Purification and active site modification studies on glyoxalase I from monkey intestinal mucosa

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Glyoxalase I ((R)-S-lactoylglutathione methylglyoxal-lyase (isomerizing), EC 4.4.1.5) from monkey intestinal mucosa was purified to homogeneity. The purified enzyme had a molecular weight of 48000, composed of two apparently identical subunits. Active-site modification was carried out on the purified enzyme in presence and absence of S-hexylglutathione, a reversible competitive inhibitor of glyoxalase I. Modification by tetranitromethane and N-acetylimidazole caused inactivation of the enzyme. Inactivation by N-acetyl-imidazole was reversible with hydroxylamine treatment, suggesting the importance of tyrosine residues for the activity of the enzyme. The enzyme was inactivated by diethyl pyrocarbonate and the activity was not restored by hydroxylamine treatment, suggesting that histidine residues may not be important for activity. Modification by N-ethylmaleimide and p-hydroxymercuribenzoate did not affect its activity, indicating that sulphydryl groups may not be important for activity. These studies indicated that the amino acids present in the active site of glyoxalase I from intestinal mucosa which may be important for activity are tyrosine, tryptophan, lysine and glutamic acid/aspartic acid residues.

Introduction

Glyoxalase enzyme is ubiquitous in living cells catalysing conversion of methylglyoxal to D-lactic acid [1,2]. This system consists of two enzymes, glyoxalase I and glyoxalase II with reduced glutathione as co-factor [3,4]. Glyoxalase I (S-

lactoylglutathione lyase, EC 4.4.1.5) converts methylglyoxal to S-D-lactoyl glutathione in presence of GSH. Glyoxalase II (hydroxycylgluta-thione hydrolyase, EC 3.1.2.6) hydrolyses lactoyl glutathione to D-lactic acid. The significance of the reaction catalysed by glyoxalase I is that it is a detoxication mechanism for methylglyoxal, a cytotoxic substance acting as an endogenous inhibitor of cell division [5]. Methylglyoxal is formed from dihydroxyacetone phosphate by the action of methylglyoxal synthetase enzyme. This enzyme has been purified and characterised from various enterobacteria such as Escherichia coli, Pseudomonas saccharophila and Proteus vulgaris [6–8]. Eucaryotic cells are not capable of producing

Abbreviations: DPC, diethyl pyrocarbonate; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; HNBB, 2-hydroxy-5-nitrobenzyl bromide; SDS, sodium dodecyl sulphate; TNBS, 2,4,6-trinitrobenzenesulphonic acid.

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methylglyoxal although they contain the glyoxalase enzyme to detoxify this compound. This led Aronson and Mannervik to suggest that mammalian glyoxalase I may have a protective function against toxic substances produced by enterobacteria in the gastrointestinal tract of mammals [9]. There are reports on the purification of glyoxalase I from various tissues of mammals but no report has been made on purification from mammalian intestine. Since the mitotic activity of mucosal cells in intestine is very high, methylglyoxal detoxification by glyoxalase system is of more significance in this tissue.

Methylglyoxal and other α-ketoaldehydes are carcinostatic agents [10,11], and it has been suggested that inhibitors of the glyoxalase enzyme might increase concentrations of methylglyoxal in cells and inhibit cell proliferation [12]. A modern approach to the design of anticancer drugs necessitates a complete study of active-site structure of glyoxalase I. However, there are only a few reports regarding the active site structure of glyoxalase I. Hence, we carried out a chemical modification study on purified glyoxalase I from monkey intestinal mucosa, and present it in this report.

Materials and Methods

Reduced glutathione (GSH), methylglyoxal (40% w/v), Tris, imidazole, N-ethylmaleimide, bovine serum albumin, iodoacetate, iodoacetamide, p-hydroxymercuribenzoate, molecular weight markers for gel filtration, 1,4-butanediol diglycidyl ether, N-acetylimidazole, tetranitromethane, diethyl pyrocarbonate, N-bromosuccinimide, 2-hydroxy-5-nitrobenzyl bromide, 2,4,6-trinitrobenzene sulfonic acid, pyridoxal phosphate, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide and Sepharose 6B were purchased from Sigma Chemical, U.S.A. Chemicals for polyacrylamide gel electrophoresis were purchased from Eastman Kodak, New York. Sephadex G-150, Sepharose 4B and Blue dextran were purchased from Pharmacia Fine Chemicals, Uppsala, Sweden; DEAE-cellulose from Whatman, Maidstone, U.K.; Chelex-100 from Bio-Rad Laboratories, Richmond, CA; cyanogen bromide from Pierce Chemical, Rockford, IL; 1-iodoexane and hydriodic acid 47% were obtained from Aldrich Chemical, U.K. All other chemicals were of analytical grade. S-Hexylglutathione was synthesised according to the method of Vince et al. [13]. Epoxy-activated Sepharose was prepared [14], and S-hexylglutathione was coupled to the epoxy-activated Sepharose [15]. Blue dextran-Sepharose 4B was prepared according to the procedure of Ryan and Vestling [16].

Assay of glyoxalase I activity

Increased absorbance at 240 nm due to thiol ester formation from GSH and methylglyoxal was measured spectrophotometrically as described by Racker with minor modification [3]. One unit of glyoxalase I is defined as the amount of the enzyme catalysing the formation of one micromole of S-carboxyglutathione per minute in the standard assay system.

Determination of protein concentration

Protein concentration was determined by the method of Lowry et al., with bovine serum albumin as standard [17].

Purification of glyoxalase I

Monkeys (Macaca radiata) were anaesthetised with Nembutal before being killed, and the entire length of the intestine was removed. Mucosa was scraped from intestine after washing it with ice-cold 1.15% KCl. All purification steps were carried out at 4°C.

Step I – Homogenisation. 20 g of mucosa was homogenised in a Potter-Elvehjem homogeniser with 9 vol. of 20 mM sodium phosphate buffer (pH 7.0), and centrifuged at 51,000 x g for 1 h.

Step II – Ammonium sulphate fractionation. The supernatant from the previous step was fractionated with solid ammonium sulphate and the enzyme was collected between a saturation of 40 and 80%. The precipitate was dissolved in the minimum volume of 10 mM Tris-HCl buffer (pH 7.8), and dialysed against the same buffer.

Step III – S-Hexylglutathione-Sepharose 6B chromatography. Solid NaCl was added to the dialysed solution from step II to a final concentration of 200 mM and the solution loaded on to a S-hexylglutathione-Sepharose 6B column (1 x 2.5 cm), equilibrated with 10 mM Tris-HCl buffer (pH 7.8), containing 200 mM NaCl. The column
was washed with equilibration buffer and then with 5 mM GSH in the same buffer. The enzyme was eluted with the buffer containing 3 mM S-hexylglutathione. The active fractions were pooled and dialysed against 10 mM Tris- HCl buffer (pH 7.8).

Step IV – DEAE-cellulose chromatography. The dialysed preparation from step III was applied to a DEAE-cellulose column (1 x 2.5 cm), equilibrated with 10 mM Tris- HCl buffer (pH 7.8). The column was washed with the equilibration buffer and then with the buffer containing 60 mM NaCl. The enzyme was eluted with 100 mM NaCl in the buffer. Active fractions were pooled and dialysed against the equilibration buffer.

Step V – Blue dextran-Sepharose 4B chromatography. The sample from the previous step was applied to a Blue dextran-Sepharose 4B column (1 x 2.5 cm), equilibrated with 10 mM Tris- HCl buffer (pH 7.8). The column was washed with the same buffer, and then with the buffer containing 200 mM KCl. The enzyme was eluted with 2 M KCl in the buffer. Active fractions were pooled and dialysed against the buffer.

Electrophoresis
SDS-polyacrylamide gel electrophoresis was carried out according to Laemmli [18], and protein staining was done by the silver-staining method [19].

Molecular weight determination
Molecular weight of the purified enzyme was determined by gel filtration on a Sephadex G-150 column [20]. The standard proteins used were human γ-globulin, bovine serum albumin, ovalbumin, cytochrome c and myoglobin.

Subunit molecular weight was determined by SDS-polyacrylamide gel electrophoresis, using bovine serum albumin, ovalbumin, carbonic anhydrase, trypsinogen, lactoglobulin and lysozyme as standards [18].

Preparation of metal free glyoxalase I
Purified enzyme was utilised to prepare apoglyoxalase I by the method of Uotila and Koivusalo [21]. The metal contaminants of the dialysis bag and glassware were minimised by methods described by Aronsson and Mannervik [9].

Reactivation of metal free glyoxalase I
The usual glyoxalase I assay system in 100 mM imidazole-HCl buffer (pH 7.0) was used. Different concentrations of metal ions ranging from 0.1 mM to 10 mM were added to the assay mixture.

Effect of sulphhydryl group reagents
The purified glyoxalase I was preincubated with different concentrations ranging from 2 to 5 mM sulphhydryl reagents such as N-ethylmaleimide,
iodoacetate, iodoacetamide and 1–5 mM p-hydroxymercuribenzoate. Excess reagents were removed by dialysis against 10 mM Tris-HCl buffer (pH 7.8) for 15 h and the activity was checked.

Active site modification

Chemical modification of the enzyme was carried out by the addition of protein modifying reagents to the purified enzyme and incubating the mixture for a specified time and temperature (Table I). The reaction mixture was diluted 2-fold with 20 mM potassium phosphate buffer (pH 7.0), and chilled in an ice-bath to stop the reaction. In the case of diethyl pyrocarbonate, 1 mM imidazole and in the case of trinitrobenzenesulphonic acid, 0.5 mM sodium sulphite were included in the buffer. The diluted reaction mixture was dialysed at 4°C against the same buffer for 15 h with three changes. After dialysis, enzyme activity was checked. Every experiment was done with a control subjected to the same treatment without modifying reagents.

Active-site protection study

Protection study was done by pre-incubating the enzyme with 2 or 5 mM S-hexylglutathione, a competitive reversible inhibitor of glyoxalase I [12], at 37°C for 5 min, followed by the addition of protein-modifying reagent, and the enzyme activity was checked after dilution and dialysis as described.

Hydroxylamine treatment

In some experiments, the modified inactivated enzyme was treated with 100 mM NH₂OH (pH 7.5) at 37°C for 30 min or 4 h [22,23]. The reaction mixture was dialysed against 20 mM potassium phosphate buffer (pH 7.0) at 4°C for 15 h, and the enzyme activity was checked.

Spectral study

Chemical modification of tyrosine residue by N-acetyl-l-imidazole, and modification of histidine by diethyl pyrocarbonate were also determined spectrophotometrically by ultraviolet absorption at 278 and 242 nm, respectively [22–24].

Results

A summary of purification of glyoxalase I from monkey intestinal mucosa is presented in Table II. The enzyme was purified 5000-fold with an overall recovery of 20% and the purified enzyme had a specific activity of 925 units/mg protein. Silver staining after electrophoresis indicated that the purified enzyme was homogeneous (Fig. 1).

Table III summarises some of the molecular and kinetic properties of the purified glyoxalase I. The purified enzyme had a molecular weight of 48 000 and was composed of two identical subunits of molecular weight 24 000.

Effect of cations on native enzyme

Effect of different concentrations of metal ions ranging from 0.1 to 10 mM were studied. It was observed that Co²⁺ at 1 mM concentration increased the enzyme activity more than 100%, and other metal ions such as Mg²⁺ and Mn²⁺ also increased the activity at 1 and 10 mM, respectively, whereas Ca²⁺ and Zn²⁺ decreased the activity.

<table>
<thead>
<tr>
<th>TABLE II</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>PURIFICATION OF GLYOXALASE I FROM MONKEY INTESTINAL MUCOSA</strong></td>
</tr>
<tr>
<td>Step</td>
</tr>
<tr>
<td>---</td>
</tr>
<tr>
<td>1</td>
</tr>
<tr>
<td>2</td>
</tr>
<tr>
<td>3</td>
</tr>
<tr>
<td>4</td>
</tr>
<tr>
<td>5</td>
</tr>
</tbody>
</table>
Effect of various anions on native enzyme
Effect of different concentrations of various anions ranging from 1 to 10 mM were also studied. It was found that acetate, carbonate, fluoride, phosphate and sulphate had no significant effect, whereas 10 mM nitrate decreased the activity by 20%, and 5 mM iodide inhibited the activity completely.

Reactivation of metal free glyoxalase I by divalent cations
Apo-glyoxalase I was essentially inactive catalytically, and the activity was restored by different divalent cations. 80% of activity was restored in the presence of 1 mM Co²⁺, and 1 mM Mg²⁺, Mn²⁺ and Ca²⁺ restored 60, 35 and 25% activity, respectively. 25% of activity was restored by Zn²⁺ at 0.1 mM concentration.

Effect of sulfhydryl group reagents
Sulphydryl group reagents such as N-ethylmaleimide, p-hydroxymercuribenzoate, iodoacetate and iodoacetamide had no significant effect on glyoxalase I.

Active-site modification
Modification by N-acetylimidazole and tetranitromethane. The activity of the enzyme was decreased when it was treated with N-acetylimidazole (Table IV). The activity was restored completely when the modified enzyme was treated with hydroxylamine. When the enzyme was preincubated with 2 mM S-hexylglutathione there was no loss of activity by N-acetylimidazole (Table V). After modification with N-acetylimidazole, the modified enzyme showed decrease in absorbance at 278 nm.

Tetranitromethane also decreased the activity (Table IV). There was no decrease in activity when the enzyme was preincubated with 5 mM S-hexylglutathione (Table V). This indicated that tyrosine is involved in the active site.

Modification by N-bromosuccinimide and HNBB
N-Bromosuccinimide inactivated the enzyme (Table IV). Pretreatment of the enzyme with 2 mM S-hexylglutathione prevented loss of activity (Table V). HNBB also inhibited the enzyme activity and 100% inhibition was observed at 10 mM HNBB (Table IV). When the enzyme was pre-

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TABLE III
MOLECULAR AND KINETIC PROPERTIES OF GLYOXALASE I PURIFIED FROM MONKEY INTESTINAL MUCOSA

<table>
<thead>
<tr>
<th>Property</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molecular weight</td>
<td>48,000</td>
</tr>
<tr>
<td>Number of subunits</td>
<td>2</td>
</tr>
<tr>
<td>pH optimum of activity</td>
<td>Constant between 6.0 and 7.0</td>
</tr>
<tr>
<td>Specific activity (measured with methylglyoxal as substrate)</td>
<td>925 µmol/min per mg protein</td>
</tr>
<tr>
<td>Apparent $K_m$ for methylglyoxal</td>
<td>0.5 mM</td>
</tr>
<tr>
<td>Apparent $K_i$ for S-hexylglutathione</td>
<td>0.035 mM</td>
</tr>
<tr>
<td>Protein modifying agent</td>
<td>Control</td>
</tr>
<tr>
<td>------------------------</td>
<td>---------</td>
</tr>
<tr>
<td>N-Acetilidiamidazolo</td>
<td>100</td>
</tr>
<tr>
<td>Tetranitromethane</td>
<td>100</td>
</tr>
<tr>
<td>N-Bromosuccinimide</td>
<td>100</td>
</tr>
<tr>
<td>HNBB</td>
<td>100</td>
</tr>
<tr>
<td>TNBS</td>
<td>100</td>
</tr>
<tr>
<td>Pyridoxal phosphate</td>
<td>100</td>
</tr>
<tr>
<td>EDC</td>
<td>100</td>
</tr>
<tr>
<td>DPC</td>
<td>100</td>
</tr>
<tr>
<td>N-Ethylmaleimide</td>
<td>100</td>
</tr>
<tr>
<td>p-Hydroxymercaptir-</td>
<td>100</td>
</tr>
<tr>
<td>benzoate</td>
<td></td>
</tr>
</tbody>
</table>

* n.a., no activity.

Treated with 2 mM S-hexylglutathione it was protected to the extent of 80% from HNBB (Table V). This showed that tryptophan is part of the active site of the enzyme.

**Modification by TNBS and pyridoxal phosphate.** Treatment of the enzyme with different concentrations of TNBS resulted in a decrease in activity (Table IV). The enzyme was protected to the extent of 90% when it was preincubated with 2 mM S-hexylglutathione (Table V). The activity was not restored when modified enzyme was treated with hydroxylamine. Pyridoxal phosphate was also found to decrease the enzyme activity at different concentrations (Table IV). Complete protection of the enzyme from inactivation by pyridoxal phosphate was achieved by pretreatment with 2 mM S-hexylglutathione (Table V), indicating the involvement of the amino group in the active site.

**Modification by EDC.** The activity of the enzyme was diminished by this carboxyl group reagent, and 95% inhibition was observed in presence of 100 mM EDC (Table IV). When the modified enzyme was treated with hydroxylamine there was no restoration of activity. The enzyme was protected to the extent of 95% by 2 mM S-hexylglutathione (Table V). This showed that either glutamic or aspartic acid is involved in the active site.

**Modification by DPC.** The enzyme was inactivated completely by 5 mM DPC (Table IV). Pretreatment of the enzyme with 2 mM S-hexylglutathione protected the enzyme to the extent of 70% (Table V). Activity of the enzyme lost by DPC could not be restored by hydroxylamine treatment. It was also observed that there was no absorbance at 242 nm when the enzyme was subjected to spectral study after its treatment with DPC.

**Table V**

<table>
<thead>
<tr>
<th>Modifying reagent</th>
<th>Concentration (mM)</th>
<th>Activity without pretreatment with hexylglutathione (%)</th>
<th>Activity after treatment with 2/5 mM S-hexylglutathione (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-Acetilidiamidazolo</td>
<td>100</td>
<td>10</td>
<td>95</td>
</tr>
<tr>
<td>Tetranitromethane</td>
<td>1</td>
<td>50</td>
<td>100</td>
</tr>
<tr>
<td>N-Bromosuccinimide</td>
<td>0.025</td>
<td>33</td>
<td>100</td>
</tr>
<tr>
<td>HNBB</td>
<td>5</td>
<td>15</td>
<td>80</td>
</tr>
<tr>
<td>TNBS</td>
<td>10</td>
<td>27</td>
<td>90</td>
</tr>
<tr>
<td>Pyridoxal phosphate</td>
<td>4</td>
<td>38</td>
<td>100</td>
</tr>
<tr>
<td>EDC</td>
<td>100</td>
<td>5</td>
<td>95</td>
</tr>
<tr>
<td>DPC</td>
<td>5</td>
<td>0</td>
<td>70</td>
</tr>
<tr>
<td>N-Ethylmaleimide</td>
<td>10</td>
<td>70</td>
<td>n.d.</td>
</tr>
<tr>
<td>p-Hydroxymercap-</td>
<td>2</td>
<td>96</td>
<td>n.d.</td>
</tr>
<tr>
<td>benzoate</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Activity of the enzyme without modifying reagent is 100%. n.d., not done.
There was no significant change in activity of the enzyme by sulphhydryl group reagents such as \( p \)-hydroxycarbonyl benzilic acid and \( N \)-ethylmaleimide (Table IV).

**Discussion**

Glyoxalase I was purified using the affinity chromatographic method, and the purified enzyme was homogenous, as revealed by the silver staining procedure which is much more sensitive than the Coomassie Brilliant Blue staining method [19]. S-Hexylglutathione-Sepharose affinity chromatography gave 300-fold purification in a single step. Approx. 200 units of the enzyme could be loaded on to 1 ml of this column which could be eluted with 3 mM S-hexylglutathione. This affinity gel was used repeatedly without any loss in capacity by regenerating the gel with 3 M KCl. Use of this affinity matrix was reported earlier by Aronson et al. [9,30].

Molecular weight of glyoxalase I reported for other mammalian sources ranges from 42,000 to 52,000 [21,25–28] and the molecular weight of the glyoxalase I from intestinal mucosa is well within this range. Glyoxalase I from mammal and yeast has been shown to contain tightly bound zinc which is essential for the activity of the enzyme [15,29]. The metal ion can be removed by dialysis against EDTA and the apo-enzyme obtained is inactive catalytically. Restoration of activity of mammalian apo-glyoxalase I has been achieved by the addition of metal ions such as zinc, magnesium and manganese [15,27,29], whereas the activity of yeast apo-glyoxalase I could not be restored [15,30]. The present study has shown that the apo-glyoxalase I from monkey intestinal mucosa could also be reactivated with different metal ions such as \( Co^{2+} \), \( Mg^{2+} \), \( Mn^{2+} \) and \( Zn^{2+} \) and the maximum activity of 80% was restored with \( Co^{2+} \). This observation is in agreement with an earlier report on sheep liver glyoxalase I by Uotila and Koivusalo [21].

**Active-site modification**

Both \( N \)-acetylimidazole and tetranitromethane were used as modifying reagents for tyrosine [23,31] and it was found that the activity was inhibited by these reagents. A progressive fall in absorbance at 278 nm was also observed after modification with \( N \)-acetylimidazol. The activity of the enzyme was restored when the modified enzyme was treated with hydroxylamine [23]. \( N \)-Acetylimidazole can also acylate lysine or histidine residues, but \( N \)-acetyllysine cannot be decarboxylated by hydroxylamine and \( N \)-acetylhistidine is unstable, so acylation of histidine is reversible [32,33]. In our experiments we observed that the loss of activity was stable and the activity was not restored by hydroxylamine treatment indicating that the loss of activity was due to tyrosine residue modification. All these observations indicate the existence of important tyrosine residues.

HNBB was used to modify tryptophan residues and observed that the enzyme was inactivated. Besides tryptophan, cysteine and tyrosine can also be modified by this reagent but under mild conditions and acidic pH, this reagent appears to be completely specific to tyrosine [34]. Hence, loss of activity by HNBB might be due to tryptophan modification. This was further supported by the fact that the enzyme was also inhibited by another tryptophan modifying reagent, \( N \)-bromosuccinimide.

Trinitrobenzene sulfonate and pyridoxal phosphate were used to modify lysine residues. TNBS inhibited the enzyme markedly and the activity was not restored with hydroxylamine, suggesting that the inactivation was not due to a tyrosine residue. Pyridoxal phosphate, another amino-group-modifying reagent, also inactivated the enzyme. This result provides further support for the importance of lysine residues for the activity of the enzyme.

The enzyme was inactivated by EDC, a carboxylic-group-modifying reagent. The activity was not restored when the modified enzyme was treated with hydroxylamine, indicating non-involvement of tyrosine residue in the inactivation [35]. This result indicates the importance of glutamic acid/aspartic acid residues for the activity of the enzyme.

Diethyl pyrocarbonate was used to check whether the histidine residue is important for the activity. We found that the enzyme lost its activity completely by DPC. The activity was not restored when the DPC-treated enzyme was treated with
hydroxylamine. This result indicates that the loss of activity was not due to the histidine residue because histidine modification with DPC is reversible [36]. Loss of activity might be due to tyrosine or lysine residues, because modification of these residues is irreversible [35]. When the modified enzyme was subjected to spectral study at 242 nm, which is characteristic of N-carbethoxyhistidine [24], there was no change in absorbance. This result ruled out histidine modification by DPC. However, we observed a change in absorbance at 278 nm, indicating possible involvement of tyrosine modification by DPC [23]. All these observations indicate that the histidine residue was not affected by DPC. The enzyme was also not affected by sulphhydril reagents such as N-ethylmaleimide and p-hydroxymercuribenzoate.

It was observed that the rate of inactivation by all modifying reagents was reduced markedly, or abolished completely, when the enzyme was preincubated with S-hexylglutathione. This result indicated that the loss of activity was due to modification of amino-acid residues at the active site of the enzyme.

Importance of tyrosine residues in yeast glyoxalase I and lysine residues in mammalian glyoxalase I has already been reported [37,38]. These reports show the possible involvement of some of the amino-acid residues in the active site of glyoxalase I from a single source. The present study has shown that tyrosine, tryptophan, lysine and glutamic acid/aspartic acid residues may be important for the activity of glyoxalase I from monkey intestinal mucosa.

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