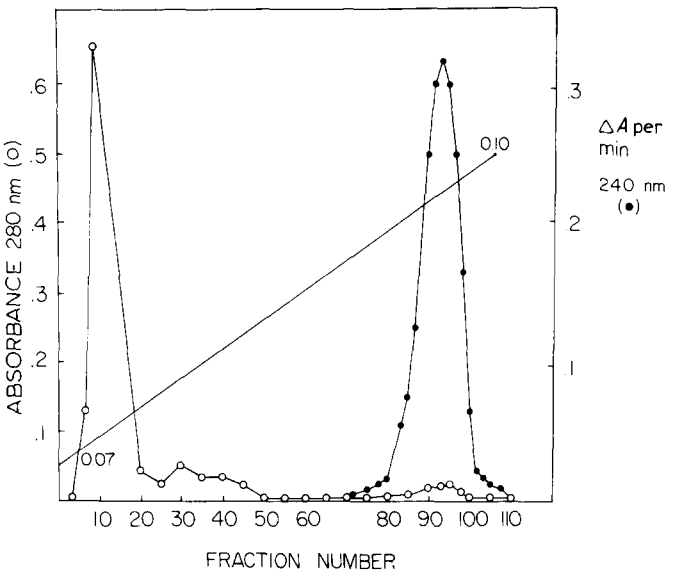


Fig. 3. Purification of rat liver glyoxalase-I on DEAE-Sephadex A-50, pH 6, with linear NaCl gradient from I = 0.07 to 0.10. ○, protein profile, 280 nm; ●, activity profile; 9 ml/fraction. Activity was measured using 10-μl aliquots. Glyoxalase-I activity in fractions 85—100 was pooled and concentrated.

zyme, specific activity 983 I.U. The overall purification was 1170 and the isolated yield was 23%.

Purification of rat erythrocyte glyoxalase-I

Rat erythrocyte glyoxalase-I was purified using a scheme similar to that used for the rat liver enzyme. After removal of hemoglobin by treatment with organic solvents, the erythrocyte enzyme was purified by chromatography on CM-Sephadex C-50, Sephadex G-100, and DEAE-Sephadex A-50. The activity profiles were identical to those in Figs. 1—3 for the liver enzyme except for the DEAE-Sephadex A-50 step where in addition to the main activity peak, similar to Fig. 3, a second activity peak was sometimes observed to come immediately after the main peak. These were designated Forms I and II and showed specific activities of 861 and 783 I.U., respectively. This represents overall purification of 3310 and 3010 for Forms I and II and a combined isolated yield of 17%. The major problem step is the DEAE-Sephadex A-50 step where the recovery is low. This column was useful only after preliminary treatment with a concentrated protein solution. Use of fresh column material invariably inactivated most of the glyoxalase-I. In addition, the amount of Form II was highly variable. Consequently, there is some question about the true existence of these two forms, at least in the whole cell. In order to study this further and also to improve on the isolated yield, the following purification scheme was developed.

Step 1. Fresh rat blood, 500 ml, obtained from animals killed by decapitation was mixed with heparin and was centrifuged at 4000 × g for 5 min. The plasma and buffy layer were removed by aspiration, and the erythrocytes were washed three times with 250 ml of 0.9% NaCl, each time followed by centrifugation at 4000 × g for 5 min and aspiration of the supernatant fraction. The

washed cells were lysed overnight at 4°C by addition of distilled water to give a final volume of 500 ml. Before beginning Step 2, 5 ml of 0.5 M $MgCl_2$ was added to the hemolysate (Table II).

Step 2. To each 100 ml portion of hemolysate in a glass centrifuge bottle was added 40 ml of 3 : 5 (v/v) mixture of chloroform/ethanol. The bottles were capped tightly and were shaken gently for 5 min and then were kept in an ice bath for 25 min. The bottles were centrifuged at 10 000 × *g* for 30 min, after which the supernatant fractions were combined and concentrated by ultrafiltration to 50 ml (Table II).

Step 3. The concentrate was treated with 25 ml of chloroform/ethanol as in Step 2 except that the final supernatant fraction was diluted to 100 ml with pH 7 phosphate buffer (I = 0.02) containing 10 mM $MgCl_2$ before concentration to a volume of 10 ml (Table II).

Step 4. Concentrate after the second chloroform/ethanol treatment, 2 ml, was placed on a blue dextran affinity column (1 × 20 cm) equilibrated with pH 7 phosphate buffer (I = 0.02) containing 5 mM $MgCl_2$. Proteins were eluted with this buffer until the effluent appeared free of protein. Most of the protein came immediately after the void volume. The column then was developed with a KCl gradient from I = 0.2 to 1.6 over a 50 ml volume. Glyoxalase-I activity was collected in a volume of 47 ml (Fig. 4) with most of the activity in a 10 ml volume (Table II).

Step 5. The most active fractions from the blue dextran column (Fig. 4) were concentrated by ultrafiltration to a volume of 1.75 ml, and the concentrate was placed on a hydroxyapatite column (0.75 × 4 cm) equilibrated with pH 7 phosphate buffer (I = 0.01) containing 5 mM $MgCl_2$. The column was washed first with this buffer and then with a linear phosphate gradient from I = 0.01 to 0.6 over a 50 ml volume (Fig. 5). Glyoxalase-I activity appeared as a single peak of activity, with some tailing. Activity was collected in a volume of 20 ml. This convenient purification scheme, summarized in Table II, gave one form of the enzyme with specific activity of 2415 I.U. The overall purification factor is 9300 for the most active fractions, with an isolate yield of 60%.

Purification of rat kidney and rat brain glyoxalase-I

The scheme in Table II is especially useful for rapid purification of glyoxal-

TABLE II

PURIFICATION SCHEME FOR RAT ERYTHROCYTE GLYOXALASE-I

Step		Volume (ml)	Protein (mg/ml)	Specific activity (I.U.)	Yield (step) (%)	Overall purification
1	Hemolysate	500	190	0.26	100	1
2	First chloroform/ ethanol fractionation	50	23	20.8	96	80
3	Second chloroform/ ethanol fractionation	10	55.1	39	90	150
4	Blue dextran affinity column	47	0.035	1650	70	6350
5	Hydroxyapatite	20	0.011	2415	100	9300

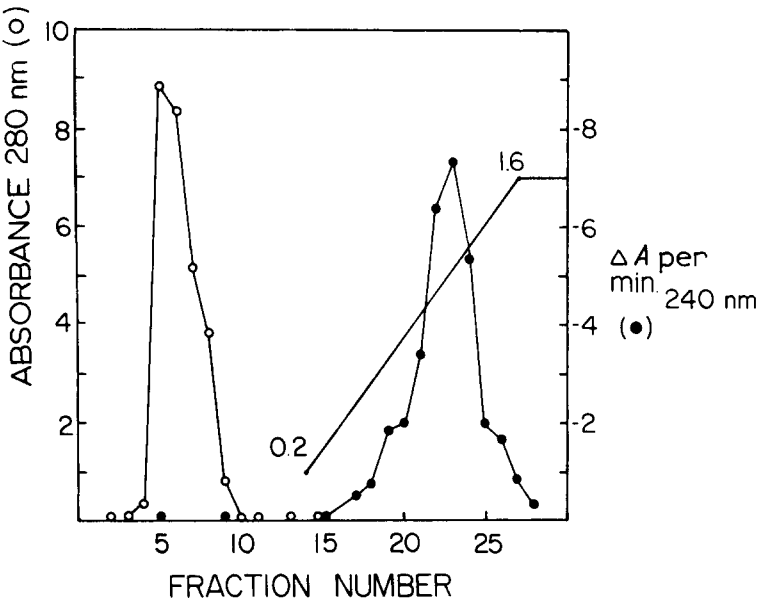


Fig. 4. Purification of rat erythrocyte glyoxalase-I by blue dextran affinity chromatography, pH 7, with linear KCl gradient from I = 0.2 to 1.6. ○, protein profile, 280 nm; ●, activity profile; 3.7 ml/fraction. Activity was measured using 10-μl aliquots. Fractions 17—21, 22—24, and 25—28 (total volume 47 ml) were collected separately. The material in fractions 22—24 was used for the hydroxyapatite column, Fig. 5.

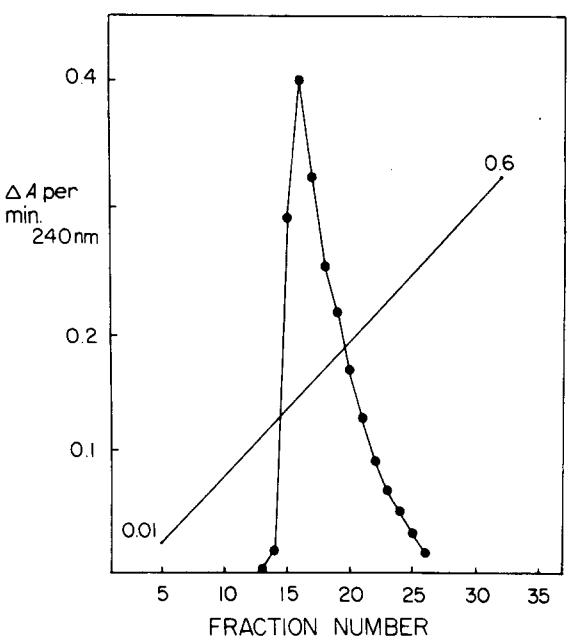


Fig. 5. Purification of rat erythrocyte glyoxalase-I on hydroxyapatite, pH 7, with a linear phosphate gradient from I = 0.01 to 0.6; 1.7 ml/fraction. Activity was measured using 10-μl aliquots. The most active fractions showed specific activity of 2415 I.U.

ase-I from erythrocytes and gives high isolated yields. However, it is also useful for the partial purification of glyoxalase-I from other tissue. Table III summarizes the purification scheme for glyoxalase-I from rat brain and rat kidney, using only the organic solvent treatment and the blue dextran affinity column. In both cases, the activity profiles obtained were identical to that in Fig. 4.

Comparison of glyoxalase-I isolated from various tissues; gel electrophoresis of glyoxalase-I

Electrophoresis of the various preparations of glyoxalase-I was carried out on 7% polyacrylamide gels at pH 8.9 [24]. Glyoxalase-I moved toward the anode at this pH. Gels were fixed for 30 min in 20% sulfosalicylic acid, stained with Coomassie blue for 30 min and then destained with 7% acetic acid. Glyoxalase-I from liver, erythrocyte and brain showed one main band with several minor bands. Glyoxalase-I activity could be recovered from the gels if pre-electrophoresis of the gels for 3 h was carried out. Gels were cut into thin sections and placed in pH 7 phosphate buffer overnight. Analysis for glyoxalase-I activity indicated that for each of these three samples, the activity band corresponded to the main protein band, and the location of the bands on the gels was the same for these samples, suggesting that the same form of glyoxalase-I is present in liver, erythrocyte and brain. The kidney enzyme has not been purified as extensively as the others, but its location on the gel also is identical to the others. The two forms of glyoxalase-I purified from rat erythrocytes by the scheme used for the liver enzyme (Table I) have quite similar mobilities. Form I appears to be free of Form II, while Form II has some residual Form I, even though a fairly conservative cut was taken from the DEAE-Sephadex column.

TABLE III

PURIFICATION SCHEME FOR RAT KIDNEY AND RAT BRAIN GLYOXALASE-I

Step		Volume (ml)	Protein (mg/ml)	Specific activity (I.U.)	Yield (step) (%)	Overall purification
Kidney						
1	27 000 × *g* supernatant fraction	115	35.1	0.62	100	1
2	Chloroform/ethanol fractionation	117	8.5	2.5	98	3.9
3	Blue dextran affinity column	2.6	0.03	101	69	163
Brain						
1	27 000 × *g* supernatant fraction	82	14.6	1.6	100	1
2	Chloroform/ethanol fractionation	109	0.9	16.3	84	10
3	Blue dextran affinity column	3.5	0.10	814	71	503

Tentatively, we conclude that a single glyoxalase-I exists in all of the rat tissues examined, and that the isolation of two forms from the erythrocyte by the scheme in Table I is the result of the purification procedure.

Molecular weight determination by gel chromatography

The purification of rat liver glyoxalase-I and rat erythrocyte glyoxalase-I by the scheme in Table I included a Sephadex G-100 column for gel chromatography. To obtain an indication of the molecular weights, the Sephadex G-100 column was calibrated using purified samples of ribonuclease, chymotrypsinogen, bovine carbonic anhydrase, ovalbumin, and bovine serum albumin. The void volume was measured using blue dextran. Glyoxalase-I from rat liver and rat erythrocytes showed molecular weight 50 000 by this procedure, similar to the molecular weights reported for other mammalian sources of the enzyme which range from 43 000 to 52 000 [15,16,25].

Effects of dinucleotides on the activity of glyoxalase-I

The purification schemes for rat erythrocyte, kidney and brain glyoxalase-I include a blue dextran affinity column. Thompson et al. [26] have investigated the use of this affinity column procedure for the purification of proteins requiring dinucleotides and have suggested that blue dextran may mimic dinucleotides, such as NAD^+. These affinity columns are useful for the purification of proteins which possess the dinucleotide fold [27], which probably includes most dehydrogenases.

Recently, the purification and characterization of formaldehyde dehydrogenase was reported [28]. This enzyme is similar to glyoxalase-I in many respects, as shown in Scheme 1. Both glyoxalase-I and formaldehyde dehydrogenase catalyze the oxidation of an aldehyde to the thiol ester of glutathione and a carboxylic acid. The main difference is that glyoxalase-I catalyzes an intramolecular redox reaction where the α-keto group of the substrate functions as the hydrogen acceptor, whereas formaldehyde dehydrogenase catalyzes a redox reaction with NAD^+ as the hydrogen acceptor. Although glyoxalase-I is not a typical dehydrogenase, its similarity to formaldehyde dehydrogenase suggested that on an evolutionary basis glyoxalase-I may possess a dinucleotide fold. The salt concentration required to displace glyoxalase-I from the affinity column (Fig. 4) is similar to that required for many dehydrogenases [26]. Use of dinucelotides to displace the enzyme is not a useful procedure owing to interference by the dinucleotides with the spectrophotometric assay for glyoxalase-I. At low concentrations (0.2 mM), neither NAD^+ nor NADH alter the activity of glyoxalase-I, suggesting that if there is a dinucleotide fold on the enzyme, it is not an important regulatory site of glyoxalase-I.

Substrate specificity of glyoxalase-I

To test further that the two forms of rat erythrocyte glyoxalase-I purified by the scheme of Table I are the result of the purification procedure, these two were compared with the rat liver enzyme in an examination of substrate specificity using a variety of aliphatic and aromatic α-ketoaldehydes. Table IV shows K_m and relative V values for the disproportionation of the hemimercaptals of GSH and these α-ketoaldehydes, assuming a one-substrate pathway (Scheme 1).

TABLE IV

SUBSTRATE SPECIFICITY FOR THE GLYOXALASE-I REACTION WITH ALIPHATIC AND AROMATIC α-KETOALDEHYDES, USING RAT LIVER OR RAT ERYTHROCYTE GLYOXALASE-I

Both Form I and Form II of the erythrocyte enzyme were studied. V values are relative to methylglyoxal, pH 7 phosphate buffer, 25°C, I = 0.2.

α-Ketoaldehyde	K_m (M)	V (rel)
Methylglyoxal	$9 \cdot 10^{-5}$	1.0
Kethoxal	$2 \cdot 15^{-4}$	1.5
Phenylglyoxal	$4 \cdot 10^{-5}$	0.5
p-Chlorophenylglyoxal	$2 \cdot 10^{-5}$	0.7
p-Hydroxyphenylglyoxal	$3 \cdot 10^{-5}$	0.4
p-Bromophenylglyoxal	$2 \cdot 10^{-5}$	1.1
p-Methylphenylglyoxal	$2 \cdot 10^{-5}$	0.2
p-Phenylphenylglyoxal	$2 \cdot 10^{-5}$	0.4
p-Methoxyphenylglyoxal	$1 \cdot 10^{-5}$	0.3
m-Methoxyphenylglyoxal	$3 \cdot 10^{-5}$	1.5
2,4-Dimethylphenylglyoxal	$7 \cdot 10^{-5}$	1.8

No variations in the kinetic parameters were observed for all the α-ketoaldehydes examined with these three enzymes. Previous studies on the substrate specificity of yeast glyoxalase-I revealed a broad substrate specificity [12] similar to the results in Table IV. For the yeast enzyme, all V values were smaller than the V for methylglyoxal, although only a 4-fold range was observed; K_m values decreased with increasing apolar character of the side chain of the α-ketoaldehyde. For rat glyoxalase-I (Table IV), V values show a similar insensitivity to the nature of the α-ketoaldehyde. K_m values for the rat enzyme appear less sensitive to the apolar character of the side chain of the α-ketoaldehyde than was observed with the yeast enzyme. If the side group of the α-ketoaldehyde is sterically crowded, the broad substrate specificity falls off abruptly. 2,4,6-Trimethylphenylglyoxal is not a substrate even though the related compound 2,4-dimethylphenylglyoxal is normal (Table IV). This same behavior was observed with the yeast enzyme [14].

Deuterium isotope effects in the glyoxalase-I reaction

As an additional test that a single rat glyoxalase-I exists, the liver enzyme and the two erythrocyte forms of the enzyme were examined for deuterium isotope effects on V and K_m using methylglyoxal and perdeuteriomethylglyoxal and using phenylglyoxal and α-deuteriophenylglyoxal as substrates (Table V). The same isotope effects were observed with these three enzymes. Using the methylglyoxal series, the isotope effect on V is less than the effect on K_m while for the phenylglyoxal series the effects on these parameters are similar. The phenylglyoxal series is a better probe of the nature of this isotope effect because only a single deuterium has been introduced into the substrate. It is known that the glyoxalase-I reaction involves intramolecular hydride migration of the aldehyde hydrogen to the α-ketone group [29,30]. The isotope effect on V (Table V) supports the conclusion that this is the rate-determining step. The observation that the isotope effect, at least for the phenylglyoxal series, is ob-

TABLE V

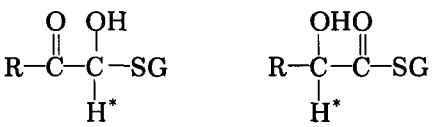
DEUTERIUM ISOTOPE EFFECTS FOR THE GLYOXALASE-I REACTION WITH METHYLGLYOXAL AND PHENYLGLYOXAL, USING RAT LIVER OR RAT ERYTHROCYTE GLYOXALASE-I

Both Form I and Form II of the erythrocyte enzyme were studied; pH 7 phosphate buffer, 25° C, I = 0.2.

α-Ketoaldehyde	K_m (H)/K_m (^{2}H)	V (H)/V (^{2}H)
Methylglyoxal	2.8 ± 0.3	1.7 ± 0.1
Perdeuteriomethylglyoxal		
Phenylglyoxal	3.9 ± 0.8	4.8 ± 0.3
α-Deuteriophenylglyoxal		

served to a similar extent in both V and K_m suggests that the catalytic rate constant for the intramolecular disproportionation is considerably larger than the rate constant for dissociation of the enzyme-substrate complex, i.e. $k_3 > k_2$, and consequently $k_m \simeq k_3/k_1$ (eqn. 1). Similar isotope effects were observed with the yeast enzyme [13].

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_3} E + P \qquad (1)$$



(S)

(P)

Discussion

It has been reported that tumor-bearing animals exhibit an altered form of liver glyoxalase-I [18]. Interestingly, the tumor itself was shown to possess a form of glyoxalase-I indistinguishable from the liver enzyme of a non-tumor bearing animal. This appearance of an altered enzyme in tissue separate from the tumor raises a number of questions: the altered glyoxalase-I may represent the appearance of a fetal protein; the altered form in the liver may be identical to the normal form in some other tissue; if this apparent change in gene expression occurs shortly after infection, it might be diagnostically useful. Answers to these questions require a knowledge of the normal distribution of glyoxalase-I. The present study of the properties of glyoxalase-I from various tissues of the rat suggests that a single form of the enzyme exists in a variety of tissues under normal conditions. This conclusion is based upon a number of observations: (1) purification of glyoxalase-I from erythrocytes and liver using a purification scheme which employed several column chromatography steps indicated very similar behavior on ion-exchange columns and identical size as determined by gel chromatography; (2) the question of a second form of the enzyme in erythrocytes was further investigated by use of an alternative purification procedure which indicated only a single form was present; (3) this rapid, high-yield alternative procedure employing blue dextran affinity chromatogra-

phy was used in the partial purification of rat kidney and rat brain glyoxalase-I which chromatographed identically to the erythrocyte enzyme on this column; (4) glyoxalase-I from all the tissues examined showed indistinguishable mobilities on polyacrylamide gel electrophoresis; (5) a detailed kinetic analysis of the liver and erythrocyte enzymes using a broad range of substrates showed experimentally identical behavior for both of these enzymes. Although the kidney and brain enzymes were not studied kinetically in detail, they were tested with several of the substrates used in Tables IV and V, and both of these also were indistinguishable from the liver and erythrocyte enzymes. We have no data to suggest that distinct isozymes exist in these various tissues under normal conditions. The glyoxalase-I reported in tumor-bearing animals [18] was distinguishable from the normal enzyme both on the basis of size and on the basis of behavior on ion-exchange column chromatography.

The mechanism of glyoxalase-I remains a challenging problem. As shown in Scheme 1, both one- and two-substrate pathways are possible for the disproportionation reaction. Mannervik has suggested [10] that both pathways operate at low concentrations of glutathione and hemimercaptal, based upon his nonlinear regression analysis. At high concentrations of glutathione, the initial rate data are described kinetically by either a one-substrate pathway with glutathione as a competitive inhibitor of the hemimercaptal or by an ordered two-substrate pathway requiring glutathione to add before ketoaldehyde adds to the enzyme [10]. Owing to the rapid reversible formation of hemimercaptal, the expressions for the one- and two-substrate pathways are kinetically equivalent [10]. Our earlier studies on yeast glyoxalase-I suggested that the one-substrate pathway is the major pathway at high substrate concentrations [14]. In view of the similarity of the results in Tables IV and V to those obtained from studies of the yeast enzyme [13,14], we anticipate that glyoxalase-I from rat tissue also may function mainly by the one-substrate pathway. These studies are underway. In addition, studies are underway which are directed toward elucidation of the role of the metal ion in glyoxalase-I. All glyoxalase-I enzymes which have been studied contain Mg^{2+} and require either Mg^{2+} or some other divalent metal ion for activity. Metal ion exchange in the yeast enzyme has not yet been accomplished. Glyoxalase-I from mammalian sources, including the rat tissues used in the present study, is amenable to replacement of the metal ion. A comparative study of the kinetics of glyoxalase-I activated by a series of divalent metal ions is in progress.

Acknowledgements

This work was supported by U.S. Public Health Service Grant CA11850 from the National Cancer Institute and by a U.S. Public Health Service Research Career Development Award, CA70939, to David L. Vander Jagt. A portion of this work was submitted by Liang-Po B. Han to the University of New Mexico in partial fulfillment of the requirements for the Ph.D. degree.

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