Characterization of Glyoxalase I Purified from Pig Erythrocytes by Affinity Chromatography

By ANNE-CHARLOTTE ARONSSON and BENGT MANNERVIK
Department of Biochemistry, Arrhenius Laboratory, University of Stockholm,
S-10691 Stockholm, Sweden

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Glyoxalase I (EC 4.4.1.5) was purified about 10000-fold from pig erythrocytes in a yield of approx. 20%. The purification included affinity chromatography on *S*-hexylglutathione coupled to Sepharose 4B. The purified enzyme normally contained two catalytically active components which were resolved by polyacrylamide-gel electrophoresis. After treatment with reduced glutathione only one component was found. The two components were also demonstrable after isoelectric focusing or DEAE-cellulose chromatography and could also in these cases be fused into one species by preincubation with reduced glutathione. It is proposed that the most acidic form of glyoxalase I is a mixed disulphide with glutathione. Except for these interconvertible forms, the purified enzyme was homogeneous, as judged by disc electrophoresis and sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. The molecule is a dimer (48000 daltons), composed of apparently identical subunits (24000 daltons). The isoelectric point was 4.8 at 4°C. The amino acid composition was consistent with the low isoelectric point. The enzyme contained about two thiol groups per enzyme molecule. EDTA inactivated the enzyme and bivalent metal ions could restore fully or partially the catalytic activity; Mg^{2+} and Mn^{2+} gave highest activity. It is proposed that a major biological function of glyoxalase I is the detoxification of methylglyoxal formed by enterobacteria in the alimentary canal.

Glyoxalase I (EC 4.4.1.5., lactoylglutathione lyase) is almost as ubiquitous as glutathione in cells of different organisms. This enzyme serves a protective function by catalysing the transformation of α -oxo aldehydes to thiol esters of glutathione. Nevertheless the biological role of glyoxalase I remains obscure.

In recent years several investigations have been made with extensively purified glyoxalase I from mammalian sources (Davis & Williams, 1969; Mannervik *et al.*, 1972; Uotila & Koivusalo, 1975; Kester & Norton, 1975; Han & Vander Jagt, 1975), but comparatively little is known about the molecular properties and catalytic mechanism of the enzyme. The present paper describes a procedure that allows preparation of glyoxalase I from pig erythrocytes on a scale large enough to make such a characterization feasible. Some of the results have previously been reported (Marmstål *et al.*, 1975).

Materials and Methods

Reduced glutathione, Tris and imidazole were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. EDTA, ϵ -aminohexanoic acid, and salts of Mg, Mn, Zn, Ca, Cu, La and Co were products of Merck A.G. Darmstadt, Germany, and those of Fe of Baker Chemical Co., Phillipsburg, NJ, U.S.A.

N-Ethyl-*N'*-(dimethylaminopropyl)carbodi-imide and methylglyoxal [40% (w/v) in water] were obtained from Fluka A.G., Buchs, Switzerland. The latter was distilled before use. Sephadex G-75, G-100 and CNBr-activated Sepharose 4B were purchased from Pharmacia Fine Chemicals, Uppsala, Sweden, and DEAE-cellulose DE-32 was from Whatman Biochemicals, Maidstone, Kent, U.K. Cytochrome *c*, ovalbumin, bovine serum albumin and trypsin were obtained from Sigma, and alcohol dehydrogenase (horse liver), malate dehydrogenase (pig heart) and glutathione reductase (yeast) from Boehringer, Mannheim, Germany. Carbonic anhydrase from bovine erythrocytes was purified as described by Lindskog (1960). *S*-Hexylglutathione was synthesized by method A of Vince *et al.* (1971).

Assay of glyoxalase I activity

The activity was measured spectrophotometrically at 30°C by following the thiol ester formation as discussed by Racker (1951). The value of $3.37\text{ mm}^{-1}\cdot\text{cm}^{-1}$ was used for the absorption coefficient at 240 nm of the product *S*-lactoylglutathione. The standard assay system contained 25 mM-imidazole/HCl (pH 7.0), 0.66 mM-GSH,* 2 mM-methylglyoxal and a suitable amount of enzyme, giving a final volume of 1 ml.

* Abbreviation: GSH, reduced glutathione.

The reaction was initiated by addition of enzyme. A unit of glyoxalase I activity is defined as the amount of enzyme catalysing the formation of $1\mu\text{mol}$ of *S*-lactoylglutathione/min in the standard assay system. Specific activity is expressed as units/mg of protein.

Determination of protein concentration

The protein concentration was determined spectrophotometrically by measuring the absorbance at 260, 280 and 410 nm. The haemoglobin concentration was estimated from the A_{410} . After correction for the absorbance of haemoglobin at 260 and 280 nm the concentration of the remaining proteins was calculated by the equation of Kalckar (1947). In the purest preparations the concentration was also determined by the method of Lowry *et al.* (1951) (with bovine serum albumin as standard), which gave essentially the same result as the above procedure. The pure enzyme had an absorbance of 1.37 cm^{-1} at 280 nm at a concentration of 1 mg/ml.

Preparation of S-hexylglutathione-Sepharose 4B

For preparation of the affinity matrix, CNBr-activated Sepharose 4B was used. The gel was pretreated according to the instructions of the manufacturer. *S*-Hexylglutathione was coupled to the gel via an arm of ϵ -aminohexanoic acid essentially as described by Cuatrecasas (1970). ϵ -Aminohexanoic acid (0.4 g/g of gel) was dissolved in 0.1 M NaHCO_3 containing 0.5 M NaCl (pH 8.5) in a volume equal to the volume of packed Sepharose. The solution was mixed with the gel and stirred by gentle rotation overnight at 4°C . *S*-Hexylglutathione (0.04 g/g of gel) was dissolved in water by titration with 2 M NaOH and the solution was then adjusted to pH 5.0 with HCl . The ϵ -aminohexanoic acid-Sepharose was washed thoroughly and mixed with a solution of *N*-ethyl-*N'*-(dimethylaminopropyl)carbodi-imide (0.04 g/g of gel) in deionized water (pH 5.0) for about 5 min before adding the *S*-hexylglutathione solution. After reaction overnight at room temperature (22°C) the gel was washed with three cycles consisting of 0.1 M sodium acetate buffer containing 0.5 M NaCl (pH 4.0) and 0.1 M NaHCO_3 containing 0.5 M NaCl to remove all material that was not covalently bound. Finally the gel was washed with a large volume of 10 mM-Tris/HCl (pH 7.8).

Enzyme purification

Fresh pig blood containing sodium citrate to prevent clotting was obtained from a local slaughterhouse. The erythrocytes were collected by centrifugation (6000 g for 20 min) and washed twice with 0.9% NaCl . During the purification the enzyme was kept at about 4°C .

Step 1: haemolysis. A sample of 750 ml of erythrocytes was stirred with 600 ml of deionized water for 1 h.

Step 2: precipitation of haemoglobin. A mixture of 960 ml of ethanol (40%) and 480 ml of chloroform was added to the haemolysate. The resulting mixture was stirred for 40 min and then centrifuged for 20 min at 6000 g. The sediment was discarded.

Step 3: Sephadex G-25 gel filtration. A Sephadex G-25 (coarse grade) column (9 cm $\times$ 90 cm) equilibrated with 10 mM-Tris/HCl (pH 7.8) was used to remove chloroform and ethanol remaining from the previous step. Fractions containing glyoxalase I activity were collected.

Step 4: DEAE-cellulose chromatography. The pooled fractions from step 3 were applied to a DEAE-cellulose column (4 cm $\times$ 5 cm) equilibrated with 10 mM-Tris/HCl (pH 7.8). Elution was carried out with a linear ionic-strength gradient (0–0.3 M NaCl in 10 mM-Tris/HCl, pH 7.8, total volume 1000 ml) at a flow rate of 40 ml/h.

Step 5: S-hexylglutathione-Sepharose chromatography. The enzyme preparation from the DEAE-cellulose column was placed on the *S*-hexylglutathione-Sepharose column (1 cm $\times$ 25 cm) in portions containing up to 3000 units of glyoxalase I. The column material was packed in 10 mM-Tris/HCl (pH 7.8). After sample application the column was washed with 10 mM-Tris/HCl (pH 7.8) containing 0.2 M NaCl . The enzyme was then eluted with a gradient of 0–5 mM-GSH in 10 mM-Tris/HCl, pH 7.8 (total volume 200 ml). The flow rate during the whole procedure was 30 ml/h. The active fractions were pooled and concentrated to about 10 ml by ultrafiltration on a PM10 Diaflo filter.

Step 6: Sephadex G-100 gel filtration. The preparation from step 5 was chromatographed on a Sephadex G-100 (superfine grade) column (4 cm $\times$ 35 cm) equilibrated with 10 mM-Tris/HCl (pH 7.8).

Electrophoretic systems

(a) Disc electrophoresis was performed essentially as described by Ornstein (1964) and Davis (1964). The electrolyte buffer was 5 mM-Tris/34 mM-glycine (pH 8.3). For detection of protein the gels were fixed in 12.5% (w/v) trichloroacetic acid for 20 min and stained with 0.1% Coomassie Brilliant Blue G-250, dissolved in 12.5% trichloroacetic acid. After 20 min the gels were destained in 5% (v/v) acetic acid. Staining for enzymic activity of glyoxalase I was carried out essentially as discussed by Kömpf *et al.* (1975). The gels were incubated with 1 mM-3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT tetrazolium), 20 mM-GSH and 60 mM-methylglyoxal in 0.2 M-sodium phosphate buffer (pH 6.8). Enzyme activity appears as colourless bands against a blue background.

(b) Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis was performed by the method of Weber & Osborn (1969). Solutions containing 0.09 mg of protein were placed on the gels and a current of 8 mA/gel was applied for 6 h.

(c) Isoelectric focusing was carried out at 4°C in a 110 ml column according to the instructions of the manufacturer (LKB Produkter AB, Stockholm, Sweden). Ampholytes (pH 4–6) were used at 1% concentration to create a pH gradient in a sucrose density gradient. The isoelectric focusing was run for 70 h with a voltage of 300 V at the beginning and 500 V at the end of the experiment. The pH-meter was calibrated at 4°C against standard buffer solutions for measurement of pH at this temperature.

Determination of molecular weight

The molecular weight of the purified enzyme was determined by gel filtration on a Sephadex G-100 column (1.5 cm × 82 cm) equilibrated with 10 mM, Tris/HCl (pH 7.8) containing 0.1 M-NaCl (Andrews, 1970). The reference proteins were mixed with 0.4 ml of glyoxalase I solution to give a total sample volume of 1 ml. Identification of the reference proteins was carried out by use of standard assay techniques, and the mol.wts. were assumed to be: glutathione reductase (118 000), alcohol dehydrogenase (80 000), bovine serum albumin (68 000), malate dehydrogenase (70 000), ovalbumin (43 000), carbonic anhydrase (29 000) and cytochrome *c* (12 400).

The molecular weights of the subunits of the purified glyoxalase I were estimated by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis by using carbonic anhydrase, ovalbumin, trypsin (mol.wt. 23 300) and cytochrome *c* as standards.

Preparation of apo-(glyoxalase I)

Apo-(glyoxalase I) was obtained by treatment with EDTA essentially as described by Uotila & Koivusalo (1975). The enzyme sample used had been purified to step 4. The dialysis tubing was cleaned by immersion sequentially for periods of 1 h in 3 litres each of 50% (v/v) ethanol, 10 mM-NaHCO₃ and deionized water (two changes of each bath). The glassware was soaked in 2 M-HNO₃ for 12 h to remove contaminating metal ions.

Amino acid analysis

Amino acid analyses were carried out by Dr. D. Eaker, Institute of Biochemistry, University of Uppsala. Samples (0.1 mg) were hydrolysed for 24 and 72 h and analysed on a Durrum D-500 amino acid analyser with norleucine as an internal standard.

Determination of thiol groups

For the determination of total protein thiol-group content, an enzyme solution containing 10–20 mg of protein/ml was used. The assay system contained 0.1 M-Tris/HCl buffer (pH 8.0), 0.01 M-EDTA, 8 M-urea and 0.4 mM-5,5'-dithiobis-(2-nitrobenzoic acid) (Glazer *et al.*, 1975). A suitable volume of enzyme solution (about 50 µl) was added to a final volume of 1 ml. The A_{412} was recorded with time for about 15 min. After development of maximal absorbance the downward-sloping line was extrapolated to zero time to determine the concentration of thiol. The value used for ϵ_{412} in 8 M-urea was 14 290 M⁻¹·cm⁻¹ (Glazer *et al.*, 1975).

Results

Purification of glyoxalase I

Table 1 summarizes the results of the purification of glyoxalase I from pig erythrocytes. After haemolysis of the erythrocytes the haemoglobin was denatured with a mixture of chloroform and ethanol. About 60% of the enzyme activity remained after precipitation of haemoglobin. A better yield could be achieved by using a more dilute haemolysate (1500 ml of water instead of 600 ml), but for routine preparation of enzyme it was easier to handle a smaller sample volume. Affinity chromatography was normally carried out on *S*-hexylglutathione coupled to Sepharose 4B by means of an arm of ϵ -aminohexanoic acid. An amount of 2500–3000 units of the enzyme preparation after DEAE-cellulose chromatography was used to saturate the affinity column. The capacity of the affinity gel was found to be related to glyoxalase I activity rather than to the amount of protein present.

S-Hexylglutathione was also coupled without a spacer to the gel (cf. Koivusalo & Uotila, 1975). The capacity was the same as for gel prepared with an arm of ϵ -aminohexanoic acid. However, the specific activity of the enzyme (measured after the Sephadex G-100 chromatography) seemed to be somewhat lower after chromatography on this gel. When the gel preparation without arm was used, the enzyme was eluted in less than 10 ml (elution profile Fig. 1), compared with a volume of about 30 ml when an arm of six carbon atoms was introduced between the gel and the ligand. The affinity columns were used about three times a week for 6 months without any apparent loss in capacity.

The enzyme solution was not analysed for protein content after the *S*-hexylglutathione-Sepharose column because of the interference of GSH in the measurements, but after removal of GSH by gel filtration on Sephadex G-25 it could be estimated that the purification by affinity chromatography was more than 10-fold.

Table 1. *Purification of glyoxalase I from pig erythrocytes*
After step 4 the enzyme was divided into three portions, which were purified consecutively.

Step	Volume (ml)	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Yield (%)	Purification factor
(1) Haemolysate	1250	281 000	23 400	0.083	100	1
(2) Chloroform/ethanol precipitation	1250	3860	15 800	4.09	67.5	49.3
(3) Sephadex G-25	1810	1840	13 300	7.23	56.8	87.1
(4) DEAE-cellulose	149	116	6590	56.8	28.2	684
(5) <i>S</i> -Hexylglutathione-Sepharose	3 × 28.0	*	3 × 1580		20.3	
(6) Sephadex G-100	3 × 29.4	3 × 1.38	3 × 1320	957	16.9	11 500

* The protein concentration was not determined, owing to interference by GSH, but the purification obtained in this step was estimated to be more than 10-fold.

Fig. 1. *Purification of glyoxalase I by chromatography on an S-hexylglutathione-Sepharose column (step 5)*

An enzyme sample (about 3000 units) obtained after the DEAE-cellulose chromatography (step 4) was applied to a column (1 cm × 2.5 cm) equilibrated with 10 mM-Tris/HCl (pH 7.8). The column was washed with 10 mM-Tris/HCl (pH 7.8) containing 0.2 M-NaCl before elution with a linear gradient of 0–5 mM-GSH in 10 mM-Tris/HCl, pH 7.8. The arrow marks the start of the gradient. ○, Glyoxalase I activity; ----, A_{280} .

After the final Sephadex G-100 chromatography the enzyme had a specific activity of about 1000 units/mg, which corresponds to a purification of about 10000-fold.

Analysis of the concentrated Sephadex G-100 pool by disc electrophoresis showed two protein bands, both containing active enzyme as demonstrated by staining for enzymic activity. If the enzyme was pre-incubated with 0.1 M-GSH, only one protein band was detected after disc electrophoresis. The incubation with GSH caused the more rapidly moving band to become transformed into the slower-moving band.

The two forms of the enzyme could also be separated by using a small DEAE-cellulose column (5 ml bed volume). The column was equilibrated with 10 mM-Tris/HCl buffer (pH 7.8) and the enzyme was eluted with a linear gradient of 0–0.3 M-NaCl in 10 mM-Tris/HCl (pH 7.8) (total volume 1000 ml). The

Fig. 2. *Separation of two forms of glyoxalase I by column chromatography on DEAE-cellulose*

A column of DEAE-cellulose (1.0 cm × 6.5 cm) was equilibrated with 10 mM-Tris/HCl (pH 7.8). About 1500 units of pure enzyme was applied to the column. Elution was carried out with a linear gradient of NaCl (0–0.3 M, 1000 ml). One sample (○) was chromatographed without pretreatment. The second (●) was incubated with 0.1 M-GSH before application to the column. The elution was in the latter case carried out with buffer solutions containing 2 mM-GSH.

elution profile (Fig. 2, open symbols) showed two activity peaks. The peaks were analysed by disc electrophoresis and each was found to contain only one protein band. The protein band of the first and second activity peaks corresponded to the slower and the faster bands, respectively, obtained after gel electrophoresis. When material from the two peaks was mixed before electrophoresis, the gels showed two separate protein bands.

The same DEAE-cellulose column was used for chromatography of enzyme preincubated with 0.1 M-GSH (adjusted to pH 7.0). After about 30 min the sample was diluted with an equal volume of buffer (to decrease the ionic strength) and applied to the DEAE-cellulose column. The elution was carried out in the same way as in the previous case, but 2 mM-GSH was included in the buffer solutions. The elution profile showed only one activity peak (Fig. 2, filled symbols) and analysis of this peak by disc electrophoresis demonstrated a single protein band.

Fig. 3. Determination of the isoelectric point of glyoxalase I by isoelectric focusing

The experimental details are described in the Materials and Methods section. The pH values refer to 4°C. (a) An enzyme sample (about 500 units) purified to step 4 was dialysed against 10 mM-Tris/HCl (pH 7.8) and then applied to the focusing column. After focusing (70 h) the column was eluted and the fractions were analysed for glyoxalase I activity (○) and pH (△). (b) The focusing was carried out as described for (a) but GSH was added to the enzyme solution to a concentration of 0.1 M before application to the column.

Isoelectric focusing

For isoelectric focusing, enzyme obtained after the DEAE-cellulose chromatography (step 4) was used. About 500 units of enzyme was applied to the column. To avoid interference by Ampholine in the activity measurements, owing to spontaneous reaction of methylglyoxal with amino groups of the ampholytes, the sample solution was first incubated with methylglyoxal before initiating the enzymic reaction with GSH. Two separate peaks of glyoxalase I with pI values of 4.4 and 4.8 were demonstrated (Fig. 3a). If GSH was added, to a final concentration of 0.1 M, to the enzyme solution before the focusing, all the activity appeared in one peak with pI 4.8 (Fig. 3b).

Molecular weight and subunit structure

The mol.wt. of glyoxalase I from pig erythrocytes was estimated by gel filtration to be 48000 ± 700 ($\pm$ s.d., $n = 3$).

Electrophoresis in sodium dodecyl sulphate/polyacrylamide gels demonstrated a single protein band at a position corresponding to a subunit mol.wt. of 24000.

Re-activation of apo-(glyoxalase I)

Apo-(glyoxalase I) from pig erythrocytes was essentially inactive but could be re-activated by several bivalent metal ions. The apoenzyme was incubated with the different metal ions in concentrations ranging from $0.01 \mu\text{M}$ to 10 mM. Maximum activities were reached after incubation for 2 h. In one experiment the apoenzyme had 2.5% residual activity, and treatment for 2 h with 1 mM- Mg^{2+} , - Mn^{2+} , - Co^{2+} or - Zn^{2+} gave respectively 65, 59, 62 and 46% of the activity of untreated holoenzyme. At 10 mM concentration of the metal ions only Mg^{2+} gave higher activity (103%) than that obtained at 1 mM. Concentrations of 0.1 mM and below of the above-mentioned ions gave no significant activation of the apoenzyme, and Fe^{2+} , La^{3+} , Ca^{2+} or Cu^{2+} did not restore significant activity at any of the concentrations investigated.

Amino acid analysis

The amino acid composition of glyoxalase I from pig erythrocytes is presented in Table 2. A relatively large fraction of the amino acids in the hydrolysate was glutamic acid or aspartic acid. Although the proportion of the acids which are in amide form in the intact protein has not been determined, the results are consistent with the low isoelectric point of glyoxalase I, considering also the amount of basic amino acids in the enzyme. The amino acid composition of glyoxalase I has previously been determined in a preparation from mouse liver (Kester & Norton, 1975) (cf. Table 2).

Table 2. *Amino acid composition of glyoxalase I from pig erythrocytes*

Amino acid	Content (residues/mol)	Nearest integer	Mouse liver (Kester & Norton, 1975)
Aspartic acid	56.2	56	53
Threonine	23.1*	23	34
Serine	28.5*	29	32
Glutamic acid	42.9	43	42
Proline	26.9	27	27
Glycine	32.1	32	31
Alanine	30.9	31	19
Half-cystine	9.25†	9	
Valine	10.7	11	14
Methionine	8.81	9	7
Isoleucine	25.5	26	17
Leucine	40.6	41	47
Tyrosine	12.1	12	13
Phenylalanine	25.1	25	25
Histidine	8.90	9	7
Lysine	38.9	39	23
Tryptophan	6.44‡	6	
Arginine	12.9	13	14

* Values obtained by extrapolation to zero time.

† Value determined after performic acid oxidation.

‡ Value determined after hydrolysis in 3M-mercaptoethanesulphonic acid (Penke *et al.*, 1974).

Determination of thiol groups

The number of thiol groups titratable with 5,5'-dithiobis-(2-nitrobenzoic acid) in the presence of 8M-urea was 1.56 ± 0.15 ($\pm$ S.D., $n = 7$), per enzyme molecule. After treatment with 0.1 M-GSH for 60 min, followed by gel filtration on a Sephadex G-25 column to remove GSH, the titre was 1.82 ± 0.11 ($\pm$ S.D., $n = 5$). This finding and the results of electrophoretic and chromatographic characterization of the two enzyme forms are explicable by the assumption that the most acidic form of glyoxalase I (isoelectric point 4.4) is a mixed disulphide of the enzyme and GSH. The implication that blocking of a thiol group on the enzyme will not cause elimination of activity is consistent with earlier findings of the relative insensitivity of glyoxalase I from pig erythrocytes to thiol-blocking reagents (Mannervik *et al.*, 1975).

If all thiol groups have been titrated under the conditions used, the half-cystine value (Table 2) would be consistent with four disulphide bonds per molecule. This corresponds to one thiol and two disulphide groups per subunit on the assumption that the subunits are alike.

Discussion

The purification procedure described gives an apparently homogeneous enzyme, as judged by electrophoretic and chromatographic analyses. How-

ever, the pure enzyme was very unstable and lost most of its activity within a few days at 4°C. Therefore only the amount of enzyme required for a particular experiment was taken through affinity chromatography and the following purification step. The modification of the enzyme appeared to be of two kinds, namely generation of a more acidic form and inactivation of the enzyme. The two forms which could be detected by isoelectric focusing, gel electrophoresis and ion-exchange chromatography were enzymically active and were consequently not at all or only partially inactivated. We suggest that the most acidic form is a mixed disulphide of glyoxalase I (Enz) and GSH, which may be formed under oxidizing conditions (in the presence of GSH). Treatment with GSH regenerates the less acidic (pI 4.8) form of the enzyme having a free thiol group:

The inactivated enzyme seemed to be less easily renatured, but the catalytic activity could be at least partially restored by treatment with GSH.

Affinity chromatography has previously been used by Kester & Norton (1975), and in parallel with our studies, by Koivusalo & Uotila (1975). We have in addition to *S*-hexylglutathione used the corresponding octyl derivative as a ligand, but this was not significantly better than the hexyl derivative and was more difficult to handle in the preparation of the adsorbent, owing to its low solubility.

The specific activity of the purified glyoxalase I from pig erythrocytes was somewhat lower than previously reported for the enzyme from mouse liver (Kester & Norton, 1975) and sheep liver (Uotila & Koivusalo, 1975). However, our standard assay system is different from those earlier reported and gives activity values about 30% lower than those obtained in the system of Uotila & Koivusalo (1975). The specific activity could not be improved by additional purification steps and the value reported in Table 1 has consistently been obtained in a large number of preparations.

The subunit structure and molecular weight of the pig enzyme are similar to those of the sheep liver enzyme (Uotila & Koivusalo, 1975). The molecular weight and amino acid composition of the mouse liver enzyme (Kester & Norton, 1975) are also similar to the results reported in the present paper.

The studies on the metal-dependence of glyoxalase I from the purified glyoxalase I from pig erythrocytes confirm and extend the previous findings (Mannervik *et al.*, 1972). Similar results for the sheep liver enzyme have been obtained (Uotila & Koivusalo, 1975). It appears probable that the metal in native glyoxalase I is Mg, because this is the most effective metal in restoring catalytic activity.

The physiological function of glyoxalase I in erythrocytes has not been established. Neither has the

formation of methylglyoxal in erythrocytes been demonstrated. Preliminary investigations on the presence of methylglyoxal synthase in erythrocytes were negative (T. Bartfai & B. Mannervik, unpublished work). However, methylglyoxal synthase, utilizing dihydroxyacetone phosphate, which is produced in the glycolytic pathway, has been demonstrated in, e.g., *Escherichia coli* and *Proteus vulgaris* (cf. Tsai & Gracy, 1976). If indeed methylglyoxal is the physiological substrate for glyoxalase I, it seems reasonable to assume that this compound could be released by enterobacteria in the alimentary canal and be taken up in the blood and transported via the portal vein to the liver. A function of the enzyme in the detoxification of the strongly electrophilic methylglyoxal would explain the high activity demonstrated both in erythrocytes and in liver. Evidence for a corresponding function of alcohol dehydrogenase in detoxification of ethanol produced by enterobacteria has been put forward by Krebs & Perkins (1970).

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