Nonstereospecific Substrate Usage by Glyoxalase I†

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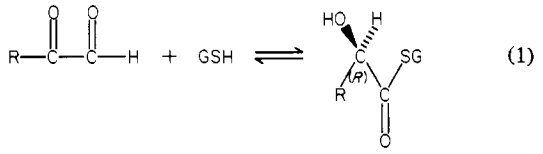
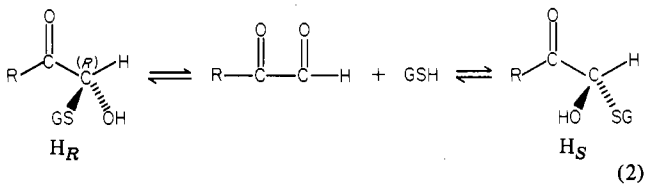
ABSTRACT: Glyoxalase I operates on a mixture of rapidly interconverting diasteriomeric thiohemiacetals, formed in a preequilibrium step between glutathione and α-ketoaldehyde. That both diasteriomers are directly used as substrates by the enzyme from yeast and from porcine erythrocytes is an outcome of a series of isotope-trapping experiments in which pulse solutions composed of the two diasteriomeric thiohemiacetals, due to [^{3}H]glutathione and phenylglyoxal, are rapidly mixed with chase solutions containing excess unlabeled glutathione and successively increasing concentrations of glyoxalase I. As the enzyme approaches infinite concentration in the chase solution, the radioactivity incorporated into the *S*-mandeloylglutathione product approaches 100% of the total radioactivity due to both diasteriomers from the pulse solution. The special properties of the active site that allow the enzyme to accommodate both diasteriomeric substrate forms may also account for the fact that the cis and the trans isomers of various para-substituted *S*-(phenylethenyl)glutathione derivatives are both strong competitive inhibitors of the enzyme. A catalytic mechanism is proposed for glyoxalase I involving catalyzed interconversion of the bound diasteriomeric thiohemiacetals before transformation to final product.

Glyoxalase I (EC 4.4.1.5) is a ubiquitous, GSH[1]-dependent enzyme that catalyzes the conversion of a variety of aromatic and aliphatic α-ketoaldehydes to α-hydroxy thio esters having the *R* configuration at C-2 (Vander Jagt et al., 1975; Ekwall & Mannervik, 1973; Alexander & Boyer, 1971; Racker, 1952) (eq 1). The favored substrate for the enzyme is actually the



(1)

thiohemiacetal formed in a preequilibrium step between GSH and α-ketoaldehyde (Vander Jagt et al., 1975). Since the thiohemiacetal carbon atom is chiral, the enzyme must operate on a mixture of rapidly interconverting diasteriomers (eq 2).



(2)

Although enzymes are generally highly stereospecific with respect to the utilization of substrate, we were prompted to test whether glyoxalase I distinguishes between H_R and H_S on the basis of the finding that the cis and trans isomers of various para-substituted *S*-(phenylethenyl)glutathione derivatives both bind to the active site of glyoxalase I with high and nearly equal affinity, as reported here and elsewhere (Creighton et al., 1980). This suggested a high degree of latitude in binding diasteriomeric substrate and/or intermediate forms.

Indeed, both H_R and H_S can be directly converted to product by glyoxalase I from yeast and from porcine erythrocytes, on the basis of the results of isotope-trapping experiments. The significance of this observation is that enzymic catalysis is not limited by the relatively slow nonenzymic rate of interconversion of the diasteriomers, a second outcome of these experiments. Molecular mechanisms that could account for the capacity of the enzyme to use both diasteriomers as substrates are compared.

Experimental Procedures

Materials. Yeast glyoxalase I was obtained from Sigma (type IV) and used without further purification. No more than one form of the yeast enzyme has been observed (Vander Jagt & Han, 1973). Porcine erythrocyte glyoxalase I was purified to apparent homogeneity according to Aronsson & Mannervik (1977) and stored at liquid-nitrogen temperatures before use. Under these conditions, the enzyme does not undergo any significant loss in activity over time. Methylglyoxal was purified by vacuum distillation of the commercial, 40% aqueous solution (Aldrich) (Kermack & Matheson, 1957). Contaminating lactic acid was removed from the distillate by filtration through a Dowex-1 carbonate column. Phenylglyoxal was an Aldrich product.

Commercial GSH from Sigma (type IV) was used without further purification and assayed to be ~95% pure by weight, on the basis of the spectrophotometric sulfhydryl group assays using either 4-pyridine disulfide (Aldrich) (Grassetti & Murray, 1967) or *N*-ethylmaleimide (Alexander, 1958). L-[*glycine*-2-^{3}H]Glutathione (New England Nuclear, NET-282) was routinely purified by high-performance liquid chromatography, since the radiopurity of the commercial material can be highly variable. This was done by isocratic elution of the commercial material, combined with 1 mg of carrier GSH, from a Waters μBondapak C_{18} column (7.8-mm i.d. × 30 cm, 10 μm) with a running solvent composed of 0.25% acetic acid in water. The effluent from the column was monitored at 206 nm. The labeled GSH appears between 12 and 15 mL, followed by a second smaller peak at ~19 mL, the magnitude of which varies depending on the extent of oxidation of the carrier GSH. (Analysis of several different lots of the commercial, tritiated GSH showed that in some cases less than 50% of the total tritium comigrated with GSH while the re-

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[1] Abbreviations: GSH, glutathione; BSA, bovine serum albumin; H_R and H_S, thiohemiacetals formed from GSH and phenylglyoxal in which the chirality at the thiohemiacetal carbon is *R* and *S*, respectively; FT, Fourier transform; NMR, nuclear magnetic resonance; DSS, sodium 4,4-dimethyl-4-silapentanesulfonate.

0006-2960/83/0422-2945$01.50/0

Table I: Aromatic and Vinyl Proton Resonances (360 MHz) Due to Cis and Trans Isomers of Para-Substituted *S*-(Phenylethenyl)glutathione Derivatives, p-X–C_6H_4–CH_A=CH_B–SG[a]

X (isomer)	chemical shift (ppm)[b] H_A	H_B	aromatic ring protons	coupling constant ($J_{CH=CH}$, Hz)	% cis isomer in sample[c]
methyl					
cis	6.60	6.29	7.25–7.45	10.5	93
trans	6.78	6.64	7.15–7.35	15.5	0
chloro					
cis	6.60	6.38	7.40–7.55	10.5	94
trans	6.85	6.62	7.30–7.40	15.5	17
phenyl					
cis	6.71	6.44	7.42–7.88	10.8	100
trans	6.96	6.76	7.42–7.84	14.4	48

[a] Samples measured in 100% D_2O. [b] vs. DSS. [c] From relative integrated intensites of *cis*- and *trans*-vinyl proton resonances.

Table II: Competitive Inhibition of Yeast and Porcine Erythrocyte Glyoxalase I by Cis and Trans Isomers of Para-Substituted *S*-(Phenylethenyl)glutathione Derivatives, p-X–C_6H_4–CH=CHSG, vs. Thiohemiacetal Substrate Due to Methylglyoxal and GSH

X	inhibition constant (mM)[a] yeast[b] cis	yeast[b] trans	erythrocyte[c] cis	erythrocyte[c] trans
H	0.114 ± 0.015[d]	0.136 ± 0.013[d]	nd[e]	nd
CH_3	0.050 ± 0.022	0.069 ± 0.029	0.294 ± 0.058	0.050 ± 0.008
Cl	0.012 ± 0.002	0.021 ± 0.004	0.076 ± 0.018	0.026 ± 0.004
C_6H_5	0.012 ± 0.003	0.029 ± 0.009	0.064 ± 0.009	0.034 ± 0.005

[a] The inhibition constants correspond to that of the pure cis and the pure trans isomers of the derivatives calculated by solving simultaneous forms of the following equation: $K_i^{obsd} = K_i^{cis}K_i^{trans}/[f_c K_i^{trans} + (1-f_c)K_i^{cis}]$ where K_i^{obsd} = observed inhibition constants due to preparations of inhibitors alternately enriched in the cis and trans isomers, K_i^{cis} = intrinsic inhibition constant due to the pure cis isomer, K_i^{trans} = intrinsic inhibition constant due to the pure trans isomer, and f_c = mole fraction of cis isomer in the inhibitor preparations calculated from the percent compositions given in Table I. [b] Assay conditions: phosphate buffer, 89.4 mM (pH 7); KCl, 100 mM; free [GSH], 0.3 mM; 25 °C. [c] Assay conditions: imidazole buffer, 90 mM (pH 6.8); $MgCl_2$, 10 mM; free [GSH], 0.2 mM; 25 °C. [d] Taken from Creighton et al. (1980). [e] nd, not determined.

maining radioactivity comigrated with the second peak. Apparently, the small mass quantities in which radiolabeled GSH is normally supplied makes oxidation a particular problem.) The labeled GSH purified in this way was stored as a lyophilized powder below 0 °C.

All other materials were of the highest purity commercially available.

Synthetic Methods. The para-substituted *S*-(phenylethenyl)glutathione derivatives were synthesized by the photochemical addition of GSH to the corresponding para-substituted phenylacetylene by a modification of the method of Oswald et al. (1964). The cis and trans isomers of these derivatives were resolved by reverse-phase liquid chromatography. The NMR spectra of the cis and trans isomers were obtained at 360 MHz with the Bruker WH 360/180 superconducting FT spectrometer at the Middle Atlantic NMR Research Facility at the University of Pennsylvania. The chemical shifts and splitting constants for the vinyl and aromatic protons of these derivatives are given in Table I along with the corresponding isomeric purity of each preparation of the derivatives. All other features of the spectra were as expected on the basis of comparisons with published chemical shift and coupling constant data for amino acids and peptides (Wuthrich, 1976) and by comparison with a previous NMR study of GSH derivatives in trifluoroacetic acid (Eriksson & Eriksson, 1967).

The para-substituted phenylacetylenes used as starting materials in the photochemical coupling reactions were prepared by dichlorination of para-substituted acetophenone with phosphorus pentachloride (Iwai & Yura, 1958), followed by base elimination with sodium amide in liquid ammonia. Detailed syntheses of the above compounds are available in the supplementary material (see paragraph at end of paper regarding supplementary material).

Inhibition Studies. Kinetic measurements were carried out on a Gilford 2400-2 spectrophotometer equipped with a thermostatically controlled cuvette carriage. The competitive inhibition of yeast and porcine erythrocyte glyoxalase I by the cis and the trans isomers of the above-named GSH derivatives was monitored vs. the substrate thiohemiacetal due to methylglyoxal and GSH. Different buffer conditions were used for each enzyme in order to maximize stability, Table II (Vander Jagt et al., 1972; Mannervik et al., 1972). Methylglyoxal and GSH were introduced into assay cuvettes (±inhibitor) in a proportion that kept the concentration of free GSH constant, independent of the concentration of thiohemiacetal; [GSH] = 0.3 mM (yeast enzyme); [GSH] = 0.2 mM (erythrocyte enzyme). The concentrations of total methylglyoxal and GSH used to produce a desired concentration of thiohemiacetal at a fixed [GSH] were calculated from the published dissociation constant of the thiohemiacetal, K_{diss} = (3.0 ± 0.5) × 10^{-3} M (Vander Jagt et al., 1972). Thirty minutes were allowed for the thiohemiacetal equilibrium to be established before introducing the enzyme and following the initial rate of appearance of product at 250 nm in an 0.5-cm cuvette. Under these conditions, spectral interference due to the inhibitor is minimized. For the erythrocyte enzyme, highly reproducible initial rate kinetics could only be obtained after the freshly thawed enzyme was incubated in assay buffer containing 5% BSA at 0 °C for ~1–2 h. Competitive inhibition constants were calculated from the increase in slope of reciprocal plots of [thiohemiacetal] vs. initial velocity due to concentrations of inhibitor that produced from 40 to 70% inhibition. The estimated errors in the slopes of the inhibited and uninhibited runs were propagated through to the error in the inhibition constant by a partial derivative error analysis

on the equation for linear competitive inhibition. In order to maximize the comparative precision of the inhibition constants due to the cis and the trans isomers of any given derivative, inhibition studies using the two isomers were always done on the same day with the same stock solutions of reagents.

Isotope-Trapping Experiments. These experiments consisted of rapidly introducing a small-volume pulse solution (0.05 mL), containing an equilibrium mixture of [^{3}H]GSH and phenylglyoxal, into a rapidly stirring chase solution (~1 mL), containing excess unlabeled GSH and glyoxalase I. The product, mandeloylglutathione, is isolated and its specific radioactivity determined.

Before being mixed, the pulse and chase solutions are temperature equilibrated to 25 °C in a water bath for 40 min. This also allows sufficient time for the thiohemiacetal equilibrium to be established. The chase solution is contained in a flat-bottom, cylindrical glass tube (1-cm diameter × 1.5-cm height) equipped with a Teflon-covered stirring bar (0.9-cm length × 0.25-cm diameter) and a rubber septum seal with a 1-mm hole in the center. The chase solution is magnetically stirred at ~2000 rpm as measured with a strobe light. The septum seal is positioned ~2 mm from the surface of the chase solution in order to suppress vortexing. The pulse solution is rapidly introduced into the stirring chase solution through the hole in the septum seal with a Drummond Wiretrol pipet. One minute later, 0.5 equiv of $HgCl_2$/equiv of GSH is added to the pulse-chase mixture to convert the excess GSH to $(GS)_2Hg$. This prevents a slow exchange reaction between the excess free GSH and the glutathionyl moiety of the mandeloylglutathione that slowly reduced the specific radioactivity of the product over time. After ultrafiltration through a PM-10 membrane, the pulse-chase mixture is brought to 0.25% acetic acid and immediately resolved by liquid chromatography on a Waters μBondapak C_{18} column (7.8-mm i.d. × 30 cm, 10 μm). The reaction mixture must be resolved soon after acidification in order to minimize acid-catalyzed hydrolysis of the mandeloylglutathione. The column is first washed with 0.25% acetic acid in water in order to elute $(GS)_2Hg$, having an elution volume between 6 and 10 mL. The same solvent containing 20% methanol is then used to elute mandeloylglutathione from the column with an elution volume between 12 and 13 mL, when referenced against the point at which the second eluting buffer is started. As a check that all of the optical density (206 nm) appearing in this region of the elution profile is due to mandeloylglutathione, a portion of the contents of the peak was neutralized to pH 7 and then treated with glyoxalase II (Sigma) in order to hydrolyze the mandeloylglutathione to GSH and phenylglyoxal. Resolution of this reaction mixture as described above showed that no detectable optical density appeared in the region of the elution profile attributed to mandeloylglutathione. Finally, the mandeloylglutathione in the pooled peak tubes is quantitated on the basis of the optical density at 240.5 nm (ϵ = 8700). For determination of specific radioactivity, an aliquot of the sample is counted in Scint-A cocktail on a Prias Tri-Carb scintillation counter.

Kinetic and Equilibrium Constants Used in Isotope-Trapping Calculations. The K_m and V_{max} values for the thiohemiacetal, due to phenylglyoxal and GSH, with glyoxalase I from yeast and erythrocytes, were determined from reciprocal plots of [thiohemiacetal] vs. the initial rate of loss of thiohemiacetal followed at 263 nm (ϵ = 5690) (Vander Jagt et al., 1972). Assay conditions for each enzyme were equivalent to those of the chase solutions described in the legends to Table III and Figure 2. The concentrations of total phenylglyoxal and GSH in the assay cuvettes were such that the free [GSH] was ~6 mM (chase solution conditions) on the basis of the thiohemiacetal dissociation constants also given in the legends to Table III and Figure 2 and evaluated as described below.

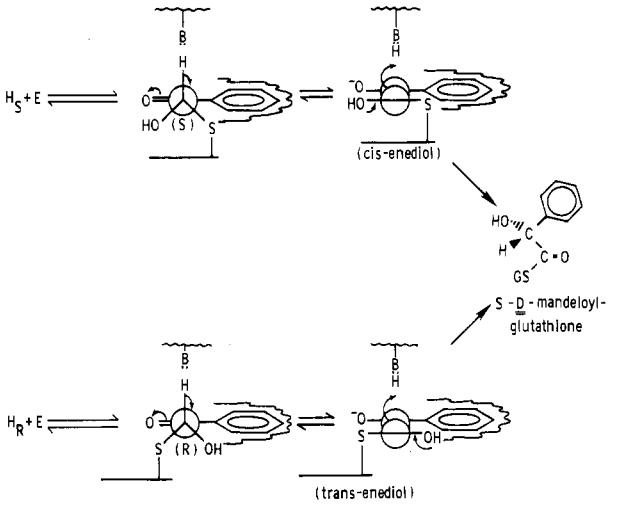


FIGURE 1: Stereochemistry of glyoxalase I reaction. Two possible stereochemical pathways are shown to *S*-D-mandeloylglutathione by an enediol-proton-transfer mechanism, depending on whether H_S or H_R is the true substrate for the enzyme. The bound diasteriomeric thiohemiacetals, due to phenylglyoxal and GSH, are shown as viewed along the C-1 and C-2 bond axis. In both pathways, a single active site base is envisioned to catalyze intramolecular proton transfer between C-1 and C-2 (Rose, 1957; Franzen, 1956; Hall et al., 1978a,b). The transferred proton must add to the *si* face of the carbonyl of bound substrate in order to generate the correct stereochemistry of product, thus establishing a direct relationship between the stereochemistry of substrate and that of the enediol intermediate.

The dissociation constant of the thiohemiacetal under different chase solution conditions was determined as follows: Known amounts of GSH were sequentially introduced into a cuvette containing 0.1 mM phenylglyoxal and the buffer components of the chase solution. The dissociation constant was determined as the ratio of the slope and intercept of a reciprocal plot of the observed increase in optical density at 280 nm (ΔOD_{280}) (Vander Jagt et al., 1972) vs. the analytical concentrations of GSH (0.17–1.13 mM). GSH does not absorb significantly at 280 nm.

Rationale and Results

Inhibition Studies with Cis and Trans Isomers of Para-Substituted S-(Phenylethenyl)glutathione. An enediol-proton-transfer mechanism for the glyoxalase I reaction is now well supported (Hall et al., 1978a,b; Shinkai et al., 1981; Kozarich et al., 1981; Chari & Kozarich, 1981). That the enzyme is completely stereospecific for either H_S or H_R is consistent with, although not proven by, the observation that the two methine proton resonances due to the two diasteriomers are lost at different rates in the presence of yeast glyoxalase I (Brown et al., 1981). Two possible stereochemical pathways from substrate to product can be envisioned, assuming absolute substrate stereospecificity and given that the product of the glyoxalase I reaction is optically pure, Figure 1. If H_S is the true substrate for the enzyme, a *cis*-enediol must form along the reaction pathway; alternatively, a *trans*-enediol intermediate must form if H_R is the substrate.

In an attempt to experimentally distinguish between these two possible stereochemical pathways, the enzymes from yeast and from porcine erythrocytes were tested for differential inhibition by the cis and the trans isomers of the title compounds. These derivatives were viewed as apolar analogues of the *cis*- and the *trans*-enediol intermediates that could form

along the reaction pathway from various para-substituted phenylglyoxal derivatives known to be good substrates for the enzyme (Vander Jagt et al., 1972). Thus, differential inhibition was to indicate whether the active site is designed to accommodate a *cis*- or a *trans*-enediol intermediate. However, no dramatic discrimination was found experimentally, Table II. This observation prompted an experimental test of whether glyoxalase I is indeed stereospecific for only one of the two diastereomeric thiohemiacetals.

Substrate Stereospecificity Based on Isotope Trapping. The substrate stereospecificity of yeast and of porcine erythrocyte glyoxalase I was tested by an isotope-trapping method that monitors the ability of the enzyme to convert a diasteriomeric mixture of thiohemiacetal substrates directly to product before nonenzymic interconversion of the diasteriomers. In this procedure, a pulse solution composed of an equilibrium mixture of [^{3}H]GSH, phenylglyoxal, and the two diasteriomeric ^{3}H-labeled thiohemiacetals is introduced into a rapidly stirring chase solution composed of excess unlabeled GSH and enzyme. The concentration conditions of the pulse solution are such that >90% of the total phenylglyoxal is present as the thiohemiacetals. The kinetic scheme describing the pulse-chase reaction mixture is as follows:

$$^*\text{GSH} + \text{Ph} \overset{k_x}{\underset{}{\leftarrow}} H_S^* \underset{E}{\overset{E}{\rightleftharpoons}} E{\bullet}H_S^* \xrightarrow{k_2} E + P_S^* \quad (3)$$

$$^*\text{GSH} + \text{Ph} \overset{k_x'}{\underset{}{\leftarrow}} H_R^* \underset{E}{\overset{E}{\rightleftharpoons}} E{\bullet}H_R^* \xrightarrow{k_2'} E + P_R^* \quad (4)$$

where H_S^* and H_R^* are the two ^{3}H-labeled diasteriomers originating from the pulse solution; Ph = phenylglyoxal, *GSH = [^{3}H]GSH due to dissociation of the diasteriomers, E = enzyme, and P_S^* and P_R^* are the stereochemically identical mandeloylglutathione products originating from H_S^* and H_R^*, respectively.

The central feature of this kinetic scheme is that H_S^* and/or H_R^* can either be trapped by enzyme to form ^{3}H-labeled product or dissociate to *GSH and phenylglyoxal. Any phenylglyoxal formed in this way will most likely react with the large excess of unlabeled GSH originally in the chase solution, forming unlabeled thiohemiacetal that will then be converted by enzyme to unlabeled product. Hence, the dissociation of H_S^* and H_R^* is depicted as an irreversible process from the standpoint of radioactivity than can potentially be trapped in product.

The reactions shown in eq 3 and 4 can be treated as two independent simultaneous processes under conditions where total enzyme is approximately equal to free, unbound enzyme. This condition is met since the K_m values for the yeast enzyme (K_m = 0.24 mM, pH 5) and erythrocyte enzyme (K_m = 0.14 mM, pH 6), determined under the conditions of the pulse-chase mixture, substantially exceed the total initial concentration of substrate thiohemiacetals, $[H_S^*]_0 + [H_R^*]_0$ = 0.02 mM. The fractional trapping of the diasteriomers by the enzyme due to each of these reactions is derived from the fact that the rate of loss of thiohemiacetal is equal to the sum of the Michaelis–Menten term at the limit $K_m \gg$ [substrate] and the unimolecular dissociation rate term for the thiohemiacetal. Thus, from eq 3

$$-dH_S^*/dt = \left(\frac{k_2}{K_m^S}[E_t] + k_x\right)[H_S^*] \quad (5)$$

which gives

$$[H_S^*] = [H_S^*]_0 \exp\left[-\left(\frac{k_2}{K_m^S}[E_t] + k_x\right)t\right] \quad (6)$$

The rate of production of P_S^* is

$$dP_S^*/dt = (k_2/K_m^S)[E_t][H_S^*] \quad (7)$$

Integrating after substitution of $[H_S^*]$ from eq 6 gives

$$[P_S^*]_t = \frac{[H_S^*]_0[E_t]}{[E_t] + k_x(K_m^S/k_2)}\left(1 - \exp\left[-\left(\frac{k_2}{K_m^S}[E_t] + k_x\right)t\right]\right) \quad (8)$$

The final yield of P_S^* is given by

$$[P_S^*]_\infty = \frac{[H_S^*]_0[E_t]}{[E_t] + k_x(K_m^S/k_2)} \quad (9)$$

By the same reasoning from eq 4

$$[P_R^*]_\infty = \frac{[H_R^*]_0[E_t]}{[E_t] + k_x'(K_m^R/k_2')} \quad (10)$$

Defining F as $([P_S^*]_\infty + [P_R^*]_\infty)/([H_S^*]_0 + [H_R^*]_0)$ and given that $[H_S^*]_0 \simeq [H_R^*]_0$ at equilibrium (Brown et al., 1981)

$$F^{-1} = 2([1 + T(1/[E_t])]^{-1} + [1 + T'(1/[E_t])]^{-1})^{-1} \quad (11)$$

where $T = k_x(K_m^S/k_2)$ and $T' = k_x'(K_m^R/k_2')$.

The nonlinear plot of F^{-1} vs. $[E_t]^{-1}$ has the following important properties with respect to analyzing the isotope-trapping results: (1) If only one of the diasteriomeric thiohemiacetals serves as substrate for the enzyme or if both are used, then, as $[E_t]^{-1} \rightarrow 0$, the intercept on the F^{-1} axis approaches either ~2 or unity, respectively. (2) The curvature of the plot depends on the magnitude of T/T'. (3) The slope of the curve at the intercept on the F^{-1} axis is $(T + T')/2$ since this is the limit of the first derivative of eq 11 with respect to $[E_t]^{-1}$ when $[E_t]^{-1} = 0$. (4) Equation 11 reduces to a linear function when $T = T'$. A special case of this condition is when $k_x = k_x'$ and $K_m^S/k_2 = K_m^R/k_2'$

$$F^{-1} = k_x K_m\left(\frac{1}{k_2[E_t]}\right)\left(\frac{1}{F_i}\right) + \frac{1}{F_i} \quad (12)$$

where F_i is the "intrinsic" trapping when $[E_t]^{-1} = 0$ and is equal to unity or ~0.5 in the nonstereospecific and stereospecific cases, respectively.

Both yeast and porcine erythrocyte glyoxalase I conform to the nonstereospecific case. Table III illustrates the method used to calculate F from the specific radioactivity of mandeloylglutathione isolated from the pulse-chase mixtures. The plot of Figure 2 clearly shows that the enzyme from both sources can directly use both H_R and H_S as substrates, since the intercept values on the F^{-1} axis are near unity. In order to reduce the length of the extrapolation to the F^{-1} axis, the isotope-trapping experiments were run in dilute buffer at the lowest possible pH where the yeast (pH 5) and erythrocyte (pH 6) enzymes are still highly active. This maximizes the trapping at any given $[E_t]$ by minimizing base-catalyzed dissociation rate of the thiohemiacetals (Barnett & Jencks, 1969).

The nonenzymic dissociation rate constant of the thiohemiacetal substrates can also be estimated from Figure 2. To a first approximation, the data for both enzymes can be fit with eq 12, since there is no compelling evidence for deviations from linearity in the data. However, the figure also shows the theoretical deviations from linearity calculated with eq 11 if $T \neq T'$. Conservatively, the magnitudes of T and

Table III: Results of Isotope-Trapping Experiments with Yeast Glyoxalase I Showing Variation of Fractional Radioactivity Trapped in Mandeloylglutathione (F) vs. V_{max} Units of Enzyme/mL ($[E_t]$) in Pulse-Chase Mixture[a]

		mandeloylglutathione[b]			
run no.	$[E_t]$ (units/mL)	μmol/mL	cpm/mL	sp radioactivity (SpA_x) (cpm/μmol)	F[e]
min[c]		0.018	622	34 556	0
1	3.04	0.021	2780	132 380	0.404
2	5.07	0.0177	3133	177 006	0.588
3	10.10	0.0166	3297	198 614	0.677
4	20.30	0.0145	3343	230 552	0.809
5	101.30	0.0161	4027	250 124	0.890
max[d]		0.0183	5553	303 443	1.000

[a] Pulse solutions (0.05 mL) contained [^{3}H]GSH (12 mM, pH 5, ~3 × 10^5 cpm/μmol) and phenylglyoxal (~0.4 mM) dissolved in a buffer system composed of KCl (0.1 M), sodium phosphate (1 mM, pH 5), and BSA (0.1 mg/ml); chase solutions (0.95 mL) contained GSH (~6 mM, pH 5) and enzyme dissolved in the same buffer system. [b] Mandeloylglutathione was isolated from the pulse-chase mixture by high-performance liquid chromatography in a total volume of ~1 mL and the specific radioactivity determined as described under Experimental Procedures. [c] This is a control for the minimum specific radioactivity of mandeloylglutathione (SpA_{min}) expected if there is *no trapping* of the diasteriomeric thiohemiacetals by the enzyme before their nonenzymic interconversion in the pulse-chase mixture. SpA_{min} is taken as the specific radioactivity of mandeloylglutathione isolated from an incubation mixture composed of 0.95 mL of chase solution (minus enzyme) plus 0.05 mL of pulse solution followed by ~9 units of enzyme. [d] This is a control for the maximum specific radioactivity of mandeloylglutathione (SpA_{max}) expected if both diasteriomeric thiohemiacetals from the pulse solution are *completely trapped* by the enzyme before nonenzymic interconversion of the diasteriomers. SpA_{max} is taken as the specific radioactivity of mandeloylglutathione isolated from a mixture of 0.95 mL of buffer plus 0.05 mL of pulse solution and ~9 units of enzyme. [e] Fractional trapping was calculated from the following equation: $F = [(SpA_x - SpA_{min})/(SpA_{max} - SpA_{min})](1 + K_{diss}/[GSH]_{pulse})$. The first term of the equation is the observed fractional trapping (F_{obsd}) defined as $F_{obsd} = X/(X + Y + Z)$ where X = product formed by direct trapping of thiohemiacetal by enzyme, Y = product formed by enzyme *after* thiohemiacetal from the pulse solution has equilibrated with the free GSH in the chase solution (untrapped), and Z = the small amount of free phenylglyoxal (~10%) in the pulse solution that ultimately ends up as product. Thus, $SpA_x = F_{obsd}(SpA_{max}) + (1 - F_{obsd})(SpA_{min})$, which is then solved for F_{obsd}. The second term of the equation is the relationship between F_{obsd} and the desired quantity F, defined as $X/(X + Y)$, given that $K_{diss} \cong [GSH]_{pulse}[Z/(X + Y)]$. K_{diss} = 1.32 mM, the thiohemiacetal dissociation constant determined under the conditions of the pulse solution, and $[GSH]_{pulse}$ = 12 mM.

T' appear to be within a factor of 5 for the yeast enzyme and probably within a factor of 2 for the erythrocyte enzyme. The thiohemiacetal dissociation rate constants were calculated under the different conditions of the pulse-chase mixtures, using the yeast and the erythrocyte enzymes, from the slopes of the lines through the data and the independently determined values of K_m with eq 12: k_x(yeast, pH 5) = 7.63 × 10^{-5} M s^{-1}/(0.24 × 10^{-3} M) = 0.32 s^{-1}; k_x(erythrocyte, pH 6) = 1.98 × 10^{-4} M s^{-1}/(0.14 × 10^{-3} M) = 1.4 s^{-1}.

Discussion

Isotope-Trapping Studies. The central outcome of these studies is that glyoxalase I can convert both diasteriomers of the thiohemiacetal formed from phenylglyoxal and GSH directly to product before the nonenzymic interconversion of the diasteriomers. That this is a property of a single active site is supported by (a) equilibrium binding studies indicating one active site in monomeric yeast glyoxalase I (Marmstal & Mannervik, 1979) and (b) the fact that the erythrocyte glyoxalase I used in the isotope-trapping studies was purified to apparent homogeneity by affinity chromatography (Aronsson & Mannervik, 1977).

The possibility that the two diasteriomers are used with different efficiencies by the enzyme was previously suggested from the fact that they are lost at different rates in the presence of yeast glyoxalase I (Brown et al., 1981). However, the absolute efficiency with which each diasteriomer was used could not be calculated in the absence of knowing the nonenzymic rate of interconversion of the diasteriomers. The isotope-trapping studies do not offer any compelling evidence for unequal usage of the diasteriomers by either yeast or porcine erythrocyte glyoxalase I, since there is no clear indication of curvature in the plot of Figure 2. On the other hand, the error in the data of this plot could conceivably accommodate curvatures due to unequal usage in which K_m/k_2 for each diasteriomer differ by a factor of about 2 and about 5 for the erythrocyte and yeast enzymes, respectively. Furthermore, any potential curvature of this plot reflects not only the relative magnitudes of K_m/k_2 for each diasteriomer but also the relative magnitudes of the nonenzymic dissociation rate constants for each diasteriomer, since T and T' are composites of the rate constants describing both kinetic processes, eq 11. A nonunity value of $(K_m^S/k_2)/(K_m^R/k_2')$ could be concealed by an equal but opposite effect on k_x/k_x', resulting in a linear plot. Thus, the question of the efficiency with which each diasteriomer is used by the enzyme must remain open until k_x/k_x' can be independently evaluated.

The capacity of glyoxalase I to use both diasteriomers as substrates may well have evolved in response to the need to efficiently remove cytotoxic α-ketoaldehydes from the cell. If under physiological conditions the formation of the thiohemiacetal from α-ketoaldehyde and GSH is under nonenzymic control, glyoxalase I is constrained to always operate on an equilibrium mixture of both diasteriomers. In this case nonstereospecific usage of the diasteriomers is more rapid than stereospecific usage by $2((k_x + k_2[E_t]/K_m)/k_x)$ under the conditions where substrate concentration is much less than K_m and assuming that $k_x \simeq k_x'$ and $K_m^S/k_2 \simeq K_m^R/k_2'$. The apparent advantage of the nonstereospecific case is that the nonenzymic rate of interconversion of the diasteriomers does not play a role in determining the rate of substrate usage by the enzyme. For example, in human blood, the rate advantage would be a factor of 2–3, given that there are ~38 units/mL of glyoxalase I in whole blood with methylglyoxal–thiohemiacetal as substrate (K_m = 0.13 mM; Aronsson et al., 1979) and, if at physiological pH, k_x is roughly in the range 10–40 s^{-1}. The latter range of values was extrapolated from the experimentally determined values of k_x at pH 5 and at pH 6, assuming that the magnitude of k_x is primarily determined by hydroxide ion concentration (Barnett & Jencks, 1969).

Two general mechanisms can be envisioned to account for the ability of glyoxalase I to use both diasteriomeric thiohemiacetals as substrates. One possibility is that either one of the two stereochemical pathways depicted in Figure 1 is operative, depending on which diasteriomer binds to the active

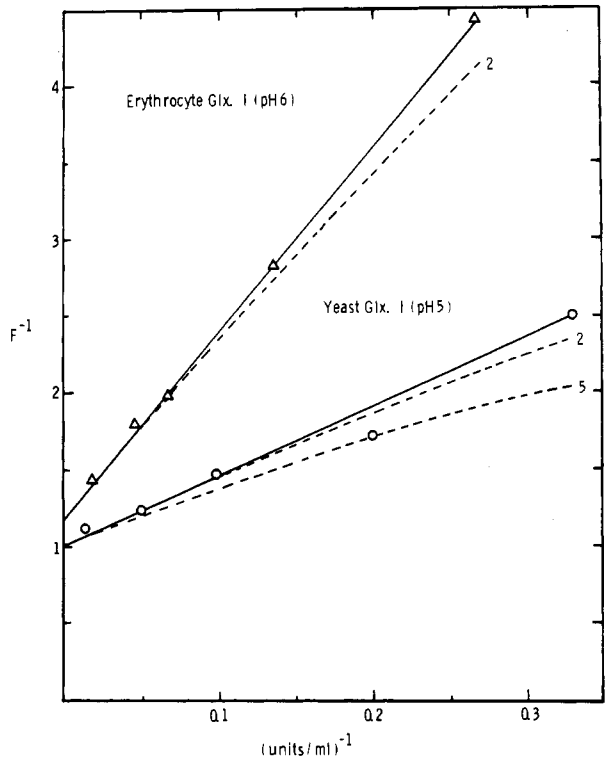


FIGURE 2: Isotope-trapping results using yeast (O) and porcine erythrocyte (Δ) glyoxalase I. The reciprocal of fractional radioactivity trapped in mandeloylglutathione (F^{-1}) vs. the reciprocal of V_{max} units of enzyme/mL $[(k_2[E_t])^{-1}]$ is plotted according to eq 12. The conditions of the experiment using yeast glyoxalase I are given in Table III. For the experiment using erythrocyte glyoxalase I, a different buffer system was employed in the pulse and chase solutions in order to maximize the stability of the enzyme: KCl (90 mM), $MgCl_2$ (10 mM), sodium phosphate (1 mM, pH 6), and BSA (0.1 mg/mL). Under these conditions, the thiohemiacetal dissociation constant is 1.94 mM. Also shown in the figure are the expected deviations from linearity if $T \neq T'$, eq 11. The deviations were calculated by first assuming that the solid line through the data gives the limiting slope $[(T + T')/2]$. Unique values of T and T' were calculated when $T/T' = 2$ and 5 (– –). These values were then inserted into eq 11, and the variation of F^{-1} vs. $[E_t]^{-1}$ was determined.

site. A clear disadvantage of this mechanism is that the same active site must be capable of accommodating *both* a *cis*- and a *trans*-enediol intermediate and catalyzing their conversion to final product by a mechanism that presumably involves different active site residues. A seemingly more likely possibility is that the enzyme catalyzes the interconversion of the bound diasteriomers so that only one of them is directly transformed to product by a single stereochemical pathway involving either a *cis*- or a *trans*-enediol intermediate but not both. By this mechanism, the interconversion of the bound diasteriomers *must* be catalyzed since k_x is significantly less than the catalytic turnover number of glyoxalase I from yeast [k_{cat}(methylglyoxal) = 700 s^{-1}, k_{cat}(phenylglyoxal) = 665 s^{-1}, pH 7 and 25 °C; Vander Jagt & Han, 1973] and from porcine erythrocytes [k_{cat}(methylglyoxal) = 547 s^{-1}, pH 7, 30 °C; Aronsson & Mannervik, 1977]. Phosphoglucose isomerase represents an analogous situation in that both the α and β anomers of glucose 6-phosphate are used as substrates (Rose, 1975). The enzyme appears to catalyze the interconversion of the bound anomers, and a *cis*-enediol is the exclusive intermediate along the reaction pathway.

Inhibition Studies. The high affinity of glyoxalase I for both the cis and the trans isomers of the (phenylethenyl)glutathione derivatives may be an important clue to the mechanism that allows the enzyme to use both diasteriomeric thiohemiacetals as substrates, Table II.

Chart I

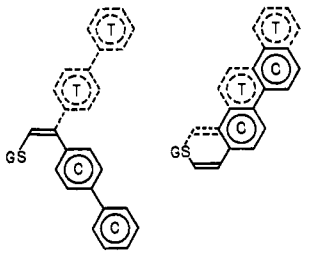
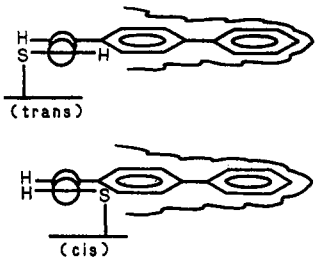


Chart II



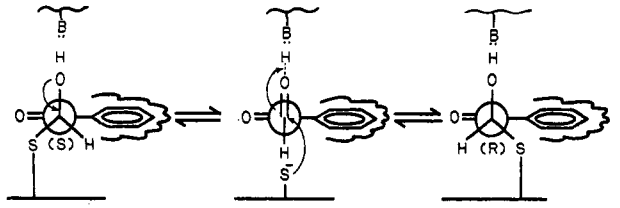
For all of the GSH derivatives listed in Table II, the decreasing inhibition constants observed down the table roughly parallel the size and hydrophobic nature of the substituent. This accords well with a previous conclusion that hydrophobic interactions in the region of the active site near the glutathionyl sulfur may be an important determinant of binding, on the basis of the finding that as the hydrophobicity of various *S*-alkyl and para-substituted *S*-(arylalkyl)glutathione derivatives increase, their inhibition constants with yeast glyoxalase I decrease (Vince & Wadd, 1969).

In addition, the cis and the trans isomers for any given derivative bind with nearly equal affinity. In the case of yeast glyoxalase I the K_i's for the different derivatives tested differ by almost an order of magnitude, yet the K_i's for the cis and the trans isomers for any given derivative differ by a factor of only 1–2. For the erythrocyte enzyme, the K_i's for the cis and the trans isomers are also similar in magnitude. The *p*-methyl derivative appears to be an exception in that the inhibition constants for the cis and the trans isomers differ by a factor of 6.

A broad hydrophobic site of nearly uniform properties would be required to explain nearly equal binding by the cis and the trans isomers, if the glutathionyl sulfur atom is fixed in one position in the active site. This was previously argued to be a reasonable explanation for the nearly equal inhibition constants observed with *cis*- and *trans*-(phenylethenyl)glutathione for yeast glyoxalase I (Creighton et al., 1980). Shown in Chart I are the minimum and maximum dimensions of the hydrophobic binding site required in order to accommodate both *cis*- and *trans*-*S*-[(*p*-phenylphenyl)ethenyl]glutathione, depending on whether the vinyl bond is free to occupy one or two positions in the active site. On the other hand, such a broad and uniform site seems less likely in view of the quantitative structure–activity relationship deduced (Silipo & Hansch, 1979) from the inhibition of yeast glyoxalase I by 37 different S-substituted GSH derivatives (Vince et al., 1971). According to this analysis, polar as well as apolar residues compose the "hydrophobic" site.

An alternative and perhaps simpler explanation for the equality of binding is that the aromatic rings of the isomers occupy nearly identical positions in the active site. This would be possible if the glutathionyl moiety of the bound isomers had sufficient conformational flexibility so as to allow the sulfur atom to occupy two positions in the active site (Chart II). The

Scheme I



same kind of positional mobility can be envisioned for the bound diasteriomers of substrate and may be of underlying importance for allowing general base catalyzed interconversion of H_R and H_S, provided this partial reaction has been correctly deduced to take place. Thus, either the C-1 hydroxyl or C-1 proton of bound H_R or H_S could be positioned near a single active site base that catalyzes either interconversion of the diasteriomers or formation of an enediol intermediate (Scheme I).

Supplementary Material Available

Detailed syntheses of para-substituted *S*-(phenylethenyl)-glutathione derivatives (7 pages). Ordering information is given on any current masthead page.

Registry No. *cis*-C_6H_5—CH=CHSG, 73951-41-4; *trans*-C_6H_5—CH=CHSG, 74006-56-7; *cis-p*-CH_3—C_6H_4—CH=CHSG, 85320-64-5; *trans-p*-CH_3—C_6H_4—CH=CHSG, 85354-22-9; *cis-p*-Cl—C_6H_4—CH=CHSG, 85320-65-6; *trans-p*-Cl—C_6H_4—CH=CHSG, 85404-33-7; *cis-p*-C_6H_5—C_6H_4—CH=CHSG, 85320-66-7; *trans-p*-C_6H_5—C_6H_4—CH=CHSG, 85354-23-0; H_R, 85354-27-4; H_S, 85354-24-1; glyoxalase I, 9033-12-9; *S*-D-mandeloylglutathione, 79813-64-2; glutathione (*R*)-methylglyoxalthiohemiacetal, 85354-25-2; glutathione (*S*)-methylglyoxalthiohemiacetal, 85354-26-3.

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