Role of the *N*-terminus of glutathione in the action of yeast glyoxalase I

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A number of *S*-substituted glutathiones and the corresponding *N*-substituted *S*-substituted analogues have been found to be linear competitive inhibitors of yeast glyoxalase I at 26°C over the pH range 4.6–8.5. *N*-Acetylation of *S*-(*p*-bromobenzyl)glutathione weakens binding by 13.7-fold, *N*-benzoylation by 25.6-fold, *N*-trimethylacetylation by 53.3-fold and *N*-carbobenzoxylation by 7.8-fold, indicating a minor steric component in the binding at the *N*-site. The K_i -weakening effect of *N*-substitution of glutathione depends on the chemical nature of the *S*-substituent, indicating flexibility in the glutathione and/or glyoxalase I contributions to the binding site for glutathione derivatives. The effect of *N*-acylation on K_i is in accord with a charge interaction of the free enzyme with *S*-blocked glutathione in a region of reasonably high dielectric constant. There is a slight pH effect on K_i for *S*-(*m*-trifluoromethylbenzyl)glutathione but not for *S*-(*p*-bromobenzyl)glutathione.

The glyoxalase system, discovered in 1913 (Dakin & Dudley, 1913*a,b*; Neuberg, 1913), consists of two enzymes and glutathione (GSH). Glyoxalase I (lactoylglutathione lyase, EC 4.4.1.5) converts the hemithiolacetal formed between GSH and methylglyoxal or other α -oxoaldehydes into *S*-lactoylglutathione etc. (Racker, 1951). This thioester is then hydrolysed under the catalytic influence of glyoxalase II (hydroxyacylglutathione hydrolase, EC 3.1.2.6):

In the glyoxalase I reaction, *S*-D-lactoylglutathione is produced stereospecifically (Ekwall & Mannervik, 1973; Mannervik, 1980) by selective reaction of one of the diastereomeric forms of the hemithiolacetal substrate (Brown *et al.*, 1981).

Nevertheless, little is known of the normal physiological role(s) of the ubiquitous (Mannervik, 1980) glyoxalase system, although a variety of suggestions have been made, including functions in the enhancement of anti-(immunoglobulin E)-induced histamine release (Gillespie, 1979), cell proliferation (the retine–promine hypothesis of Szent-Gyorgyi, 1968) and microtubule assembly (Gillespie, 1975, 1978). The α -oxoaldehyde co-

substrates are markedly cytotoxic and thus a protective function against intestinal bacteria has been ascribed to glyoxalase I (Aronsson & Mannervik, 1977). The glyoxalase system has also been suggested to be involved in a postulated cycle for the degradation of glycine and threonine, as methylglyoxal is a deamination product of aminoacetone

(Elliott, 1958; Urata & Granick, 1963). The demonstration that γ,δ -dioxovalerate is a competent substrate for glyoxalase, being converted into D- α -hydroxyglutarate, has led to a suggested connection with the haem-biosynthetic pathway (Jerzykowski *et al.*, 1973).

The reaction catalysed by glyoxalase I is an internal redox process, which for some time has been regarded as an enzymic analogue of the Cannizzaro reaction. However, recent evidence (Hall *et al.*, 1976, 1978; Kozarich *et al.*, 1981; Chari & Kozarich, 1981) has been more in favour of the 1,2-proton-shift enediolate mechanism originally proposed by Racker (1951) rather than a hydride transfer (Rose, 1957; Franzen, 1956).

Abbreviation used: GSH, reduced glutathione.

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Scheme 1. Nomenclature of binding subsites for glutathione derivatives

Our interest in the enzyme stems from many reports that glyoxalase I inhibitors often possess significant anti-cancer activity, sometimes lost *in vivo* (Vince & Daluge, 1971; Lyon & Vince, 1977; Vince *et al.*, 1973; Takeuchi *et al.*, 1975; Chimura *et al.*, 1975; Kurasawa *et al.*, 1975; Iio *et al.*, 1976). The enzyme is also of interest in the study of glutathione binding sites, many examples of which have evolved over the years.

It is known from studies with *S*-blocked glutathiones (Vince *et al.*, 1971) that there is an extensive hydrophobic binding site which we shall call the 'S-site' in the approximate region of the α -oxoaldehyde side chain component of the substrate (see Scheme 1). Little detailed analysis is available of the region on the enzyme which would be close, perhaps bound, to the *N*-terminus of the GSH moiety of the substrate (the N-site). It has been suggested, on the basis of $[I]_{50}$ (concentration of inhibitor causing 50% inhibition) values for some *N*-acetyl derivatives, but without detailed inhibition studies (Vince *et al.*, 1971), that a free amine site is not essential, but does contribute to binding of *S*-blocked inhibitors. One cannot assume competitive binding of *N*-blocked GSH analogues on the basis of the competitive inhibition observed for some *S*-blocked glutathiones (Marmstål & Mannervik, 1979), as *S*-blocked cysteinylglycine residues with *N*-glutaryl and *N*- ω -aminopropionyl groups exhibit non-competitive inhibition (Lyon & Vince, 1977) and *S*-(ω -aminoalkyl)glutathiones showed mixed inhibition (Kester *et al.*, 1974). *N*-Acetylglutathione can substitute as a substrate for GSH with glyoxalase I with no change in V_{max} , but an increase in K_m (Van der Jagt & Han, 1973).

To clarify further the nature of the N-site we have undertaken detailed inhibition studies for a number of *N*-acylated *S*-blocked glutathione derivatives with yeast glyoxalase I.

Experimental

Materials

Glyoxalase I (grade IV from yeast) and substrates were purchased from Sigma Chemical Co.

Methylglyoxal was purified by removal of acidic contaminants by ion-exchange chromatography, and stock concentrations were determined by a modification of the method of Bergmeyer (1974). Enzyme stock solutions contained 0.1% bovine serum albumin as stabilizing agent (Racker, 1951). Substrate stock solutions were prepared fresh daily and kept on ice. *N*-acylated *S*-(substituted-benzyl)-glutathiones were prepared by dimethylamino-pyridine-catalysed acylations of the respective *S*-blocked glutathione. They were purified by combinations of recrystallization and Sephadex LH-20 chromatography. Structures were confirmed by n.m.r. spectroscopy. Full analytical and synthetic details are given in the Appendix.

Methods

Enzyme assay. The enzyme assay was carried out (25°C, 10mm-pathlength cuvettes, 0.05M-phosphate buffer, pH6.60) as follows. For each assay, the cuvette contained appropriate amounts of GSH and methylglyoxal [i.e. arranged to minimize the levels of inhibitory (Bartfai *et al.*, 1973) free GSH present in the medium] in a total volume of 3.0ml, along with inhibitor etc. as appropriate. The components were kept together at 25°C for 5–8 min to ensure complete equilibration to hemithiolacetal. Addition of enzyme led to an increase in A_{240} , which was used to monitor the kinetics of the reaction.

Determination of K_i etc. Hemithiolacetal concentrations present in the equilibrated mixture of GSH and methylglyoxal were calculated by using a value of 3.1 mM for the dissociation constant of the hemithiolacetal at pH6.60 (Vince *et al.*, 1971). Substrate concentrations were taken as 0.5 times the appropriate hemithiolacetal concentrations to allow for the diastereomeric selectivity of glyoxalase I (Brown *et al.*, 1981), assuming that this selectivity is present at pH6.6.

To diagnose inhibition type, both Lineweaver–Burk ($1/v_0$ versus $1/s_0$) and Dixon ($1/v_0$ versus $[I]$) plots were scrutinized. For calculation of K_i values the Dixon plot was used along with the value of $1/V_{max}$, determined in the absence of inhibitor. In

this way, the K_i value was taken as the mean of the intersections of the $1/v_0 = 1/V_{\max}$ line with the best lines (by linear least-squares regression) through the $1/v_0$ -versus-[I] data at the various substrate concentrations used (usually four different substrate concentrations). The errors quoted on K_i are standard deviations of these intersections from their mean. Values of K_i determined by a replot of $1/(K_m)_{\text{app}}$ (from the Lineweaver–Burk plot) versus [I] were in good agreement with such values.

Computations were carried out on an Exidy Sorcerer Minicomputer.

Kinetic procedures and buffers. Buffer solutions were prepared from Analar quality-materials, with glass-distilled deionized water and a Philips PW 9409 digital pH-meter.

All rate measurements were conducted at 25°C in the thermostatically-controlled compartment of a Pye–Unicam SP.8–100 u.v./visible spectrophotometer.

Stock inhibitors were prepared in purified dimethyl sulphoxide; final concentrations of this co-solvent in assay media were kept to $\leq 1\%$ (v/v) at which value there is no detectable rate effect in the assay of yeast glyoxalase I.

Results

In agreement with previous reports for *S*-blocked glutathiones (Marmstål & Mannervik, 1979) we

have found that *S*-(*p*-bromobenzyl)-, *S*-(carboxybenzoxy)- and *S*-(*m*-trifluoromethyl)-glutathione are linear competitive inhibitors of yeast glyoxalase I at pH 6.60, 25°C. As can be seen from Fig. 1, both Lineweaver–Burk and Dixon criteria support this interpretation of these experimental data. The K_i value determined from the Dixon plot was $10.1 \pm 2.3 \mu\text{M}$.

We also found linear competitive inhibition of yeast glyoxalase I at pH 6.60, 25°C by the *N*-acetyl *S*-blocked glutathiones 2–6 and 9 (for example, see Fig. 2; numbering of glutathiones as in Table 1). Again internal agreement in K_i values calculated from the Dixon and Lineweaver–Burk plots was good. Values of K_i for a number of derivatives are collected in Table 1. These K_i values were calculated, as described above, by using the Dixon plot.

The dependence of K_i on pH was determined for *S*-(*m*-trifluoromethylbenzyl)glutathione and for *N*-acetyl-*S*-(*p*-bromobenzyl)glutathione with yeast glyoxalase I at 25°C. The results are summarized in Table 2. As can be seen for *N*-acetyl-*S*-(*p*-bromobenzyl)glutathione agreement is good between Lineweaver–Burk [$(K_m)_{\text{app}}$ replot] and Dixon methods of K_i determination, again arguing for the applicability of the linear-competitive-inhibition equation for *N,S*-blocked glutathiones. The K_i data for compound (see Table 1) 2 (Dixon analysis) are plotted in Fig. 3 as a pH profile. There is a slight, but significant (relative to the error bars shown),

Fig. 1. (a) Dixon plot of $1/v_0$ versus inhibitor concentration at pH 6.60, 25°C for *S*-(3-trifluoromethylbenzyl)glutathione with yeast glyoxalase I and (b) Lineweaver–Burk plot of the data from (a)

(a) Points are experimental; lines, marked with the substrate concentration used, are theoretical and derived by least-squares linear-regression analysis of the data, assuming linear competitive inhibition with $K_i = 10.1 \pm 2.3 \mu\text{M}$. (b) Points are experimental; lines are theoretical (marked with the appropriate inhibitor concentration used) for linear competitive inhibition. Substrate (adduct) concentrations are $0.5 \times$ total hemithiolacetal adduct concentration to allow for the diastereomeric selectivity of glyoxalase I.

Fig. 2. (a) Dixon plot of $1/v_0$ versus inhibitor concentration at pH 6.60, 25°C for *N*-acetyl-*S*-(*p*-bromobenzyl)glutathione with yeast glyoxalase I and (b) Lineweaver-Burk plot of the data in (a) for linear competitive inhibition of yeast glyoxalase I at pH 6.60, 25°C by *N*-acetyl-*S*-(*p*-bromobenzyl) glutathione with $1/V_{\max} = 211.4 \Delta A^{-1} \cdot s$

(a) Points are experimental; lines are theoretical, assuming linear competitive inhibition with $K_i = 2.29 \pm 0.40 \times 10^{-5} \text{ M}$. Each line is marked with the hemithiolacetal substrate used ($\approx 0.5 \times$ total hemithiolacetal concentration to allow for the diastereomeric selectivity of glyoxalase I). (b) Lines are annotated with the appropriate concentrations of inhibitor used. The replot of $(K_m)_{\text{app}}$ (determined by least-squares linear-regression analysis) gives $K_i = 2.46 \pm 0.12 \times 10^{-5} \text{ M}$.

dependence of K_i on pH, binding being about 2-fold tighter at lower pH values. It is possible that the K_i values weakly reflect an ionization of pK_{app} in the region of pH 6–7, but the data do not warrant the drawing of a theoretical ionization curve through them. There appears to be no detectable pH-dependence of K_i for compound 8, the mean K_i over the pH range studied being $1.32 (\pm 0.37) \times 10^{-5} \text{ M}$ (data from Table 2).

Discussion

From the results in Table 1 and Fig. 2, acylation of the *N*-terminus of *S*-blocked derivatives of GSH

Fig. 3. Plot of K_i versus pH for the inhibition of yeast glyoxalase I by *N*-acetyl-*S*-(*p*-bromobenzyl)glutathione (compound 2) at 25°C

The K_i values and error bars are from Table 2 (Dixon-plot procedure).

does not destroy binding to glyoxalase I, in agreement with previous results (Vince *et al.*, 1971; Van der Jagt & Han, 1973), which were limited to *N*-acetyl substitution. Vince *et al.* (1971) reported that *N*-acetylation of *S*-hexyl-, *S*-*n*-propyl- and *S*-(*p*-bromobenzyl)-glutathione increased $[I]_{50}$ values by 5–8-fold, but did not report detailed inhibition kinetics. The present report of linear competitive inhibition for compounds 2–6 and 9 (Table 1) indicates that these molecules probably bind to the same site as simple *S*-blocked glutathiones, most likely the active site, unless glyoxalase I [monomeric (Marmstål *et al.*, 1971) from yeast] is flexible. Comparison of compound 1 with compound 2 indicates that, for yeast glyoxalase I, the K_i value is raised some 13.7-fold by *N*-acetylation. Van der Jagt & Han (1973) have shown that, at pH 7.0, $V_{\max}$ is unaltered by *N*-acetylation of the GSH co-substrate. In contrast, K_m values increased on *N*-acetylation by 5–10-fold, depending on the nature of the α -oxoaldehyde co-substrate (Van der Jagt & Han, 1973). More detailed analysis of these effects is difficult because of the nature of some of the parameters reported (e.g. K_m need not reflect binding). The K_i comparisons of Table 2 clearly reflect binding phenomena. On *N*-acylation of an *S*-blocked glutathione the *N*-terminus is considerably changed sterically and its charge is changed (if the pK_a of the α -amino site of GSH is not significantly altered on binding to glyoxalase I). Thus compounds 1, 7 and 8 probably bind as the $-\text{NH}_3^+$ form, in contrast with the *N*-neutral binding of compounds 2–6 and 9. The steric effect can be gauged approximately by the change in K_i on going from $-\text{NHCOCH}_3$ (2) to $-\text{NHCOC}(\text{CH}_3)_3$ (4); binding is weakened by this considerable steric change by only 3.9-fold, whereas the charge is not changed. Comparing compound 2 with compound 3

$$\text{RNH}-\text{CH}(\text{CO}_2^-)-\text{CH}_2\text{CH}_2-\text{CONH}-\text{CH}(\text{SR}')-\text{CONH}-\text{CH}_2\text{CO}_2^-$$

† See Marmstål & Mannervik, 1979.

§ The enzyme is not very stable at such pH values and the K_i values quoted are subject to error.

compound 7 with compound 6), although the inhibition weakening effect of *N*-carbobenzylation varies with the substituent at the S-site [cf. 7.8-fold weakening for the *S*-(4-bromobenzyl)

derivatives but only 1.9-fold for *S*-carbobenzoxo derivatives]. For the *S*-(*m*-trifluoromethylbenzyl) derivatives (compounds 8 and 9) the effect of *N*-benzoylation is to weaken K_i by ~10-fold; the corresponding effect for *S*-(*p*-bromobenzyl) analogues (compounds 1 and 3) being 25.6-fold. There must be a degree of glyoxalase backbone/GSH backbone-binding region flexibility in substrate binding in that, to some extent at least, effects at the *S*-site are reflected at the *N*-site. There has been discussion of potential flexibility of monomeric yeast glyoxalase I (Marmstål & Mannervik, 1979, 1981).

The gradual increase in K_i as *N*-substitution changes from $\text{CH}_3\text{CO}-$ to $\text{PhCO}-$ to $(\text{CH}_3)_3\text{CCO}-$ agrees with a minor steric effect at the *N*-site, although this is small. This effect operates in the opposite sense to hydrophobic binding. The *N*-substituted derivatives probably bind with the *N*-acyl groups protruding into solution, as in this case the size of the *N*-substituents would be relatively unimportant. There must be, however, a binding site of some description for the NH_3^+ group of the substrate, as K_i is reduced significantly on *N*-blocking for various structural effects at the *S*-site [cf. also compound 6 with compound 7, compound 8 with compound 9 and the data of Van der Jagt & Han (1973)]. Also, omitting the $\alpha\text{-NH}_3^+$ group from *S*-(*p*-bromobenzyl)glutathione, compound 1, changes inhibition type from competitive for compound 1 (Marmstål & Mannervik, 1979) to non-competitive (Lyon & Vince, 1977) and changes K_i from $1.8 \times 10^{-6}\text{M}$ to $3.2 \times 10^{-4}\text{M}$. In this non-competitive case, it was suggested that there were two binding sites (Lyon & Vince, 1977).

The simpler 14-fold decrease in linear competitive binding strength for *N*-acetylation can be interpreted in terms of a single site. For an electron and proton separated by 0.33 nm (3.3 Å) in water, the interaction energy is $5.4\text{kJ}\cdot\text{mol}^{-1}$ ($\sim 1.3\text{kcal}\cdot\text{mol}^{-1}$) (Fersht, 1977) and is greater as the local dielectric constant (D) decreases. The difference in binding free energies of *N*-acetylated (compound 2) and NH_3^+ -free (compound 1) inhibitors, using $\Delta\Delta G = 2.303 RT \cdot \Delta pK_i$, where ΔpK_i is the log of the ratio (~ 14) of K_i values (see Table 2), is estimated as $\sim 6.3\text{kJ}\cdot\text{mol}^{-1}$ at 25°C . This agrees with surface electrostatic binding at the *N*-site with a high local dielectric constant.

The results for *S*-(*m*-trifluoromethylbenzyl)glutathione (Table 2) show K_i values to be pH-independent, within experimental error, standing in contrast with the slight pH-dependence of K_i values for the *N,S*-substituted derivative (compound 2). *S*-(*p*-Bromobenzyl)glutathione also has a pH-independent K_i , but porphyrins inhibit glyoxalase I with a marked pH effect (K. T. Douglas and J. Ghotb-Sharif, unpublished work). In view of the significant effects of pH on V_{max}/K_m (Van der Jagt

& Han, 1973) such insensitivities of K_i values are surprising and possibly indicate that the enzyme can exist in more than one conformation.

In summary, *N*-acyl analogues of *S*-blocked glutathiones inhibit yeast glyoxalase I competitively, but more weakly than the *S*-blocked glutathione itself. The *N*-acyl group of the bound inhibitor is likely to protrude into solution, away from the enzyme surface, but exerts a minor steric influence on binding. The free $\alpha\text{-NH}_3^+$ groups of *S*-blocked glutathiones and substrate are probably bound electrostatically at the *N*-site of glyoxalase I.

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APPENDIX

Synthesis of N-acylglutathione derivatives by dimethylaminopyridine catalysis

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A general procedure, involving 4-dimethylaminopyridine-catalysed acylation of the amino groups in the appropriate S-blocked glutathione, is reported for the preparation of N-blocked derivatives of glutathiones.

Glutathione (GSH), ubiquitous in biochemistry, has many roles ranging from those as coenzyme or co-substrate through transport processes, detoxification, protein SH maintenance to primitive chemoreceptor activity (Jocelyn, 1972). We have been studying GSH-dependent enzymes from a variety of angles, including the use of chemically modified GSH analogues, e.g. photoaffinity labels based on GSH (Seddon & Douglas, 1980; Seddon *et al.*, 1980; D'Silva *et al.*, 1981). In the course of such work we required a range of N-modified analogues of glutathione and experienced considerable difficulty in simple Schotten–Baumann acylation, either under aqueous or non-aqueous conditions. In spite of their apparent simplicity, such derivatives have only rarely been described by routes starting with GSH (Sokolovsky *et al.*, 1964a,b; Utzinger *et al.*, 1963; Fujihara *et al.*, 1972; Vince *et al.*, 1971), and are usually the results of classical solution total peptide syntheses of GSH analogues (Goldschmidt & Jutz, 1953; Amiard *et al.*, 1958; Wieland & Wedenmuller, 1955; Kovacs *et al.*, 1972). We now

report a successful and convenient application of the catalyst 4-dimethylaminopyridine (Steglich & Vorbrüggen, 1978) to solve this problem of the low reactivity of the glutathione amino group. We also report purification procedures for such derivatives (2–7):

- (1) $R^1 = R^2 = \text{COOCH}_2\text{C}_6\text{H}_5$
- (2) $R^1 = \text{COPh}$; $R^2 = \text{CH}_2\text{C}_6\text{H}_4m\text{CF}_3$
- (3) $R^1 = \text{COC}_6\text{H}_4p\text{N}_3$; $R^2 = \text{CH}_2\text{C}_6\text{H}_4m\text{CF}_3$
- (4) $R^1 = \text{COCH}_3$; $R^2 = \text{CH}_2\text{C}_6\text{H}_4p\text{Br}$
- (5) $R^1 = \text{COC}(\text{CH}_3)_3$; $R^2 = \text{CH}_2\text{C}_6\text{H}_4p\text{Br}$
- (6) $R^1 = \text{COC}_6\text{H}_5$; $R^2 = \text{CH}_2\text{C}_6\text{H}_4p\text{Br}$
- (7) $R^1 = \text{COOCH}_2\text{C}_6\text{H}_5$; $R^2 = \text{CH}_2\text{C}_6\text{H}_4p\text{Br}$

Experimental

Elemental analyses were performed by the Micro-analytical Laboratory, Department of Chemistry,

Abbreviation used: GSH, reduced glutathione.