BINDING OF THE COMPETITIVE INHIBITOR *S*-(*p*-BROMOBENZYL)-GLUTATHIONE TO GLYOXALASE I FROM YEAST

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1 Introduction

Glyoxalase I (EC 4 4 1 5) catalyzes the formation of *S*-D-lactoylglutathione from glutathione (GSH) and methylglyoxal [1] and corresponding *S*-2-hydroxyacylglutathione products from alternative 2-oxoaldehydes and GSH It is generally assumed that the hemimercaptal adduct, which is formed spontaneously from the 2-oxoaldehyde and GSH, is the true substrate for the enzyme However, free GSH acts as an apparent competitive inhibitor of the enzyme [2], and when extended ranges of reactant concentrations are investigated under steady-state conditions, it has been shown that the kinetics are non-Michaelian and the inhibition by GSH nonlinear [3–5] A simple explanation of the rate-behaviour was a branching reaction scheme involving addition of GSH and 2-oxoaldehyde as an alternative pathway to that involving addition of their adduct to the enzyme [3–5] The rate-behaviour is qualitatively the same with methylglyoxal and phenylglyoxal and systematic errors which might have affected the kinetics have been excluded as an explanation of the observed kinetic patterns [6] However, pre-steady-state kinetics could not support the branching mechanism, even if it could not be disproved [7] and it is therefore essential to scrutinize alternative explanations of the rate-behaviour

Glyoxalase I from yeast, in contrast to the dimeric mammalian enzymes, is a monomeric enzyme and cooperative subunit interactions can therefore be excluded [8] However, more than one binding site for a ligand may exist on a monomer and it was therefore essential to investigate the equilibrium binding of suitable ligands to glyoxalase I from yeast This study shows that the binding of GSH to the enzyme is undetectable or negligible under the conditions used, but that the competitive inhibitor *S*-(*p*-bromobenzyl)-glutathione binds in a stoichiometry of 1 molecule/enzyme molecule with a K_d similar to the K_i determined in kinetic studies Neither GSH nor methylglyoxal had any detectable effect on the binding of the inhibitor

2 Materials and methods

Glyoxalase I from yeast was obtained from Boehringer Mannheim and was purified to homogeneity as in [8] Protein concentration was determined with a micro-biuret method [9] Labeled *S*-(*p*-bromobenzyl)-glutathione was synthesized from [*Gly*-2-^{3}H]glutathione (obtained from New England Nuclear) and *p*-bromobenzyl bromide [10] The purity of the synthesized glutathione derivative was ascertained by use of paper electrophoresis The derivative had spec act 50 mCi/mol Radioactivity of ligands was measured in 10 ml Aquasol (New England Nuclear) by liquid scintillation counting Equilibrium dialysis was made by use of MSE Dianorm equipment The membranes (Spectropor membrane tubing, Spectropor Medical Industries, Inc , Los Angeles, CA) were pretreated as in [11] The 2 compartments of a dialysis cell were each loaded with 200 μl 10 mM Tris/HCl (pH 7 8), one of them contained the enzyme (10–20 μM) and the other one contained the radioactive ligand Equilibration was achieved within 3 h at 22°C, after which time aliquots (100 μl) from the compartments were counted It was shown that 3 h was sufficient to reach

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equilibrium and that the inactivation of the enzyme was negligible under the conditions used.

3. Results

Binding studies were first made with labeled GSH, but no binding could be detected even at the highest concentrations of glyoxalase I used (20 μM). [^{3}H]GSH was varied in the range 15 μM–2 mM total conc. and a 10-fold excess of dithioerythritol was included to keep GSH in the reduced form. Next the strong inhibitor of glyoxalase I *S*-(*p*-bromobenzyl)glutathione [10] was used. Figure 1 shows that this is a competitive inhibitor versus hemimercaptal and an app. K_i = 1.8 ± 0.1 μM was determined by nonlinear regression. A similar experiment was performed with phenylglyoxal as the 2-oxoaldehyde. Also in this case *S*-(*p*-bromobenzyl)glutathione was a competitive inhibitor versus the hemimercaptal; the inhibition constant was 3.7 ± 0.5 μM. It has been shown that *S*-(*p*-bromobenzyl)glutathione is competitive also versus glutathione [4,5]; a finding indicating that hemimercaptal, GSH, and the inhibitor all bind to the same site. Figure 2 shows a Scatchard plot of the equilibrium binding of *S*-(*p*-bromobenzyl)glutathione (2.5–75 μM total conc.). The equilibrium (dissocia-

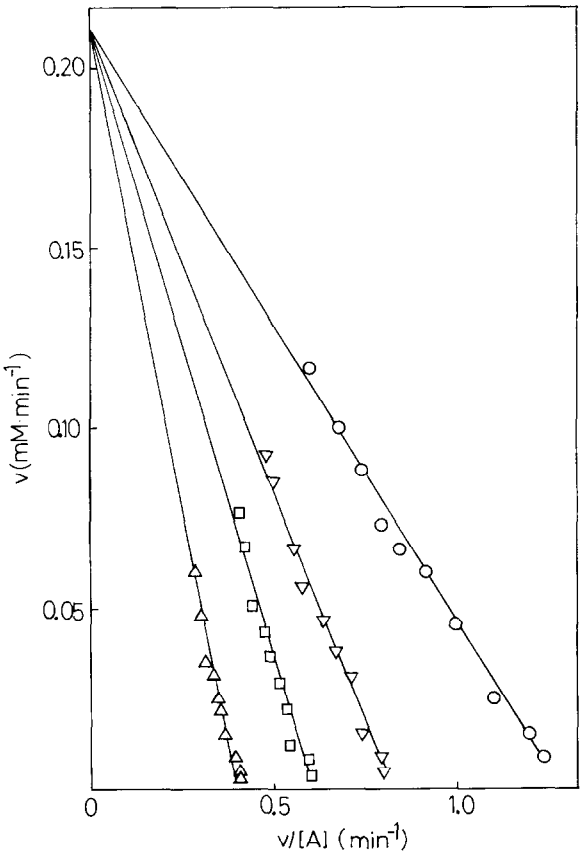

Fig.1. Inhibition of glyoxalase I from yeast by *S*-(*p*-bromobenzyl)glutathione. The assay system (30°C) contained: 10 mM Tris/HCl (pH 7.8) and various combinations of total methylglyoxal and glutathione concentrations to give a constant concentration (0.10 mM) of GSH and various concentrations (5–200 μM) of hemimercaptal adduct (A). The calculations of adduct and free glutathione concentrations were based on an equilibrium constant of 3.0 mM (cf. [3]). The inhibitor *S*-(*p*-bromobenzyl)glutathione was added in the following concentrations: zero (○); 1.0 (▽); 2.0 (□); 4.0 μM (△).

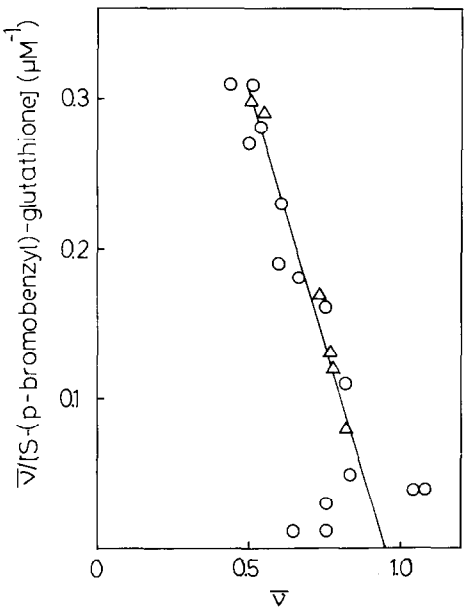

Fig.2. Equilibrium binding of *S*-(*p*-bromobenzyl)glutathione to glyoxalase I from yeast (Scatchard plot). The measurements were made by equilibrium dialysis as described in the text. The binding was determined in the absence (○) as well as in the presence (△) of 10 mM unlabeled GSH. The number of ligand molecules bound per enzyme molecule, $\bar{v}$, were calculated on the basis of a molecular weight of 32 000 for glyoxalase I from yeast [8]. The data were analyzed by use of weighted nonlinear regression; the weighting factors were based on the residuals of a provisional fit (see [12]).

tion) constant was 1 5 ± 0 1 μM as determined by non-linear regression and 3 5 μM by the non-parametric method in [13] In view of the experimental variance, the two estimates of the equilibrium constant are not significantly different from each other nor from the inhibition constants determined for the steady-state kinetics (fig 1) The binding at saturation of the enzyme was 0 95 ± 0 02 mol *S*-(*p*-bromobenzyl)glutathione/mol glyoxalase I GSH at ⩽ 100 mM and methylglyoxal at ⩽ 10 mM did not affect the binding of *S*-(*p*-bromobenzyl)glutathione when added in the equilibrium dialysis (cf , fig 2) Neither did a mixture of 2 mM GSH and 5 mM phenylglyoxal or 10 mM GSH and 10 mM methylglyoxal (which within a few minutes is enzymatically transformed to the corresponding *S*-2-hydroxyacylglutathione product) give any detectable effect However, addition of unlabeled *S*-(*p*-bromobenzyl)glutathione showed that the binding of labeled ligand was reversible and that the radioactivity bound extrapolates to nil when the concentration of the unlabeled ligand increases

4 Discussion

The equilibrium binding of *S*-(*p*-bromobenzyl)-glutathione shows that the ligand binds in a 1 1 stoichiometry to glyoxalase I from yeast with an equilibrium constant which is not significantly different from the inhibition constant determined for the same substance The competitive rate-behaviour versus GSH and hemimercaptal (fig 1, [4,5]) and the similar values of the equilibrium constant for binding and the inhibition constant indicate strongly that GSH and the two GSH derivatives all bind to the active site of the enzyme The lack of effect of GSH on the equilibrium binding of *S*-(*p*-bromobenzyl)glutathione is in contrast to the marked competitive effect of GSH on the inhibition by the same compound under steady-state conditions [4,5] This finding can be explained by the assumption that the enzyme may occur in one form under equilibrium conditions, which does not bind GSH, and mainly as another form, which does bind GSH, during catalysis Kinetic models involving different isomeric forms of an enzyme have been considered (see [14] and papers cited therein), and in the case of glyoxalase I such a model may explain also the non-Michaelian kinetics A reaction scheme of this kind requires further detailed quantitative analysis in order to be expressed in a definitive form Nevertheless, the results reported here show that under equilibrium conditions there is only one binding site on the enzyme for glutathione derivatives, and rule out the possibility that multiple binding sites might explain the rate-behaviour of glyoxalase I from yeast

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