Probing the active site of glyoxalase I from human erythrocytes by use of the strong reversible inhibitor S-p-bromobenzylglutathione and metal substitutions

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Glyoxalase I from human erythrocytes was studied by use of the strong reversible competitive inhibitor S-p-bromobenzylglutathione. Replacements of cobalt, manganese and magnesium for the essential zinc in the enzyme were made by a new procedure involving 10% methanol as a stabilizer of the enzyme. The $K_m$ value for the adduct of methylglyoxal and glutathione was essentially unchanged by the metal substitutions, whereas the inhibition constant for S-p-bromobenzylglutathione increased from 0.08 $\mu\text{M}$ for the Zn-containing enzyme to 1.3, 1.7 and 2.4 $\mu\text{M}$ for Co-, Mn- and Mg-glyoxalase I respectively. Binding of the inhibitor to the enzyme caused quenching of the tryptophan fluorescence of the protein, from which the binding parameters could be determined by the use of non-linear regression analysis. The highest dissociation constant was obtained for apoenzyme (6.9 $\mu\text{M}$). The identity of the corresponding kinetic and binding parameters of the native enzyme and the Zn$^{2+}$-re-activated apoenzyme and the clear differences from the parameters of the other metal-substituted enzyme forms give strong support to the previous identification of zinc as the natural metal cofactor of glyoxalase I. Binding to apoenzyme was also shown by the use of S-p-bromobenzylglutathione as a ligand in affinity chromatography and as a protector in chemical modification experiments. The tryptophan-modifying reagent 2-hydroxy-5-nitrobenzyl bromide caused up to 85% inactivation of the enzyme. After blocking of the thiol groups (about 8 per enzyme molecule) 6.1 2-hydroxy-5-nitrobenzyl groups were incorporated. Inclusion of S-p-bromobenzylglutathione with the modifying reagent preserved the catalytic activity of the enzyme completely and decreased the number of modified residues to 4.4 per enzyme molecule. The findings indicate the presence of one tryptophan residue in the active centre of each of the two subunits of the enzyme. Thiol groups appear not to be essential for catalytic activity. The presence of at least two categories of tryptophan residues in the protein was also shown by quenching of the fluorescence by KI.

Little is known about the biological function of the glyoxalase system. It has been suggested that it may serve in the detoxication of 2-oxo aldehydes, which may be formed from various endogenous metabolites as well as from xenobiotics (for a review see Mannervik, 1980). Another function, put forward by Szent-Györgyi (1976, and papers cited therein), may be related to a suggested role of methylglyoxal (or other 2-oxo aldehydes) in cellular regulation, especially cell proliferation. The proposal of a regulatory role of methylglyoxal is supported by the finding that this compound may modulate the transcription of capped messenger RNA by binding to 7-methylguanine (Kozarich & Deegan, 1979). Glyoxalase I, which catalyses the transformation of a 2-oxo aldehyde and reduced glutathione to an S-2-hydroxyacylglutathione, is also incompletely characterized. A comparison of the known properties of the enzyme from microbial and mammalian sources has been made (Marmstål et al., 1979), but, even if some of the structural and physical characteristics have been unravelled, little is known about the catalytic mechanism and the chemical groups effecting catalysis.

The present study has been performed on glyoxalase I from human erythrocytes. Like the enzyme...
from other mammalian sources, but contrary to the monomeric yeast enzyme, it is a dimer composed of two identical or nearly identical subunits (cf. Marmstål et al., 1979). Glyoxalase I appears to contain one essential Zn atom per subunit, i.e. 2 Zn atoms per molecule for the mammalian enzyme and 1 Zn atom per molecule for the yeast enzyme (Aronsson et al., 1978). However, the role of zinc in the catalytic function of the enzyme and the possible localization of zinc in the active site have, so far, remained unsettled. Apart from zinc, no other chemical group has with certainty been identified as essential for catalytic activity, even though some amino-group-modifying reagents inactive glyoxalase I from mammals as well as from yeast (Mannervik et al., 1975). The purpose of the present investigation was to clarify the role of the essential metal in catalysis and in binding of the competitive inhibitor S-p-bromobenzylglutathione, and to identify any amino acid residue that would be of importance for the function of glyoxalase I from human erythrocytes.

Experimental

Materials

Glyoxalase I from human erythrocytes was purified as described by Aronsson et al. (1979). Form I of the enzyme (Aronsson et al., 1979) was used in the experiments reported, but results from additional studies, in which a mixture of the three isoenzymes was employed, did not differ from the results obtained with the pure form I. S-p-Bromobenzylglutathione was synthesized by method A of Vince et al. (1971). This glutathione derivative was immobilized on epoxy-activated Sepharose 6B by covalent linkage to the amino group of the glutamic residue, as described previously for a similar derivative (Aronsson et al., 1978). All other materials used were commercial products of the highest purity available. Dialysis tubing and glassware were treated as previously described (Aronsson & Mannervik, 1977; Aronsson et al., 1978).

Assay of glyoxalase I activity

The spectrophotometric method of Racker (1951) was used under the conditions detailed by Marmstål et al. (1979). Protein concentration was measured by a micro biuret method (Goa, 1953).

Preparation of apoenzyme and metal-substituted glyoxalase I

Apoenzyme was prepared by a method modified from that described by Uotila & Koivusalo (1975), a significant difference being the inclusion of 10% (v/v) methanol, which was found to stabilize the enzyme against irreversible inactivation (Sellin et al., 1980). Native enzyme (1–2 mg/ml) was dialysed at 4°C against 1 mM-EDTA in 50 mM-imidazole/HCl buffer, pH 6.8, containing 10% methanol.

For reconstitution of active enzyme an excess (about 10-fold molar excess) of Zn²⁺, Mn²⁺, Mg²⁺ or Co²⁺ was added to apoenzyme in 10 mM-Tris/HCl buffer, pH 7.8, containing 10% methanol. Excess of metal ions was removed by dialysis for 3 x 3 h against 10 mM-Tris/HCl buffer, pH 7.8, containing 10% methanol at 4°C. The amount of metal bound to the enzyme was measured by atomic-absorption spectrometry, and was for all metalloenzymes found to be close to 2 g-atoms/mmol of enzyme (i.e. 1 g-atom/mol of subunit). In all the work involving preparation of apoenzyme and re-activation with metal ions, the buffers were as described by Willard et al. (1969), treated with Chelex 100 or extracted with dithizone in carbon tetrachloride (7 mg/l). The latter treatment was employed for concentrated salt solutions. Whenever possible, reaction vessels of polyethylene were used instead of glassware.

Kinetic studies

Steady-state kinetic studies of the enzymic reaction involving methylglyoxal or phenylglyoxal and glutathione were performed under the experimental conditions described previously (Marmstål et al., 1979). The hemimercaptil adduct was treated as substrate, and the total concentrations of 2-oxo aldehyde and glutathione were chosen to give constant concentrations of free glutathione when the adduct concentration was varied. The kinetic data were treated as described by Mannervik et al. (1973) and analysed by non-linear regression methods (Bartfai & Mannervik, 1972; Mannervik & Bartfai, 1973).

Fluorescence studies

Fluorescence was measured at 22°C on a Jasco FP-4 spectrofluorimeter with rectangular 1 cm quartz cells. The scale of wavelength of the emission monochromator was calibrated by use of a mercury lamp. The fluorescence spectra were not corrected for the wavelength-dependence of the response of the photomultiplier (Freifelder, 1976). The relative quantum yield (Q/Q₀) was determined by comparison with the fluorescence of L-tryptophan in aqueous solution by using the relation:

\[ \frac{Q}{Q_0} = \frac{I \times \lambda \times A_0 \times \text{antilog}(A_0/2)}{I_0 \times \lambda_0 \times A \times \text{antilog}(A/2)} \]

where I is the fluorescence intensity at the wavelength (λ) giving maximal emission and A is the absorbance of the substance at the wavelength used for excitation (Kirby & Steiner, 1970). The I₀, λ₀ and A₀ parameters refer to the standard (tryptophan). Evaluation of the titration curves obtained by
fluorescence quenching caused by addition of ligand was made by non-linear regression. The equation

$$F_i = F_0 - R \cdot (F_0 - F_\infty)$$  \hspace{1cm} (2)

was fitted to the measured fluorescence intensities ($F_i$) determined at different concentrations of the ligand ([LI]) used in the titration. $F_0$ and $F_\infty$ are the fluorescence intensities at zero and saturating concentrations of ligand respectively; $R$ ($0 < R < 1$) is the relative change in fluorescence. For hyperbolic binding to an enzyme having $n$ equivalent sites an explicit expression for $R$ can be obtained from the equilibrium equation:

$$R = \frac{[L]_0 + K_d + n[E]_0}{2n[E]_0} - \left( \frac{[L]_0 + K_d + n[E]_0}{2n[E]_0} \right) \begin{array}{c} \frac{[L]_0}{2n[E]_0} \\
\frac{n[E]_0}{2n[E]_0} \end{array} \right)^{\frac{1}{n}}$$  \hspace{1cm} (3)

where $[L]_0$ is the total concentration of ligand added, $K_d$ is the dissociation constant for binding of L to the enzyme, E, and $n[E]_0$ is the concentration of binding sites. This explicit formulation of $R$ (eqn. 3) was used in eqn. (2) when the latter expression was fitted in the regression analysis. A Gauss–Newton non-linear regression program BMDPAR (University of California, Los Angeles, CA, U.S.A.) was employed; $[L]_0$ was treated as the independent variable, and $F_0$, $F_\infty$, $K_d$ and $n[E]_0$ were the parameters calculated. ([E]_0 is known, which allows calculation of the number of binding sites, $n$). Unweighted regression was used, because the total variation of the dependent variable ($F_i$) was ≤15% and the experimental error could accordingly be considered as constant. The adequacy of the assumption of non-co-operative binding was verified by use of the plot described by Stockell (1959). This plot gives a straight line for simple hyperbolic binding, and no significant deviations from linearity were detected in the present investigation.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>$K_m$ (mM)</th>
<th>$K_i$ (µM)</th>
<th>$K_d$ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native</td>
<td>0.071 ± 0.007</td>
<td>0.077 ± 0.007</td>
<td>0.15 ± 0.03</td>
</tr>
<tr>
<td>Zn$^{2+}$-substituted</td>
<td>0.069 ± 0.005</td>
<td>0.080 ± 0.006</td>
<td>0.17 ± 0.01</td>
</tr>
<tr>
<td>Co$^{2+}$-substituted</td>
<td>0.065 ± 0.006</td>
<td>1.3 ± 0.1</td>
<td>0.43 ± 0.05</td>
</tr>
<tr>
<td>Mn$^{2+}$-substituted</td>
<td>0.050 ± 0.004</td>
<td>1.7 ± 0.2</td>
<td>0.54 ± 0.09</td>
</tr>
<tr>
<td>Mg$^{2+}$-substituted</td>
<td>0.097 ± 0.008</td>
<td>2.4 ± 0.3</td>
<td>1.1 ± 0.3</td>
</tr>
<tr>
<td>Apo-enzyme</td>
<td>—</td>
<td>—</td>
<td>6.9 ± 1.6</td>
</tr>
</tbody>
</table>

* Only one kinetic-data set was used.

Results

Steady-state kinetics

The steady-state kinetics of glyoxalase I from human erythrocytes were found to follow the same pattern as previously described for the enzyme from yeast (Mannervik et al., 1974), pig erythrocytes (Mannervik et al., 1973), and rat liver (Marmstål & Mannervik, 1979a). Thus the hemimercaptal adduct of glutathione and a 2-oxo aldehyde can be treated as the substrate and free glutathione as a non-linear competitive inhibitor versus the adduct. The 2-oxo aldehydes investigated were methylglyoxal and phenylglyoxal. If the concentration of the hemi-
kinetics. The 2-oxo aldehyde used was methylglyoxal. No significant differences between the $K_m$ values were discerned.

The glutathione analogue $S\cdot p$-bromobenzylglutathione was found to be a strong reversible competitive inhibitor of glyoxalase I from human erythrocytes (Fig. 1). The plot shows that the inhibition was approximately linear with respect to inhibitor concentration in the range investigated. This inhibition had previously been characterized by an inhibition constant and dissociation constant for binding to glyoxalase I from yeast of about 2$\mu$m (Marmstål & Mannervik, 1979b), whereas the enzyme from pig erythrocytes was inhibited less strongly (Mannervik, 1974). The human enzyme was considerably more sensitive to the inhibitor ($K_i = 0.08\mu$m) than was the yeast enzyme. In contrast with the lack of significant effect on the $K_m$ value, the substitution of some bivalent metals for zinc in the enzyme caused a significant increase in the inhibition constant. This finding shows that the essential metal affects the binding of the glutathione derivative. The demonstration that apoenzyme reactivated by Zn$^{2+}$ exhibits the same inhibition constant as the native enzyme provides strong additional evidence for the identification of zinc as the essential metal of glyoxalase I (Aronsson et al., 1978).

**Fluorescence of glyoxalase I**

Glyoxalase I contains 5 tryptophan residues per molecule, according to amino acid analysis (Marmstål et al., 1979). Since the two subunits appear to be identical in the isoenzyme (form 1) used in the present study (Aronsson et al., 1979), each subunit contains 2 or 3 tryptophan residues. The intrinsic fluorescence of glyoxalase I is consistent with tryptophan residues acting as fluorophores. The excitation spectrum is similar to the absorption spectrum, indicating negligible energy transfer from tyrosine to tryptophan residues. Fig. 2 shows fluorescence emission spectra of glyoxalase I obtained on excitation at 280nm. The emission maximum of the native enzyme was at 344 nm when the enzyme was excited at 280 or 270nm and at 347 nm when excited at 295 nm. The relative quantum yield was determined at 22°C by use of free tryptophan in aqueous solution as a reference (Kirby & Steiner, 1970). At the excitation wavelengths of 270, 280 and 295 nm it was found to be 0.65, 0.68 and 1.13, similar to the corresponding values of human serum albumin: 0.65, 0.69 and 1.07 (de Lauder & Wahl, 1971).

The fluorescence spectrum of the apoenzyme was very similar to that of the holoenzyme, but the emission maximum was blue-shifted by 3 nm to 341 nm (Fig. 2a). Addition of 4$\mu$m-Zn$^{2+}$ to 0.2$\mu$m-apoenzyme, which restored maximal enzymic activity, shifted the emission maximum back to 344 nm and increased the fluorescence yield by 4%. Use of other bivalent cations such as Mg$^{2+}$, Mn$^{2+}$ and Co$^{2+}$, which all restore enzymic activity when added to apo-glyoxalase I, gave the same shift of the wavelength of the emission maximum. Mg$^{2+}$ and Mn$^{2+}$, like Zn$^{2+}$, caused a small increase in fluorescence yield, whereas Co$^{2+}$ effected a quenching of a similar magnitude (5%). The increase of fluorescence caused by Mn$^{2+}$ appeared to be
somewhat less than the corresponding effects of Zn$^{2+}$ and Mg$^{2+}$.

**Binding of S-p-bromobenzylglutathione to glyoxalase I**

Binding of the competitive inhibitor S-p-bromobenzylglutathione to native glyoxalase I caused a partial quenching of the fluorescence of the enzyme (Fig. 2b). Fig. 3 shows a titration curve obtained by adding increasing concentrations of the inhibitor to the enzyme. The maximal quenching reached was about 10%. Analysis of this titration curve by non-linear regression analysis gave a dissociation constant of $0.15 \pm 0.03 \mu M$ and a binding stoichiometry of $1.60 \pm 0.16$ mol of ligand/mol of enzyme.

Enzyme that had been re-activated by addition of bivalent cations to apo-glyoxalase I also exhibited quenching of the intrinsic fluorescence on binding of S-p-bromobenzylglutathione. Table 1 shows the dissociation constants determined from titration curves similar to that in Fig. 3. It is evident that the dissociation constants reflect the values of the inhibition constants determined from the steady-state kinetics. In particular, it is noteworthy that the native zinc-containing enzyme has a significantly lower dissociation constant than the enzyme re-activated with other bivalent metal ions.
The fluorescence of the apoenzyme was quenched to the same extent as that of the holoenzyme by addition of \( S-p \)-bromobenzylglutathione, but higher concentrations were required to reach saturation. It could be excluded that the observed effect was due to inadvertent re-activation of the enzyme by metal ions in the inhibitor solution, because the maximum of the emission spectrum was not changed by addition of the inhibitor. Addition of \( \text{Zn}^{2+} \) to a cuvette containing apoenzyme and \( S-p \)-bromobenzylglutathione gave rise to a shift of the emission maximum from 341 to 344 nm, the wavelength expected for native glyoxalase I. These findings demonstrate that the inhibitor is bound less tightly to the apoenzyme than to the holoenzyme. Table 1 shows that the dissociation constant is considerably higher for the apoenzyme. Results obtained by use of equilibrium dialysis (similar to those obtained by Marmståhl & Mannervik, 1979b) also show that the affinity of the apoenzyme for the inhibitor is less than that of the holoenzyme. Owing to the low specific radioactivity of the ligand available (cf. Marmståhl & Mannervik, 1979b), accurate results could not be obtained for human glyoxalase I, which has a more than 20-fold higher affinity than the yeast enzyme.

**Accessibility of tryptophan residues**

The accessibility of the tryptophan residues of native glyoxalase I was probed by measuring the quenching of the fluorescence of these residues by various concentrations of KI in the range 0.05–1.0 M. The results of these experiments were analysed in the Stern–Volmer plot and some of its transformations (cf. Lehrer & Leavis, 1978), and it could clearly be established that the dependence of \( F_0/F \) on the concentration of KI deviates from linearity. This finding demonstrates that at least two classes of tryptophan residues are present in the glyoxalase I molecule, which are characterized by different availability to \( I^- \) ions. The quenching afforded by KI was stronger than that obtained with \( S-p \)-bromobenzylglutathione; at 1 M-KI more than 50% quenching was registered. The effect of \( S-p \)-bromobenzylglutathione was not eliminated by KI (and vice versa). Thus enzyme fluorescence quenched by KI could be further quenched by up to about 8% of the remaining fluorescence by addition of \( S-p \)-bromobenzylglutathione.

**Inactivation of glyoxalase I by a tryptophan-modifying reagent**

Native glyoxalase I (5 \( \mu \)M) was partially inactivated by treatment at 0°C with 7.5 mM-2-hydroxy-5-nitrobenzyl bromide (Fig. 4). Maximal inactivation achieved was 85%, even after prolonged times of incubation (results not shown). The inactivated enzyme contained about 8 mol of 2-hydroxy-5-nitrobenzyl groups/mol of enzyme, i.e. 4 mol/mol of subunit, as determined spectrophotometrically at 410 nm (Barman & Koshland, 1967). The competitive inhibitor \( S-p \)-bromobenzylglutathione at 1 mM concentration provided partial protection against the inactivator (Fig. 4), whereas the same concentration of \( S \)-methylglutathione afforded only a minor protection. The latter compound is a poor inhibitor of the enzyme and was not expected to protect by competing with the inactivator for the enzyme. It was used to evaluate the possibility that the amino group of the glutathione derivatives reacted with 2-hydroxy-5-nitrobenzyl bromide. Since no significant protection was obtained with \( S \)-methylglutathione, the protection observed with \( S-p \)-bromobenzylglutathione has to be ascribed to binding to the enzyme and not to a trivial deactivation of 2-hydroxy-5-nitrobenzyl bromide. The
apoenzyme was also inactivated by 2-hydroxy-5-nitrobenzyl bromide. The effect was evaluated by observing the loss of activity of apoenzyme that had been re-activated by 10 mM-Mg²⁺ after treatment with the reagent. This metal ion was used rather than Zn²⁺ because re-activation can be accomplished most rapidly by addition of a large excess of Mg²⁺. Also, the apoenzyme was partly protected against inactivation by S-p-bromobenzylglutathione.

It is known that 2-hydroxy-5-nitrobenzyl bromide may react with thiol groups in addition to tryptophan residues (Horton & Koshland, 1965). The possibility that the inactivation of glyoxalase I by the reagent was due to modification of thiol groups was therefore investigated. Native glyoxalase I from human erythrocytes contained 8–9 thiol groups/molecule of enzyme, as determined under denaturing conditions with 5,5'-dithiobis-(2-nitrobenzoate) as described by Glazer et al. (1975). (Analyses made in the absence of denaturant did not give significantly different values.) A solution of native enzyme (31 μM) in 10 mM-Tris/HCl buffer, pH 7.8, containing 10% methanol was treated with 1.3 mM-p-mercuribenzoate. No significant inhibition was obtained (1%). After dialysis against the same buffer only 0.05 thiol group/molecule was detected. This enzyme was allowed to react at 6.2 μM concentration with 6 mM-2-hydroxy-5-nitrobenzyl bromide in 0.1 m-Mops (4-morpholinepropanesulfonic acid)/NaOH buffer, pH 7.5, in the absence and in the presence of the protector S-p-bromobenzylglutathione (2 mM) in the same buffer. After 45 min the different samples were dialysed and the numbers of tryptophan residues per molecule determined (Barman & Koshland, 1967). The samples contained 6.1 and 4.4 modified tryptophan residues/molecule of enzyme after incubation in the absence and in the presence respectively of the protector. In neither sample were any thiol groups detected. Thus it appears that 1.7 tryptophan residues/molecule are protected against modification with 2-hydroxy-5-nitrobenzyl bromide. It is assumed that the tryptophan residues are monosubstituted and that no other residues are modified.

In order to determine if any other amino acid residues were modified, the contents of all amino acids except tryptophan and half-cystine were determined on an amino acid analyser after acid hydrolysis. (The analysis was performed by Dr. Hans Jörnvall, Karolinska Institutet, Stockholm, Sweden.) The native enzyme was compared with two preparations of modified enzyme prepared in the presence and in the absence of S-p-bromobenzylglutathione, but no difference in the amino acid compositions could be noted. The enzyme was inhibited by 47% in the absence of protector, but retained full catalytic activity after modification in the presence of protector. In a parallel experiment enzyme that had not been pretreated with p-mercuribenzoate was treated with 2-hydroxy-5-nitrobenzyl bromide in the absence and in the presence of protector. In the absence of protector 57% inhibition and 8.7 2-hydroxy-5-nitrobenzyl groups/molecule were obtained; in the presence of protector the corresponding values were 11% and 4.4.

**Binding of apoenzyme and holoenzyme to immobilized S-p-bromobenzylglutathione**

A chromatography column containing S-p-bromobenzylglutathione immobilized on epoxy-activated Sepharose 6B had a high affinity for glyoxalase I. It was found also that the apoenzyme was bound to this matrix under the same conditions (10 mM-Tris/HCl buffer, pH 7.8). The adsorbed enzyme could be almost quantitatively eluted by 6 mM-S-p-bromobenzylglutathione + 10 mM-glutathione in the same buffer. The apoenzyme was re-activated with 10 mM-Mg²⁺ before assay of enzymic activity. These chromatographic experiments provide further evidence that the apoenzyme has the capacity of binding the glutathione derivative.

**Discussion**

The present investigation clearly demonstrates the presence in glyoxalase I from human erythrocytes of tryptophan residues that directly or indirectly are connected with the enzymic function and the binding of a substrate analogue to the enzyme. The inhibition studies showed that S-p-bromobenzylglutathione is a strong competitive inhibitor of the human enzyme, with a more that 20-fold lower inhibition constant ($K_i = 0.08\,\mu\text{M}$) than that for the yeast enzyme ($K_i \approx 2\,\mu\text{M}$) (Marmstål & Mannervik, 1979b). The competitive effect demonstrates that the inhibitor and the substrate compete for the same enzyme form in the scheme of the enzymic reaction. The effect could arise either from competition in binding to the active site of the enzyme or from binding of the inhibitor to another site in such a way that the binding capacity of the active site is lost (e.g. by conformational changes induced by the inhibitor). The structural similarity between substrate and inhibitor and the stoichiometry of binding (about 1 inhibitor molecule/subunit) gives credence to the first explanation. If the competitive inhibitor were to bind at a site distinct from the active site it would be expected to bind, in addition, to the active site, owing to its structural similarity to the substrate. In such a case more than 1 inhibitor molecule/subunit would be expected to bind under saturating conditions. However, no evidence for a higher binding stoichiometry was found. It could be argued that it is not known if both subunits of the dimeric mammalian glyoxalase I are active and that one of the subunits...
may bind the substrate and the other one the inhibitor, but the tryptic-digest peptide 'maps', which indicate identical primary structures of the subunits (Marmstål et al., 1979), and the binding data for the monomeric yeast enzyme (Marmstål & Mannervik, 1979b) speak against this possibility. We therefore conclude that \( S-p \)-bromobenzylglutathione binds to the active site of glyoxalase I. Thus the protection afforded by this ligand, which shows that approximately 2 tryptophan residues/molecule can be shielded from modification with 2-hydroxy-5-nitrobenzyl bromide, indicates that these residues are at or close to the active site of the enzyme. It seems probable that the enzyme has two identical active sites, each containing one tryptophan residue. The evidence is the binding stoichiometry of the glutathione derivative and the similarity of the amino acid compositions of the two subunits (Marmstål et al., 1979). An alternative explanation would be that the ligand induces a conformational change that makes the tryptophan residues inaccessible. This alternative appears to be ruled out, since the same ligand does not make the tryptophan residues less available to collision-quenching by KI.

The experiments involving quenching of the tryptophan fluorescence by KI also showed that readily and less readily accessible tryptophan residues are present. The finding that \( S-p \)-bromobenzylglutathione gave further quenching in the presence of KI indicates that the residues at or close to the active site are buried in the interior of the enzyme molecule.

The effect on the tryptophan fluorescence of addition of metal ions to the apoenzyme shows that the environment of tryptophan residues is perturbed. The small red-shift of the spectra indicates that at least some residues experience a somewhat more polar milieu after addition of the metal ions. However, owing to the possibility of energy transfer between tryptophan residues in the enzyme molecule and other electronic interactions, it is difficult to make specific interpretations of the results. The shift of the emission maximum of the native enzyme on increasing the wavelength of the excitation light to 295 nm indicates that different tryptophan residues are in different environments or that additional chromophores such as tyrosine are involved in absorption of energy. Since the metal has to be present to make an active enzyme, we conclude that it is located at the active site. Thus both the metal and a tryptophan residue interact with the glutathione derivative bound to the active centre.

The binding studies involving metal-substituted enzyme and \( S-p \)-bromobenzylglutathione (Table 1) show that the strength of interaction as measured by the dissociation constants is dependent on the nature of the metal in the active site. Re-activation studies have shown that various bivalent metal ions do indeed restore catalytic activity and do not activate by addition of contaminating amounts of \( \text{Zn}^{2+} \) (Sellin et al., 1980). \( \text{Mg}^{2+} \) gives as high a catalytic activity as \( \text{Zn}^{2+} \) does, but confers a poor binding affinity (Table 1). The finding of equally high activities indicates that both metals give rise to the fully active structure of the protein. Thus it appears that the effect of the different metals on the strength of binding of the glutathione derivative is not merely structural (even if there is evidence that the apoenzyme undergoes conformational changes on binding of the bivalent metal ions: A.-C. Aronsson, S. Sellin & B. Mannervik, unpublished work), but is due to direct interaction between the metal atom and the ligand. An interpretation of the \( K_m \) values cannot be made rigorously because the kinetics are not truly Michaelian (see the legend to Fig. 1). Suffice it to state that these parameters appear to be essentially insensitive to metal replacements and do not reflect the strength of binding of glutathione derivatives. Likewise, the \( K_a \) values are not unbiased in view of the simplified kinetic model used in the regression analysis; the minor differences between the \( K_i \) and \( K_d \) values for an enzyme preparation should not be regarded as established at the present stage. The important finding is that the kinetically determined \( K_i \) values, which reflect binding strength of the inhibitor, show similar differences between the different metal-substituted enzymes to those between the \( K_d \) values determined under equilibrium conditions. The fluorescence titration conducted with apoenzyme (Table 1) shows that the metal is not essential for binding of the glutathione derivative, even if the metal increases the affinity. The binding of apoenzyme to immobilized \( S-p \)-bromobenzylglutathione leads to the same conclusion. Similarly, the protection of apoenzyme against modification with 2-hydroxy-5-nitrobenzyl bromide by the ligand shows that binding takes place.

The modification studies on enzyme with blocked thiol groups gave a value of 6.1 tryptophan residues/molecule. This value is somewhat higher than the value obtained by amino acid analysis after hydrolysis (Marmstål et al., 1979), but it should be noted that, quite apart from the usual difficulties of tryptophan analyses, it was difficult to obtain accurate values of the protein content of samples of modified enzyme owing to the presence of the coloured label. The protein determinations were therefore related to proper controls processed in the absence of 2-hydroxy-5-nitrobenzyl bromide. This uncertainty does not allow a clear conclusion, but the findings seem to indicate 3 tryptophan residues/subunit. The finding of 8.7 2-hydroxy-5-nitrobenzyl groups/molecule of enzyme after treatment of native glyoxalase I, which contains free thiol groups, shows that about 2 cysteine residues/molecule may be modified by the reagent. However, the modi-
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fication of thiol groups appears not to affect the catalytic activity significantly, because aged preparations of the native enzyme, which lacked detectable thiol groups, are fully active catalytically (results not shown). A similar conclusion about the lack of importance of thiol groups for enzymic activity was previously drawn in the case of glyoxalase from pig erythrocytes (Mannervik et al., 1975). The role of the tryptophan residues is probably not essential for catalysis, since modified enzyme is still somewhat active. It is suggested that tryptophan has a function in the binding of the substrate in the active centre of the enzyme. The present investigation also indicates that the essential metal, in addition to its catalytic role, has a function in the binding of the substrate.

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