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PHOTOAFFINITY LABELLING BY S-(p-AZIDOPHENACYL)-GLUTATHIONE OF GLYOXALASE II AND GLUTATHIONE S-TRANSFERASE

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Summary: S-(p-azidophenacyl)-glutathione, 1, is a linear competitive inhibitor at pH 7.40 of beef liver glyoxalase II with K_i = 7.96 x 10^{-4} M. On irradiation at 340 nm it covalently inhibits glyoxalase II to a level of 42 ± 5% inhibition. This photoaffinity labelling is prevented by the presence of a glyoxalase II competitive inhibitor (the hemimercaptal of glutathione and methylglyoxal). A crude preparation of sheep liver glutathione S-transferases is also irreversibly inactivated (86% ± 5% inhibition) by irradiation at 320 nm in the presence of 1.

Introduction

During evolution many functional roles have arisen for glutathione (GSH), with consequent development of a variety of glutathione-binding sites. To help explore these sites we have synthesised S-(p-azidophenacyl)-glutathione, 1, as a photoaffinity label based on the GSH structure. In a previous report, 1 was shown to covalently inhibit yeast glyoxalase I on photoactivation (1). Below we show that it can also be used with beef liver glyoxalase II and the glutathione S-transferases.

The glyoxalase system (2,3), widely distributed in cells of diverse types, consists of two enzymes. Glyoxalase I (EC 4.4.1.5) converts the hemimercaptal adducts of glutathione (GSH) and methylglyoxal, into the thiolester of the corresponding α-hydroxy acid and glyoxalase II (EC 3.1.2.6) catalyses the hydrolysis of this thiolester (equation (1)).

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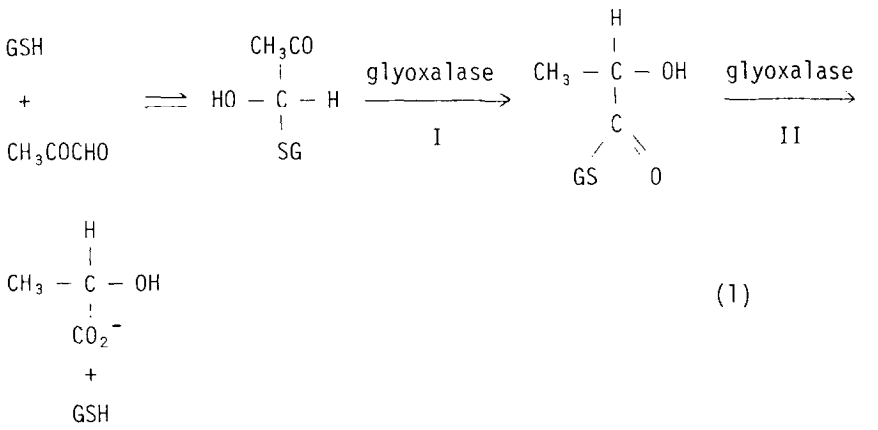


(1)

Several hypotheses have been advanced for the role of the glyoxalases. Of especial current interest is the suggestion that, for normal cell division, careful control of the level of indigenously cytotoxic methylglyoxal must be maintained (4). In accord with a regulatory role for the glyoxalase system are observations that methylglyoxal is tumour inhibitory (5), that some inhibitors of glyoxalase I are antitumour agents (6-9) and that glyoxalase II is absent from cancerous tissues or cells (10). S-lactoyl-glutathione affects anti-IgE induced histamine release from leukocytes (11) and the glyoxalase system has been linked with the regulation of tubulin aggregation (12).

A wide variety of glutathione S-transferases catalysing reaction (2), and varying in their specificity for the thiol acceptor (RX) have been

$$GSH + RX \longrightarrow GSR + HX \qquad (2)$$

described (13,14) in the detoxification of xenobiotics.

Materials and Methods

Beef liver glyoxalase II, S-lactoylglutathione, methylglyoxal and glutathione were purchased from Sigma Chemical Company, p-azidophenacyl bromide from Pierce Chemical Company and Sephadex G-25 from Pharmacia. S-(p-azidophenacyl)-glutathione was from a previous study (1). Assays of glyoxalase II activity were performed at 25.00 ± 0.05°C by following the time dependence of the decrease in absorbance at 240 nm of S-lactoylglutathione, $\Delta\varepsilon = 3370\ M^{-1}\ cm^{-1}$. The assay mixture contained 0.1 M Tris buffer at pH 7.40, 0.15 mM substrate and enzyme in a total volume of 3.00 ml. Detailed conditions for K_i determinations are described in the legends to the Figures. Initial rates were determined on a thermostatted Pye-Unicam SP8-100 UV-Visible Spectrophotometer.

The concentration of S-lactoylglutathione was determined by glyoxalase II-catalysed hydrolysis and from the amount of GSH produced by its hydrolysis (Ellman's reagent (15)), after correction for the amount of contaminating GSH present in the S-lactoylglutathione sample. Methylglyoxal, purified by distillation and acidic impurity removal (ion exchange, Dowex CG-200), was determined by a modification of the method of Bergmeyer (16). Protein concentrations were determined by a Coomassie Blue procedure (17) (commercial Bio-Rad kit).

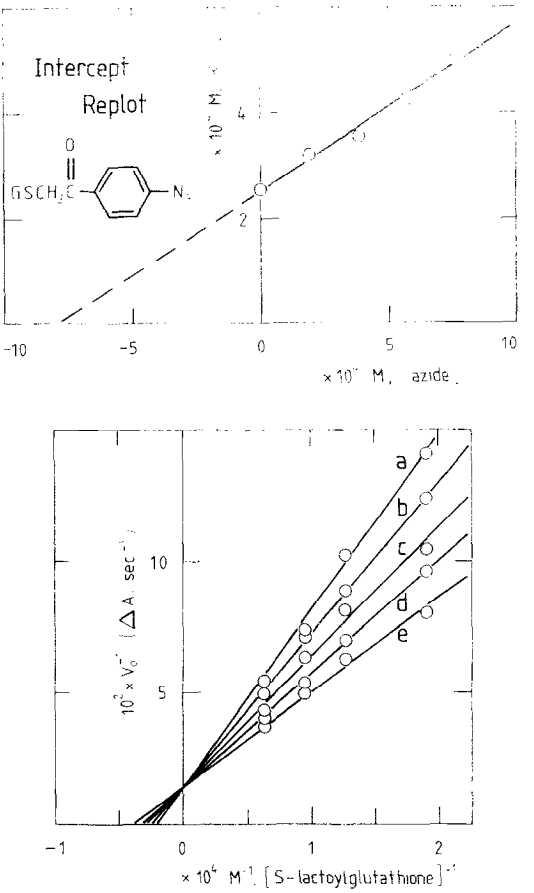
A crude preparation of glutathione S-transferases was prepared from fresh sheep liver by a modification of the procedure described by Habig et al. (14) for the rat liver enzymes. Sheep liver was homogenised, freed of lipid by glass wool filtration, chromatographed on DEAE-cellulose (DE-52) using tris buffer at pH 8.0 as equilibrant and eluant. Active fractions were pooled and treated with ammonium sulphate (600 g/l). The supernatant after centrifugation was ultrafiltered (Amicon, PM-10 membrane) and used in the experiments described below, without further purification. Assays were carried out using 1-chloro-2,4-dinitrobenzene as substrate according to the conditions described by Habig et al. (14) ([GSH] = 1 mM; [1-chloro-2,4-dinitrobenzene] = 1 mM; pH 6.5, 340 nm; $\Delta\varepsilon$, 9600 M^{-1} cm^{-1}).

Photolyses were effected, as previously described (1), for 20 min at the appropriate wavelength. Under these conditions, 1 photolysed smoothly while photodestruction of glyoxalase II was only 5%, based on enzyme activity. The glutathione S-transferase preparation, more light-sensitive, lost 23% of its activity. These conditions represent a compromise (in time and wavelength) for the smooth photolysis of 1 versus photodestruction of the enzymes).

Results

S-(p-azidophenacyl)-glutathione, prior to photoactivation inhibits glyoxalase II, displaying linear competitive inhibition kinetics (K_i = 7.96 ± 0.91 x 10^{-4} M) with S-lactoylglutathione as substrate (K_m = 2.51 ± 0.10 x 10^{-4} M), see Figure 1.

For the photoactivation experiments, 1 (2.72 mM) was incubated with glyoxalase II (0.26 µg/ml) in 1.50 ml of 0.1 M Tris buffer (pH 7.40) at 25.0°C in the presence of 2.7% (v/v) dimethylsulphoxide, for solubilizing 1. This mixture was irradiated at 340 nm as described. After separation of noncovalently bound inhibitor/photolysis products from protein by Sephadex G-25 gel filtration, the activity of the enzyme isolated in four separate experiments, including protection work described below, was 58 ± 5% of a control sample similarly treated, but in the absence of azide 1. Enzyme activities were compared per mg of total protein. Protection experiments were also conducted: in this glyoxalase II was incubated



Lower Section: Lineweaver-Burk (double reciprocal) plot of the inhibitory effect of S-(p-azidophenacyl)-glutathione on beef liver glyoxalase II activity at pH 7.40. Points are experimental, lines are theoretical for competitive inhibition with a K_i value of 7.96 x 10^{-4} M for this inhibitor. The concentrations of inhibitor used corresponding to lettered lines were: (a) 7.67 x 10^{-4} M; (b) 5.75 x 10^{-4} M; (c) 3.83 x 10^{-4} M; (d) 1.92 x 10^{-4} M; (e) no inhibitor present.

Upper Section: Replot of K_m(app) from the Lineweaver-Burk plot for beef liver glyoxalase II, described above, versus inhibitor concentration. Points are derived by least squares linear regression analysis of the Lineweaver-Burk data. The line drawn through these points is also derived by least squares linear regression analysis and indicates linear competitive inhibition with K_m = 0.251 ± 0.010 mM and K_i = 0.796 ± 0.091 mM.

as above in the presence of 1 (2.72 mM) along with GSH (8.7 mM) and methylglyoxal (6.06 mM). The remaining activity after G-25 passage was 91 ± 1% of a similarly treated control, omitting the azide. The reproducibility of the technique (i.e. incubation, irradiation, G-25 passage, rate assay) was ±2% for control enzyme.

S-(p-bromobenzyl)-glutathione is a linear competitive inhibitor of the crude glutathione S-transferase preparation ($(K_i)_{app}$ = 1.1 x 10^{-5} M). On this basis, approximate conditions for the photoactivation of 1 were chosen. The enzyme preparation was incubated at 25.0°C in pH 6.50 phosphate buffer (0.1 M) with 1 (1.67 x 10^{-3} M) and 1.64% v/v dimethylsulphoxide and irradiated (320 nm, 20 min). After this period, the mixture was G-25 gel-filtered. The eluted protein was assayed using 1-chloro-2,4-dinitrobenzene and the total concentration of protein determined. Compared to a control sample, similarly treated but with no azide (1) present, a level of 86 ± 5% covalent inhibition was obtained in these preliminary experiments on the crude mixture of transferases.

Discussion

It is apparent that S-(p-azidophenacyl)-glutathione binds to the active-site of glyoxalase II and on irradiation at 340 nm, the generated nitrene covalently labels the enzyme with concomitant destruction of activity. The photoaffinity covalent inhibition can be effectively prevented by a protection experiment, involving a GSH/methylglyoxal mixture, which is in equilibrium with a glutathione-methylglyoxal hemimercaptal adduct (reported to be a competitive inhibitor of glyoxalase II from human (18) and mouse livers (19), K_i = 0.12 mM and 0.3 mM respectively), providing circumstantial evidence that the photolabel is active-site directed.

It is, however, possible that photolabelling of an alternative enzyme conformation has occurred with the photo-inactivation process being more rapid than the equilibration between the two enzyme forms, i.e. that the photoaffinity label has trapped an inactive conformation. The value of K_m = 0.251 mM for S-D-lactoylglutathione with beef liver glyoxalase II compares closely with values for mouse liver (19) (0.27 mM) and human liver (0.19 mM) enzymes (18). For glyoxalase II from rat erythrocytes, S-

(p-chlorophenacyl)-glutathione is a competitive inhibitor (20) with K_i = 0.15 mM, a value close to that (0.796 mM) for S-(p-azidophenacyl)-glutathione and beef liver glyoxalase II.

Although the glutathione S-transferase preparation was crude, the results indicate that one or more of the components was covalently inactivated by means of azide 1.

In conclusion, photoaffinity labelling with S-(p-azidophenacyl)-glutathione (1) of three glutathione-dependent enzymes, an isomerase (1), a thiolesterase and a transferase, has been achieved. Thus, 1 is a useful photoaffinity probe of glutathione-binding sites and detailed follow-up is underway.

Acknowledgement

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