A PHOTOAFFINITY LABEL DERIVATIVE OF GLUTATHIONE AND ITS INHIBITION OF GLYOXALASE I

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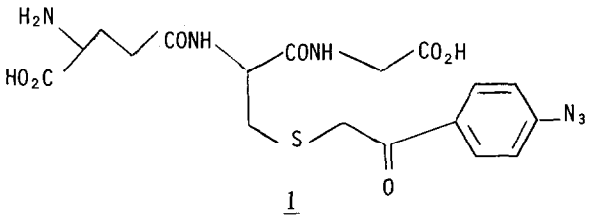
1. Introduction

Glutathione (GSH) plays many roles in biochemistry. In addition to its coenzyme functions (e.g., with glyoxalase) it is involved in the detoxification of xenobiotics, maintenance of -SH levels of proteins, disulphide exchange processes, removal of hydrogen peroxide, organic peroxides and free radicals and possibly in amino acid transport across membranes, as well as other phenomena [1,2].

With this involvement of GSH in such a myriad of biological activities in mind, we have synthesised a photoaffinity analogue of glutathione (1) based on the azidophenacyl group, introduced in [3].



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Photoaffinity labelling requires photo-induced generation of a highly reactive intermediate (in this case a nitrene) from a precursor molecule bound at the target biological site. Under suitable conditions covalent attachment of label to the biological site occurs because of the high chemical reactivity of the photo-generated intermediate [4].

Here we report the application of this technique (using 1 as photoaffinity label) to glyoxalase I (EC 4.4.1.5) for which GSH is co-substrate and S-blocked glutathiones, related to 1, are known to be competitive inhibitors [5]. There is considerable current interest in glyoxalase I as many of its inhibitors have been found to have significant antitumour activities [5a,5b,6]. For this reason, we have applied 1, as part of a series of structure–function studies, to glyoxalase I.

2. Materials and methods

Glyoxalase I (type IV, from yeast), methylglyoxal and GSH were purchased from Sigma, *p*-azidophenacyl bromide from Pierce and Sephadex G-25 from Pharmacia. *S*-(*p*-bromobenzyl)-glutathione and *S*-(*p*-azidophenacyl)-glutathione (1) were synthesised by a modification of method A in [5b]. Compound 1, m.p. 181–183°C with decomposition, was characterised by ^{13}C NMR spectroscopy and elemental analysis. ($C_{18}H_{22}N_6SO_7$: calc. – C, 46.4; H, 4.8; N, 18.0; found – C, 46.8; H, 5.1; N, 17.9). It produced no detectable coloration at 412 nm with Ellman's reagent, indicating the absence of free -SH groups, whilst the ninhydrin test was positive for the presence of $-NH_2$ groups. The ultraviolet spectrum of 1 has a band at 296 nm (ϵ = 17 440 $M^{-1} \cdot cm^{-1}$), characteristic of this type of system [3,4a]. Rate assays of glyoxalase I activity were carried out at 25°C as in [6] (but see fig.1 legend for conditions used in the inhibition study).

Photolyses were effected by irradiation of the appropriate sample in a 3 ml, quartz cuvette in a specially-constructed brass compartment through which coolant was passed to regulate the temperature of the contents of the cuvette to 25°C. Irradiation was performed for 20 min at 340 nm using a Bausch and Lomb SP-200 Mercury light source with Bausch

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and Lomb grating monochromator (2700 grooves/mm) attached. Photodestruction of glyoxalase I was negligible under these conditions, while 1 photolysed smoothly.

Acidic impurities were removed from methylglyoxal by means of ion exchange. Concentrations of methylglyoxal stock solutions were determined by a modification of the method in [7]. GSH concentrations were determined using Ellman's reagent [8]. Protein concentrations were determined by a modification of dye-binding procedures in [9] and the commercial Bio-rad protein assay kit.

3. Results

In accord with the reported inhibition of glyoxalase I by a variety of S-substituted glutathiones [5], we have found that 1 (prior to photoactivation) is an effective inhibitor (K_i = 1.05 ± 0.05 × 10^{-4} M), competitive with the hemimercaptal substrate (K_m = 1.84 ± 0.05 × 10^{-4} M, assuming both diastereomers bound). The Dixon plot is shown in fig.1.

Compound 1 (3.26 × 10^{-3} M) was incubated with glyoxalase I (65.6 μg/ml) in 1.35 ml of pH 6.6 phosphate buffer at 25°C in the presence of 7.4% (v/v) dimethyl sulphoxide and irradiated as above.

After separation of non-covalently bound inhibitor from protein by Sephadex G-25 gel filtration (1.5 × 17 cm, column), the activity remaining (per mg total protein) was 36% of a control similarly treated, but in the absence of azide inhibitor. When glyoxalase I was incubated under the above conditions and in the presence of both 1 (3.26 × 10^{-3} M) and *S*-(*p*-bromobenzyl)-glutathione (3.10 × 10^{-3} M), the remaining activity, after Sephadex G-25 passage, was 89% of a control similarly treated but omitting the azide. The reproducibility of the technique for the control enzyme was ±2%. Glyoxalase I was not covalently inhibited on incubation for 20 min with the products of photolysis of 1.

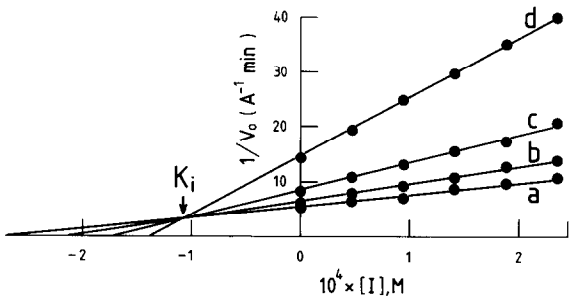

Fig.1. Dixon plot of $1/V_0$ versus [I], where I = *S*-(*p*-azidophenacyl)-glutathione, at various concentrations of hemimercaptal substrate for glyoxalase I at 25°C (pH 6.6). Initial concentrations of total hemimercaptal (both diastereomers) were: (a) 3.38 × 10^{-4} M; (b) 2.38 × 10^{-4} M; (c) 1.45 × 10^{-4} M; (d) 0.64 × 10^{-4} M. Points are experimental: lines were obtained by linear regression analysis using the values of V_{max} determined from Lineweaver-Burk analysis and of K_i from a replot of the intercepts (at $1/V_0$ = 0) of the double-reciprocal plot.

4. Discussion

It is immediately apparent from the results that 1 can be used to (photo)covalently label glyoxalase I. Labelling is likely to be occurring at or near the active-site in view of the efficient protection afforded by the powerful competitive inhibitor (K_i = 7 × 10^{-6} M, [10]) *S*-(*p*-bromobenzyl)-glutathione. In addition, the efficiency of the photolabelling process appears high. From the K_i (1.05 × 10^{-4} M) and the operational azide concentration chosen (3.25 × 10^{-3} M), we can calculate that the amount of enzyme bound to the azide at the start of the experiment was ~96%. The observed inhibition was of the order of 64%, leading to an apparent [11] efficiency of (64 × 100/96) 67%. In the presence of *S*-(*p*-bromobenzyl)-glutathione at 3.10 × 10^{-3} M, >99% of the enzyme is inhibitor-bound. The concentrations of azide, 1, and *S*-(*p*-bromobenzyl)-glutathione used were approximately equal, but the *p*-bromobenzyl derivative binds ~16-times more strongly based on K_i values. Consequently, a high degree of protection is observed under these conditions, indicating the specificity of the labelling.

We cannot tell from the present data if more than one site on the yeast enzyme has been attacked by the photolabel. It has been suggested that, although monomeric, there are two binding sites for glutathione derivatives [12]. A radioactive photolabel may yield information on this aspect of glyoxalase structure–function. This type of glutathione analogue should be applicable in a variety of situations: we have found that 1, as well as *S*-(2-nitro-4-azido)-glutathione, are effective inhibitors of γ-glutamyl transpeptidase in the dark, and are in the process of carrying out the photoactivation reaction. We have also found a 'dark inhibition' of glyoxalase II by azide 1.

Acknowledgements

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[11] The efficiency is 'apparent' because if the rate processes governing formation and dissociation of the inhibitor complex with azide are fast relative to the rate of the photolytic reactions, the potential degree of covalent inhibition from a photoaffinity label can actually be greater than the K_i would predict, because of leakage of the free enzyme to irreversibly formed, photolabelled products.
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