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Isomerization of (*R*)- and (*S*)-Glutathiolactaldehydes by Glyoxalase I: The Case for Dichotomous Stereochemical Behavior in a Single Active Site†

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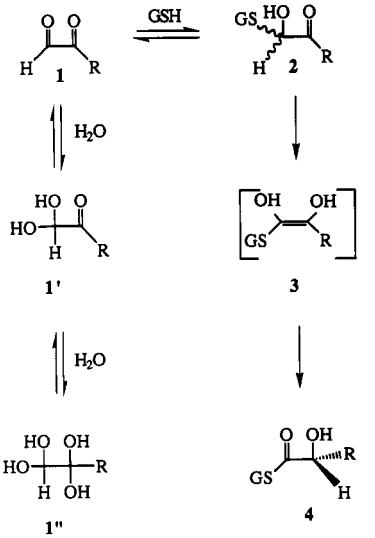
ABSTRACT: The ability of glyoxalase I to isomerize both diastereomeric thiohemiacetals formed between glutathione and α-ketoaldehydes has been probed with stereochemically "locked" substrate analogues. Both (*R*)- and (*S*)-glutathiolactaldehyde (**5** and **5'**) were unambiguously synthesized by employing the Sharpless epoxidation procedure as a key step. In the presence of human erythrocyte glyoxalase I, high-field ^{1}H NMR analysis reveals that the *R* and *S* isomers (~20 mM) are both converted to glutathiohydroxyacetone at rates of 0.8 and 0.4 s^{-1}, respectively. This reaction is characterized by a nonstereospecific proton abstraction followed by a partially shielded proton transfer to the *si* face of the *cis*-enediol intermediate. Glyoxalase I catalyzes the exchange of the *pro-S* proton of glutathiohydroxyacetone with solvent deuterium. Glutathiohydroxyacetone was found to be a good competitive inhibitor of the normal glyoxalase I reaction (K_I = 1.46 mM), suggesting that the slow processing rate of these compounds with respect to the normal thiohemiacetals is not due to poor binding. The results are consistent with a nonstereospecific proton abstraction and a stereospecific reprotonation at contiguous substrate carbons.

The stereochemical course of an enzymatic reaction is an end product of the chemical mechanism and of the spatial organization of the active site functional groups employed by the enzyme to effect catalysis. As a result of the asymmetry inherent in an enzyme active site, enzymes, in general, demonstrate little leniency for changes in substrate stereochemistry, especially at chiral centers which undergo reaction. In addition, the formation of stereochemically ambiguous products is usually not observed. Thus, the absolute stereospecificity with which substrate is converted to product is a hallmark of enzymatic catalysis. However, the selective pressure of a particular physiological condition coupled with the economy of nature may produce an enzyme with broadened substrate stereospecificity. Glyoxalase I has been studied over the years as a paradigm for this type of behavior.

Glyoxalase I [*S*-lactoylglutathione methylglyoxal-lyase (isomerizing) EC 4.4.1.5, GX I[1]] catalyzes the conversion of the thiohemiacetal **2** of α-ketoaldehyde **1** and glutathione [*N*-(*N*-L-γ-glutamyl-L-cysteinyl)glycine, GSH] to thioester

Scheme I: Glyoxalase I-Catalyzed Reaction



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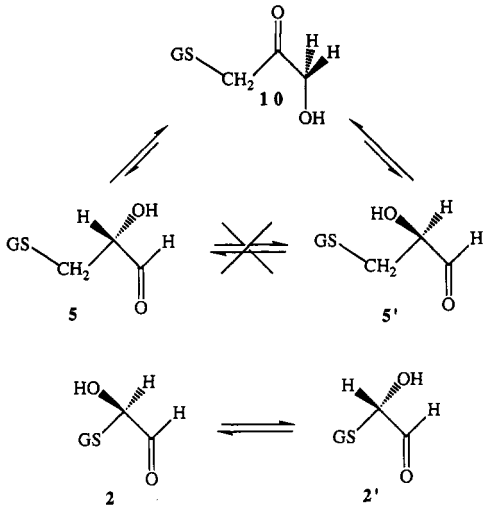
Scheme II: Comparison of the Stereochemically Ambiguous Thiohemiacetals with the Stable Diastereomeric Glutathiolactaldehydes[a]



[a] The rate of interconversion of the thiohemiacetals (**2** and **2'**), estimated to be between 10 and 40 s^{-1}, is contrasted by the configurationally stable **5** and **5'**. The chemical interconversion of **5** and **5'** via **10** is slow and favors the formation of **10**.

4 of an α-hydroxy acid and glutathione (Racker, 1961; Scheme I). The reaction proceeds via a shielded proton transfer with the presumed intermediacy of *cis*-enediol **3** (Hall et al., 1976; Shikai et al., 1981; Kozarich et al., 1981; Chari & Kozarich, 1981), and the resulting acid has been established as the D isomer (Ekwall et al., 1973; Vander Jagt et al., 1975). The substrate for the enzyme is the thiohemiacetal formed in a chemical preequilibrium step between glutathione and α-ketoaldehyde (Cliffe & Waley, 1961; Davis & Williams, 1969; Vander Jagt et al., 1975; Rae et al., 1990), the latter being in equilibrium with its monohydrate **1'** and dihydrates **1''** (Creighton et al., 1988). The enzyme, then, operates on a mixture of interconverting diastereomers (epimers). This has complicated the question of whether GX I facilitates thiohemiacetal interconversion with stereospecific proton abstraction from one favored epimer [presumably the (*S*)-thiohemiacetal] or whether GX I exhibits an unusual stereorandom proton abstraction with stereospecific protonation to give product. Previous attempts in a number of laboratories to address this issue have been inconclusive.

We report here the results of our strategy to answer this question. The approach utilizes (*R*)- and (*S*)-glutathiolactaldehydes, chemically synthesized with high stereoselectivity, as configurationally "locked" thiohemiacetal analogs (**5** and **5'**, Scheme II) to probe the stereochemical requirements of both the enzymatic abstraction and addition of the proton. The ^{1}H NMR analysis reveals that GX I catalyzes the conversion of both epimers to the same product stereoisomer with a significant degree of transfer of substrate proton to product for both epimers. The findings are consistent with a remarkable coexistence of stereorandomness and stereospecificity for proton transfers at contiguous carbons by a single active site.

Materials and Methods

Materials. All chemicals (Aldrich) used in synthetic procedures were of reagent grade or higher. Where indicated, solvents were dried using standard procedures. Commercial glutathione obtained 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 assay with 4-pyridine disulfide (Aldrich) (Grasseti & Murray, 1967) or DTNB (Aldrich) (Ellman, 1959). Methylglyoxal (Aldrich, 40% aqueous) was purified by vacuum distillation (Kermack & Matheson, 1957). Contaminating lactic acid was removed from the distillate by filtration through a Dowex 1 carbonate column. Methylglyoxal was quantitated by the procedure of Gawehn and Bergmeyer (1974) using yeast GX I (Sigma). Glutathiohydroxyacetone (**10**; GHA) was prepared by the method of Chari and Kozarich (1983).

General Methods. ^{1}H NMR spectra were recorded on a Bruker AM 400 (FT) or a Bruker AF 200 (FT) instrument, as indicated. For enzymatic reactions, the temperature of the probe was maintained at 25 °C. Chemical shifts were standardized to an HDO resonance at 4.7 ppm or a TMS resonance at 0.0 ppm in $CDCl_3$. Mass spectra were recorded on a VG 7070H spectrometer. IR spectra were recorded on a Nicolet 5DXC FT-IR spectrophotometer. Elemental analyses were performed by Galbraith Laboratories, Inc. Melting points were determined with a Thomas Hoover Uni-melt capillary melting point apparatus, and the reported values are uncorrected. Optical rotations were measured using a JASCO Model 370DIP polarimeter. UV assays were performed on a Gilford Response II spectrophotometer equipped with a Lauda M20 external circulating water bath.

Synthetic Methods. (R)-Oxiranemethanol (**6**) *and (S)-Oxiranemethanol* (**6'**). (*R*)-Oxiranemethanol (**6**) and (*S*)-oxiranemethanol (**6'**) were each prepared by the method of Sharpless and co-workers (Gao et al., 1987) by employing D-(−)- and L-(+)-diisopropyl tartrate, respectively, on a 2-fold scale. For the *S* isomer (**6'**) distillation of the crude product at reduced pressure (50–52 °C, 5 mmHg) afforded 21.7 g of a mixture containing 2-phenyl-2-propanol, cumene, cumene hydroperoxide, and 42% glycidol (as determined by ^{1}H NMR, 0.122 mol, 61% theoretical yield; the ^{1}H NMR spectrum of this compound is identical to that of the racemic mixture supplied by Aldrich). An enantiomeric excess of 88% (Gao et al., 1987; 90% ee) was determined by ^{1}H NMR ($CDCl_3$) of the complex formed between Eu(hfc)$_3$ and the diacetate of the ring-opened diol formed between glycidol and thiophenol. The *R* isomer was obtained as 40% of the crude product and found to be in an enantiomeric excess of 86%.

(R)-Oxiraneformaldehyde (**7'**) *and (S)-Oxiraneformaldehyde* (**7**). (*R*)-Oxiraneformaldehyde (**7'**) and (*S*)-oxiraneformaldehyde (**7**) were each prepared using the oxidation procedure of Moffatt (1967) with the appropriate alcohol. To a stirring solution of crude (*R*)-oxiranemethanol or (*S*)-oxiranemethanol (17.8 g, ~100 mmol) in 200 mL of anhydrous ether were made the following additions: DCC (30.95 g, 150 mmol), DMSO (7.8 mL, 8.6 g, 110 mmol), and pyridine (4.0 mL, 3.96 g, 50 mmol). This solution was cooled to 0 °C, and TFA (3.7 mL, 5.5 g, 48 mmol) was slowly added. The mixture was stirred for an additional 10 min at 0 °C and then at room

[1] Abbreviations: BHDMA, bromohydrin dimethyl acetal; DCC, 1,3-dicyclohexylcarbodiimide; DHAP, dihydroxyacetone phosphate; DMSO, dimethyl sulfoxide; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); EPR, electron paramagnetic resonance; EtOAc, ethyl acetate; Eu(hfc)$_3$, tris-[(3-heptafluoropropylhydroxymethylene)-*d*-camphorato]europium; EXAFS, extended absorption fine structure; GAP, glyceraldehyde 3-phosphate; GHA, glutathiohydroxyacetone, *N*-[*N*-L-γ-glutamyl-*S*-(3-hydroxy-2-oxopropyl)-L-cysteinyl]glycine; GLA, glutathiolactaldehyde, *N*-[*N*-L-γ-glutamyl-*S*-(2-hydroxy-3-oxopropyl)-L-cysteinyl]glycine; GLADMA, glutathiolactaldehyde dimethylacetal, *N*-[*N*-L-γ-glutamyl-*S*-(3,3-dimethoxy-2-hydroxypropyl-L-cysteinyl]glycine; GSH, glutathione, *N*-(*N*-L-γ-glutamyl-L-cysteinyl)glycine; GX I, glyoxalase I; GX II, glyoxalase II; NMR, nuclear magnetic resonance; ppm, parts per million; TFA, trifluoroacetic acid; THF, tetrahydrofuran; TIM, triosephosphate isomerase; TMS, trimethylsilane; TSP, 3-(trimethylsilyl)-tetradeuteriosodium phosphate.

temperature for 3 h. Attempts to monitor reaction progress by TLC were hindered by an apparent reaction of oxiraneformaldehyde with the silica. After 3 h, the mixture was filtered, and solvent was removed from the filtrate under reduced pressure. For the *S* isomer, vacuum distillation of the resulting mixture (32–37 °C, 40 mmHg) gave 6.3 g of a mixture containing predominantly pyridine and 31% of the desired oxiraneformaldehyde (31 mmol) as indicated by ^{1}H NMR. The *R* isomer was obtained in a similar manner in 29% yield: ^{1}H NMR ($CDCl_3$, 200 MHz) δ 8.96 (d, 1, J = 6.5 Hz), 3.37 (ddd, 1, J = 2.4, 4.5, 6.5 Hz), 3.15 (dd, 1, J = 4.5, 5.3 Hz), 3.03 (dd, 1, J = 5.3, 2.4 Hz).

3-Bromo-1,1-dimethoxy-2(R)-propanol (**8**) *and 3-Bromo-1,1-dimethoxy-2(S)-propanol* (**8′**) [*(R)-BHDMA*, *(S)-BHDMA*]. The following procedure was adapted from that noted by Behrens and Sharpless (1985). (*R*)-Oxiraneformaldehyde (**7′**) and (*S*)-oxiraneformaldehyde (**7**) were each protected as the bromohydrin dimethyl acetals. To an oven-dried, three-neck, 2-L flask was added 1 L of anhydrous methanol under a nitrogen atmosphere. Tetraethylammonium bromide (29.4 g, 0.14 mol) and either of the oxiraneformaldehyde enantiomer/pyridine mixtures (~31 mmol) were added to the methanol with stirring. This solution was cooled to −78 °C, and acetyl bromide (10.35 mL, 17.2 g, 0.14 mol) was added via an addition funnel over a period of 20 min. The system was sealed and the reaction was allowed to warm to room temperature. After stirring overnight, the solution was neutralized by the addition of solid $NaHCO_3$ and solvent was removed under reduced pressure. The bromohydrin dimethyl acetal was extracted from the resulting white solid with 200 mL of ice-cold THF. The extract was concentrated to $^1/_{10}$ of the original volume, cooled to −20 °C, and rapidly filtered. The concentrated filtrate was subjected to flash chromatography (300-mm diameter, 18-cm silica bed; 4:1 EtOAc/hexane). This procedure afforded 4.48 g (23 mmol, 73%) of (*R*)-BHDMA and 4.26 g (21.5 mmol, 70%) of (*S*)-BHDMA: $[\alpha]^{22}_D$ (*R*)-BHDMA −17.3° (1.33, $CHCl_3$); $[\alpha]^{22}_D$ (*S*)-BHDMA +17.1° (1.33, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 200 MHz) δ 4.39 (d, 1, J = 5.0 Hz), 3.83 (m, 1), 3.63 (dd, 1, J = 3.7, 10.7 Hz), 3.61 (dd, 1, J = 3.7, 10.7 Hz), 3.50 (s, 3), 3.49 (s, 3); IR ($CHCl_3$) 3582, 3340, 2910, 2830, 2397, 1740, 1449, 1265, 1126, 1076, 975 cm^{-1}; mass spectrum, m/z (relative intensity) 197 (13), 195 (M − 3), 183 (17), 181 (19), 169 (38), 167 (46), 137 (58), 135 (66), 125 (17), 123 (18), 109 (18), 107 (16), 85 (49), 83 (61), 75 (100); mass spectrum, m/z 197.9891; calcd for $C_5H_{11}BrO_3$, 197.9892.

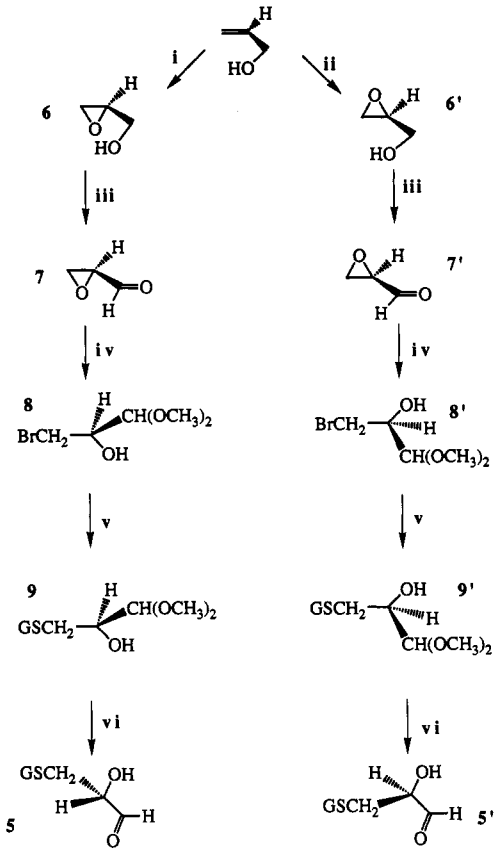
N-[N-L-γ-Glutamyl-S-(3,3-dimethoxy-2(R)-hydroxypropyl)-L-cysteinyl]glycine (**9**) *and N-[N-L-γ-glutamyl-S-(3,3-dimethoxy-2(S)-hydroxypropyl)-L-cysteinyl]glycine* (**9′**) [*(R)-Glutathiolactaldehyde Dimethyl Acetal*, *(R)-GLADMA*; *(S)-Glutathiolactaldehyde Dimethyl Acetal*, *(S)-GLADMA*]. In a three-neck 20-mL round-bottom flask, under nitrogen atmosphere, glutathione (9.22 g, 30 mmol) was added to 10.0 mL of deionized, degassed H_2O and adjusted to pH 8.2 with 1 N KOH. (*R*)-BHDMA (**8**) or (*S*)-BHDMA (**8′**) (3.96 g, 20.0 mmol) in 3.0 mL of MeOH was added to the above solution with stirring. The pH was maintained at 8.2 by the frequent addition of 1 N KOH and was allowed to stir overnight. Solvent was removed to give a yellow, hygroscopic solid which was triturated with acetone. ^{1}H NMR showed this solid to contain a substantial amount of unreacted glutathione. Purification of (*R*)-GLADMA and (*S*)-GLADMA was accomplished using anion-exchange chromatography. Crude GLADMA (1.2 g) was dissolved in 5.0 mL of deionized H_2O, and the solution was brought to pH 8.5 by the addition of 1 N KOH. The unreacted glutathione (GSH) was oxidized (GSSG) in the presence of 0.5 mM in $Fe(NH_4)_2(SO_4)_2$ and saturating oxygen. Oxidation of GSH was monitored by reduction of DTNB at 412 nm (ϵ = 13.6 mM^{-1} cm^{-1}). When oxidation was complete (≈4 h), the solution was applied to a Dowex 1×2 formate column (2.5 cm × 50 cm). After an H_2O wash, GLADMA was eluted with 1 L of a 0–0.3 M formic acid gradient. Solvent was removed from the pooled GLADMA fractions (detected with ninhydrin) under reduced pressure. Removal of residual formic acid was accomplished by repeated addition/removal of H_2O. Trituration with acetone gave analytically pure samples: $[\alpha]^{22}_D$ ((*R*)-GLADMA) −30.9° (0.33, H_2O); $[\alpha]^{22}_D$ ((*S*)-GLADMA) −4° (0.17, H_2O) [glutathione: $[\alpha]^{25}_D$ −17° (2, H_2O)]; mp 107–110 °C; ^{1}H NMR (D_2O, 400 MHz) δ 4.48 (dd, 1, J = 5.1, 8.2 Hz), 4.28 (d, 1, J = 5.53 Hz), 3.61 (m, 4), 3.37 (s, 3), 2.97 (dd, 1, J = 5.1, 13.9 Hz), 2.68 (m, 3), 2.42 (m, 2), 2.06 (m, 2). 2-D COSY NMR established that the multiplet at δ 3.61 contained resonances for the α-proton of glutamate, the methylene protons of glycine, and the C-2 proton of the adduct. The resonance at δ 2.97 corresponds to one proton of the cysteine methylene, while the multiplet at δ 2.68 corresponds to the other cysteine proton as well as the adduct methylene protons. The two diastereomers have minor chemical shift differences. Anal. Calcd for $C_{15}H_{27}N_3O_9S$: C, 42.34; H, 6.4; N, 9.88; S, 7.52. Found: C, 42.79; H, 6.61; N, 9.66; S, 6.97.

GLADMA Deprotection. Each GLADMA diastereomer (60 mg, 0.14 mmol) was added to 1.5 mL of 1 N H_2SO_4 and maintained at 30 °C for 36 h. Acid was removed by passing samples through a 10-fold excess of Amberlite IRA-68 resin in a sintered glass funnel. Potassium phosphate buffer (2.25 mL, 100 mM) was added to these samples, and the solutions were passed through a 2.0-mL Chelex 100 column. The pH was adjusted to 6.5 with 1 N HCl and the solvent then exchanged with D_2O by repeated lyophilization and reconstitution with D_2O to 2.25 mL (pD = 7.0). Sample concentration for NMR experiments was ≈20 mM as determined with TSP as an internal standard in control samples.

Enzyme Purification. Human erythrocyte glyoxalase I was purified following the procedure of Mannervik and co-workers (Mannervik et al., 1982) and used as a mixture of the three isozymes. While the isozymes have different electrophoretic and chromatographic properties, they have been reported to be kinetically indistinguishable (Mannervik et al., 1982). Protein concentration was determined using the micro-biuret assay (Bailey, 1962) with bovine serum albumin (Sigma) as a standard. For NMR experiments, enzyme was repeatedly exchanged with deuteriated 100 mM phosphate buffer, pD 7.0, by ultrafiltration (Centricon 10, Amicon). Enzyme activity was assayed by the method of Racker (1951) using methylglyoxal and glutathione; the formation of the thioester, *S*-D-lactoylglutathione, was monitored at 240 nm (ϵ = 3300 M^{-1} cm^{-1}). One unit of activity is defined as that amount of enzyme required to form 1.0 μmol of thioester/min at pH 7.0 in 100 mM phosphate buffer at 25 °C.

Inhibition Studies. The competitive inhibition of glyoxalase I by glutathiohydroxyacetone was determined vs the substrate thiohemiacetal formed between glutathione and methylglyoxal. Methylglyoxal and glutathione (100 and 33 mM stock solutions, respectively) were introduced into assay cuvettes (±glutathiohydroxyacetone) to give a final volume of 1.0 mL in 100 mM sodium phosphate (0.5 M stock; pH 7.0). The concentrations of total methylglyoxal and glutathione used to form the desired level of thiohemiacetal (0.05–0.5 mM) in the presence of a fixed level of free glutathione (0.1 mM) were

Scheme III: Synthesis of the Diastereomeric Glutathiolactaldehydes (**5**, **5'**)[a]



[a] Reagents/conditions: i, Sharpless epoxidation, L-(+)-DIPT; ii, Sharpless epoxidation, D-(+)-DIPT; iii, DCC, DMSO, pyridinium trifluoroacetate, Et_2O; iv, tetraethylammonium bromide, acetyl bromide, MeOH, −78 °C RT; v, GSH, pH 8.0; vi, 1 N H_2SO_4.

calculated from the dissociation constant (K_D) of the thiohemiacetal: 3×10^{-3} M (Vander Jagt & Han, 1973). The concentration of glutathiohydroxyacetone (100 mM stock solution) was varied between 0.5 and 5.0 mM.

Glyoxalase I Catalyzed Processing of the Diastereomeric Glutathiolactaldehydes. ^{1}H NMR was used to follow the reaction of glyoxalase I with each glutathioactaldehyde diastereomer. The temperature of the probe was maintained at 25 °C. The concentration of substrate (≈20 mM, 0.75 mL) was determined for control samples as described above. The reactions were initiated by the addition of 56 units of glyoxalase I (0.06 mg, exchanged into deuteriated phosphate buffer, pD 7.0) to each glutathiolactaldehyde sample preequilibrated at 25 °C. The reaction was monitored by recording successive ^{1}H NMR spectra with a Bruker AM 400 NMR spectrometer. The spectrum associated with each time point (16 transients with a relaxation delay of 2 s and a pulse width of 9 μs) required a total acquisition time of ≈1 min. Chemical shifts were standardized to the HDO resonance at 4.70 ppm, and the intensity of the resonances of interest represents an average value from two determinations that were typically within 5% deviation of one another. To ensure that the slow rate of processing of the glutathiolactaldehydes was not due to an instability of the enzyme in their presence, enzyme activity was determined by the UV assay subsequent to the NMR experiment and found to be without change during the time course of the experiment.

Results

Synthesis of Diastereomeric Glutathiolactaldehydes. The syntheses of the diastereomeric dimethyl acetals, (*R*)-GLADMA (**9**) and (*S*)-GLADMA (**9'**), are shown in Scheme III. Two features crucial to the successful syntheses are the high enantioselectivity observed with the Sharpless epoxidation procedure using allyl alcohol/diisopropyl tartrate and the retention of the chiral center during formation of the bromohydrin dimethyl acetal. Thus, the chirality of the carbon susceptible to proton abstraction by glyoxalase I is known unambiguously and remains intact during the entire synthesis. The integrity of chirality is substantiated by the observation of unique ^{1}H NMR resonances for the α-cysteinyl proton and the dimethyl acetal proton for the individual diastereomeric GLADMA compounds as determined by a mixture of both diastereomers (Figure 1).

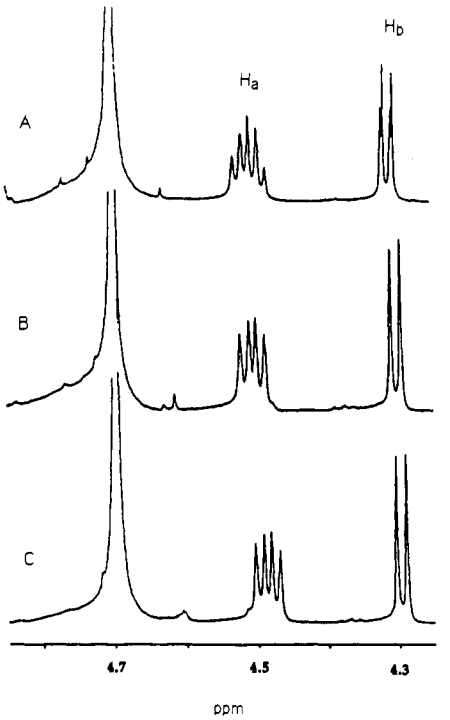


FIGURE 1: Verification of diastereomeric glutathiolacetaldehydes via ^{1}H NMR in D_2O. The resonance at 4.48 ppm, designated H_a, corresponds to the α-cysteine proton. The resonance at 4.3 ppm, designated H_b, corresponds to the dimethyl acetal methine proton. Spectrum A represents a mixture of (*R*)- and (*S*)-GLADMA; spectra B and C correspond to (*R*)- and (*S*)-GLADMA, respectively.

(*R*)- and (*S*)-GLADMAs were quite stable for months when stored at −20 °C. The corresponding (*R*)- and (*S*)-GLAs (Scheme III, **5** and **5'**, respectively) were prepared prior to use by acid-catalyzed deacetalization (1 N HCl, 30 °C, 36 h). Deacetalization yields both GLA and a minor amount of glutathiohydroxyacetone (**10**, GHA; Scheme II; Chari & Kozarich, 1983) formed from acid-catalyzed isomerization of GLA. Integration of the ^{1}H NMR resonances corresponding to the hydrated aldehydic proton at δ 4.85 of GLA to the hydroxymethylene protons at δ 4.39 of GHA indicates that the amount of GHA initially present in the reaction mixture corresponds to ~10% of the total glutathione-derivatized material.

Specificity of Glyoxalase I for Diastereomeric Glutathiolactaldehydes. The substrate specificity of human GX I was tested using (*R*)-GLA and (*S*)-GLA, generated from their respective dimethyl acetals by acid deprotection. As shown in Figure 2, addition of GX I to a deuteriated, buffered solution of each compound leads to a time-dependent change in each spectrum, demonstrating the ability of the enzyme to process *both* glutathiolactaldehydes. As shown by integration, the resonance at δ 4.85, representing the hydrated aldehydic

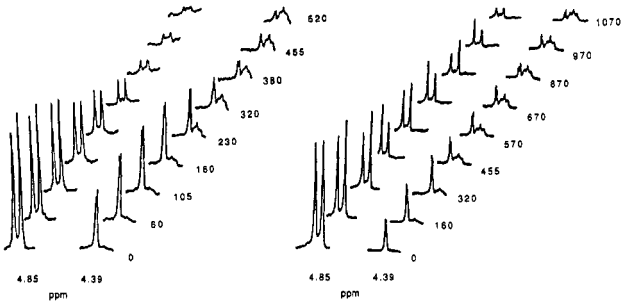


FIGURE 2: Stereospecific processing of (*R*)-GLA and (*S*)-GLA by GX I monitored by ^{1}H NMR. At time zero, 56 units of GX I was added to 20 mM solutions of (*R*)-GLA (**5**, left) and (*S*)-GLA (**5′**, right) in deuteriated phosphate buffer. The numbers to the right of each spectrum represent time, in minutes. The resonance at 4.85 ppm represents the hydrated aldehydic proton of GLA. The resonance at 4.39 ppm, initially present at time zero, represents the AB quartet corresponding to the hydroxymethylene protons of GHA (**10**) produced by chemical isomerization during acid deprotection of the GLADMA precursors. The broadened triplet upfield from the AB quartet represents GHA monodeuteriated in the *pro-S* position. Resolution of this resonance into a clean 1:1:1 triplet (J = 2.7 Hz) is problematic (Chari & Kozarich, 1983).

proton, decreases with time in a first-order manner at a different rate for each diastereomer. Initial rates for the loss of hydrated aldehyde were obtained from the slope of the plot of log percent resonance vs time subsequent to subtraction of the nonenzymatic rate (0.007 s^{-1}) from the observed rate to give final rates for (*R*)-GLA and (*S*)-GLA of 0.8 and 0.4 s^{-1}, respectively.

The rates observed for formation GHA from the GLAs reflect the enzyme-catalyzed isomerization and not the rates of dehydration of the aldehydic carbons of the GLAs. It is reasonable to assume that the unhydrated form of GLA serves as the substrate for GX I. This assumption is based on the observation that a number of glycolytic enzymes utilize the appropriate unhydrated species as substrates and that the hydrated species are not substrates and bind poorly, if at all (Trentham et al., 1969; Reynolds et al., 1971; Gray & Barker, 1970). In addition, the reaction under consideration may be compared to the reaction catalyzed by triosephosphate isomerase (TIM) due to the structural similarity of the lactaldehyde and hydroxyacetone portions of GLA and GHA with GAP and DHAP, respectively. As determined for the TIM system, only 4% of GAP and 59% of DHAP are in the unhydrated forms, and this renders the K_{eq} of the TIM-catalyzed isomerization for the unhydrated forms to be 340 (K_{eq} = total [DHAP]/total [GAP] = 22; Knowles & Albery, 1977). In addition, the rate constant for the dehydration of GAP has been shown to be 0.087 s^{-1} (Trentham et al., 1969). Plaut and Knowles (1972) have used the following argument to demonstrate that the k_{cat} measured for TIM processing of GAP to DHAP (5000 s^{-1}) is not controlled by the rate of dehydration of GAP (0.087 s^{-1}). By consideration of the simplified scheme

$$S{\cdot}H_2O \underset{k_{-h}}{\overset{k_{+h}}{\rightleftharpoons}} S + E \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} (E{\cdot}S) \xrightarrow{k_{+2}} E + P$$

where $S{\cdot}H_2O$ represents hydrated substrate, it was shown that $[E_t] = [E{\cdot}S](1 + K_m k_{+h}/[S_0]k_{-h})$, which is equivalent to the Michaelis equation for $k_{+2} = k_{cat}$ and $K_m' = K_m k_{+h}/k_{-h}$. If $k_{+2} \ll k_{-h}$ ($[S{\cdot}H_2O]/[E{\cdot}S]$) (i.e, $[S] \gg [E_t]$) and $k_{+2} = k_{cat}$, $[E{\cdot}S] \leq [E_t]$, and $[S{\cdot}H_2O] \cong [S_0]$, then $k_{cat}/k_{-h} \ll [S_0]/[E_t]$. For our system, assuming the rate of dehydration of GLA is similar to the rate of GAP dehydration and that enzyme is saturated with respect to GLA, then $k_{cat}/k_{-h} \approx 10$. For Michaelis kinetics to be followed, $[S_0]/[E_t]$ must be greater than 10. Since $[S_0]/[E_t] \sim 7000$, dehydration of GLA cannot be rate-limiting. In essence, the rate of the enzymatic process is controlled by the concentration of [E·S] and not by the rate of dehydration of GLA.

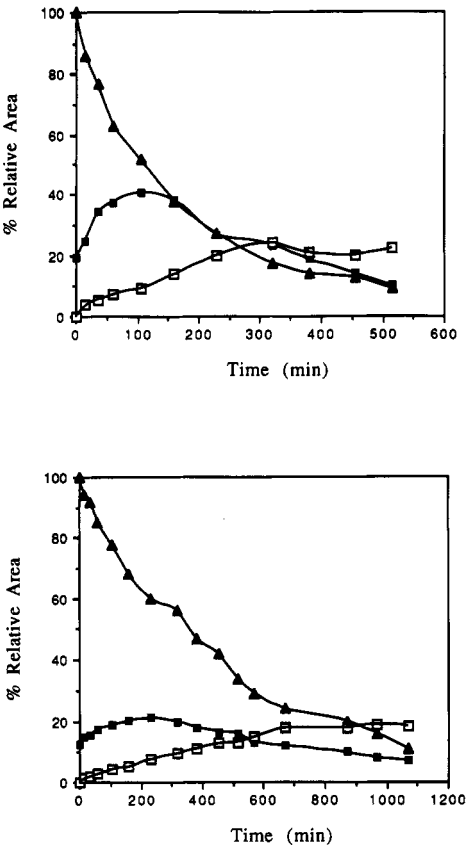
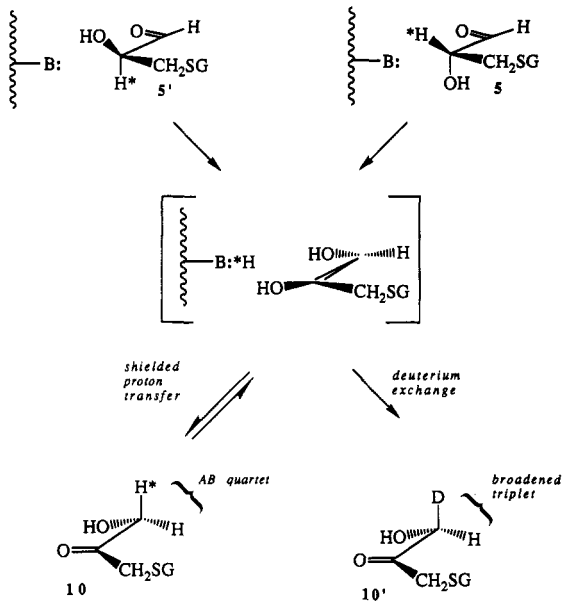


FIGURE 3: Processing of glutathiolactaldehydes by GX I via a consecutive process [(*R*)-GLA (**5**) top panel; (*S*)-GLA (**5′**) bottom panel]. The first-order decay of the hydrated aldehyde (solid triangles) is concomitant with an initial burst in the area corresponding to the Ab quartet of GHA (**10**; solid squares). The broadened triplet (open squares) corresponding to GHA monodeuteriated in the *pro-S* position (**10′**) may arise from enzyme-catalyzed exchange with diprotio GHA or from a nonshielded proton transfer. The observed initial burst of diprotio GHA arising from **5** argues for the enzyme-catalyzed formation of stereospecifically deuteriated GHA. The relative areas for all signals were calculated with respect to the intensity of the hydrated aldehyde at time zero.

Assuming proton abstraction to be the rate-determining step, this indicates nonstereospecific but preferred proton abstraction. The resonance at δ 4.39 initially present at time zero represents the hydroxymethylene protons of GHA formed by chemical isomerization during acid deprotection of the aldehydic functionality. The initial "burst" in the intensity of this resonance upon the addition of glyoxalase I is consistent with the proton transfer being shielded. Therefore, proton transfer is observed in D_2O during the isomerization of *both (R)- and (S)-GLA*. Concomitant with shielded proton transfer, solvent proton exchange at C-1 of GHA occurs to give a single broadened triplet 0.015 ppm upfield from the AB quartet of the hydroxymethylene protons, regardless of the chirality of the starting material. As shown by Chari and Kozarich (1983), this resonance represents the *pro-R* proton of GHA coupled to deuterium (spin = 1), stereospecifically exchanged into the *pro-S* position. Therefore, while GX I is *nonstereospecific with respect to proton abstraction*, it is *stereospecific with respect to proton delivery to the enediol intermediate*, as indicated by the generation of identical monodeuteriated GHAs. This sequence of events is depicted in Scheme IV. Integration of the resonances of interest for each reaction time

Scheme IV: Interpretation of the ^{1}H NMR Experiment[a]



[a] Abstraction of the hydroxymethine proton from either (*R*)- or (*S*)-GLA (**5** and **5'**, respectively) by GX I leads to a presumed *cis*-enediol intermediate (in brackets). This intermediate is further processed either via a shielded proton transfer to diprotio GHA (**10**, indicated by the AB quartet at δ 4.39) or with solvent exchange of the active site base to give the stereospecifically monodeuteriated GHA (**10'**, indicated by the broadened triplet). Enzyme-catalyzed solvent exchange of the *pro-S* hydroxymethylene proton of nascently formed diprotio GHA also yields the monodeuteriated GHA.

course is shown in Figure 3. The resonance at δ 4.39 corresponding to the AB quartet of diprotio GHA shows the initial increases in magnitude for each diastereomer followed by the expected decrease in magnitude as diprotio GHA is converted to *pro-S* deuteriated GHA by GX I. It is apparent that the integral intensity of the monodeuteriated GHA formed is less than that expected on the basis of the initial intensity of the GLA. The chemical and enzymatic stability of the *pro-S* deuteriated GHA (Chari & Kozarich, 1983) makes this difficult to explain on the basis of a slower nonstereospecific proton exchange. Error in the integration of the deuterium-broadened proton may contribute to an underestimate in the quantitation of this resonance as compared to the hydrated aldehyde resonance from which it is derived. This observation, however, does not affect the conclusions of the experiment.

Inhibition of GX I by GHA. The processing of (*R*)- and (*S*)-GLA to GHA occurs some 3 orders of magnitude more slowly than the turnover of the thiohemiacetal formed between GSH and methylglyoxal ($\approx$1100 s^{-1}; Sellin et al., 1983). The possibility of poor substrate binding was investigated by determining the inhibition constant of GHA (and by structural analogy, GLA) for the normal reaction. The observed inhibition pattern was determined to be competitive with the value K_I = 1.46 $\pm$ 0.06 mM obtained from a slope vs inhibitor concentration replot of the data (not shown). This indicates that poor binding is most reasonably not the cause of slow processing of the substrates GLA and GHA. This value lies between the values reported for the K_M of the glutathione–methylglyoxal thiohemiacetal (0.1–0.46 mM; Rae et al., 1990; Creighton et al., 1988) and the K_I most recently reported for free glutathione (7.88 mM; Rae et al., 1990), suggesting that binding to GX I is largely determined by the interaction of the glutathione moiety.

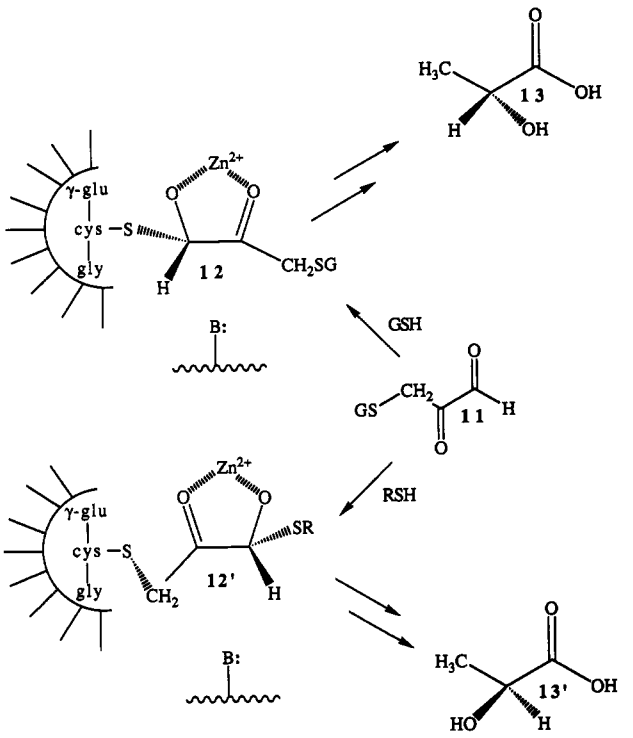
Discussion

Previous Studies. The possibility of nonstereospecific substrate processing by GX I has been previously addressed using the naturally occurring, stereochemically ambiguous thiohemiacetals. Using ^{1}H NMR Douglas and co-workers (Brown et al., 1981) observed the selective disappearance catalyzed by yeast GX I of one of the diastereotopic methine protons from an equilibrium mixture of the thiohemiacetals formed between glutathione and phenylglyoxal. The experiment was performed at low pH (pD 4.4) to minimize the rate of chemical interconversion and thereby maximize any observed diastereomeric discrimination by the enzyme. They observed that the intensity of one methine resonance decreased somewhat more rapidly than the other. Since interconversion of the isomers by elimination/addition of glutathione was relatively rapid, the apparent rate differential could only be interpreted as a stereoselectivity for one thiohemiacetal and not an absolute stereospecificity. Knowing the stereochemistry of the product (D-lactate) and assuming a *cis*-enediol intermediate, the authors concluded that the enzyme preferentially (and perhaps exclusively) processed the (*S*)-thiohemiacetal with chemical equilibration providing the favored diastereomer.

The direct processing of the diastereomeric thiohemiacetals by yeast and by porcine GX I was demonstrated by Creighton and co-workers using high concentrations of GX I to convert both [^{3}H]-(*R*)- and [^{3}H]-(*S*)-thiohemiacetals (formed between [^{3}H]GSH and phenylglyoxal) to tritiated product before exchange with unlabeled GSH (Griffis et al., 1983). They found that GX I processed both diastereomeric thiohemiacetals with virtually equal efficiencies and that the enzymatic rate of thiohemiacetal processing to product thioester was not limited by the nonenzymatic rate of thiohemiacetal interconversion (10–40 s^{-1}). They concluded that the enzyme catalyzed the interconversion of the bound diastereomers so that only one of them is directly processed to product by a single stereochemical pathway involving either the *cis*- or *trans*-enediol intermediate. However, enzyme-catalyzed thiohemiacetal interconversion is unlikely since the enzyme is known to prefer the chemically formed thiohemiacetal as substrate and there is no evidence to support enzyme-catalyzed formation of the thiohemiacetal (Vander Jagt et al., 1975; Rae et al., 1990).

The ability of glyoxalase I to directly process each diastereomeric thiohemiacetal through either *cis*- or *trans*-enediol intermediates would obviate the interconversion mechanism. Along these lines, the observation that both the cis and trans isomers of various para-substituted *S*-(phenylethenyl)glutathione derivatives bind to the active site of glyoxalase I with high and nearly equal affinity (Creighton et al., 1980; Griffis et al., 1983) suggested the intermediacy of both *cis*- and *trans*-enediols in the catalytic mechanism. However, investigations probing the catalytic role of enzyme-bound metal have partially defined the orientation of the enzyme-bound intermediate and have suggested a cis geometry. Glyoxalase I isolated from both yeast and mammalian sources contains a single catalytically indispensable zinc ion per subunit (Aronsson et al., 1978). EXAFS studies with native enzyme (Garcia-Iniquez et al., 1984) and optical and EPR studies with Co(II)-substituted enzyme (Sellin et al., 1983) suggest a distorted octahedral geometry for the active site metal. Nuclear relaxation studies with native and Co(II)-substituted enzymes indicate that *S*-D-lactoylglutathione is in the outer coordination sphere (Sellin et al., 1982). This observation is supported by distance measurements with *S*-D-lactoylglutathione enriched with C-13 at the lactoyl carbonyl moiety on Mn(II)-substituted enzyme (Rosevear et al., 1983), indicating that the carbonyl oxygen is indirectly coordinated to the metal ion. An interesting mechanism proposed by Sellin

Scheme V: Mirror-Image Processing of (Glutathiomethyl)glyoxal (11) by Glyoxalase I[a]



[a] GX I binds **11** via the glutathione moiety. Glutathione activates the C-1 proton of substrate as the thiohemiacetal which may be coupled to **12** or uncoupled from binding (**12′**). When uncoupled from binding, other thiols may replace glutathione for proton activation leading to L-lactic acid (**13′**) instead of D-lactic acid (**13**). The double arrow indicates treatment with GX II to hydrolyze the thioester and desulfurization at C-3 with Raney nickel. The coordination of Zn(II) to substrate is intended to reflect its requirement in catalysis; spectroscopic studies suggest that the coordination is outer sphere (see Discussion).

et al. (1982) places substrate in the second-coordination sphere of a hexacoordinate zinc ion coordinated to two H_2O ligands. The metal serves to polarize the carbonyl at C-2 via an intervening water molecule to generate the enediol intermediate with a proton transfer between C-1 hydroxyl and C-2 carbonyl groups necessitating a *cis*-enediol geometry. Overall, on the basis of these studies and the shielded nature of the proton transfer and the product stereochemistry, the existence of a *cis*-enediol intermediate is strongly supported.

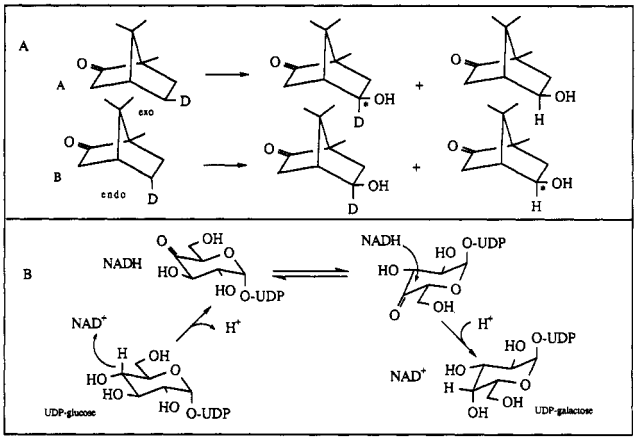
Mirror-Image Catalysis. Our study is based in part on our observations of an unusual example of inverse substrate processing by GX I (Kozarich & Chari, 1982; Scheme V). The thiohemiacetal **12** formed between GSH and (glutathiomethyl)glyoxal **11**, when processed by GX I and subsequently treated with GX II and Raney nickel (to effect thioester hydrolysis and desulfurization, respectively), was found to give the expected D-lactate (**13**) as the major, but not exclusive, product. In addition, the thiohemiacetal **12′** formed between **11** and either ethanethiol or β-mercaptoethanol was also found to be a substrate for the enzyme, suggesting a remarkable loss of specificity for GSH as the activating thiol. Moreover, the opposite stereochemical product was obtained as indicated by the L-lactate/D-lactate ratio of 4:1. The observed loss of specificity for glutathione in the processing of the thiohemiacetals **12** and **12′** suggests two roles for glutathione: activation of the C-1 proton by thiohemiacetal formation and binding of the glutathione functionality to the enzyme (Kozarich & Chari, 1982). Proton activation is, therefore, not a prerequisite for productive glutathione binding. The uncoupling of chemical and binding functions for glutathione in substrate processing was termed "mirror image catalysis", and it suggested that C-1 proton activation could be mimicked by a variety of structures intrinsically more stable than the rapidly interconverting diastereomeric thiohemiacetals. This hypothesis was tested with the mirror image substrate glutathiohydroxyacetone (Chari & Kozarich, 1983). The yeast GX I catalyzed stereospecific exchange of the *pro-S* proton of GHA to give deuteriated product (**10′** in Scheme IV) not only confirmed this hypothesis but, assuming a suprafacial proton transfer, indicated a cis geometry for the enediol intermediate. The failure to detect the formation of the glutathiolactaldehydes, **5** and **5′**, in this experiment suggested, not surprisingly, that the equilibrium of the reaction lies in favor of the hydroxyacetone species.

Glutathiolactaldehydes. We have directly assessed the stereospecificity of thiohemiacetal processing using a new class of mirror image substrates, the diastereomeric glutathiolactaldehydes (**5** and **5′**, Scheme II). These configurationally locked compounds mimic the thiohemiacetals formed between glutathione and methylglyoxal. However, they are diastereomeric alcohols and inert toward interconversion. Our ^1H NMR results unambiguously demonstrate the ability of human GX I to process both **5** and **5′** by the only mechanism available to these analogues: *direct nonstereospecific proton abstraction.* These diastereomers are also processed with nearly equal efficiencies and with a significant amount of proton retention in the initially formed product. However, both the *R* and *S* diastereomers afford the *same, chirally deuteriated product.*

A tentative hypothesis is outlined in Scheme IV. A single, monovalent base may be positioned to effect proton abstraction from either diastereomer to afford a *cis*-enediol intermediate. We suggest that active site reorientation upon formation of the intermediate renders the protonated base accessible to only the *si* face of the enediol carbon that is protonated (or deuteriated) at the *pro-S* position of the resulting hydroxymethyl group. While a two-base mechanism cannot be excluded, two observations reduce its appeal. First, the significant retention of substrate proton in product for both diastereomers is difficult to rationalize by a two-base process. While proton retention in product could be explained for the reaction of **5** by a suprafacial mechanism with a *cis*-enediol intermediate, it is difficult to rationalize in the isomerization of **5′** that would require two bases catalyzing an antarafacial proton transfer. Second, the high degree of stereospecificity in the reprotonation to product in the reaction with normal substrates (Patterson et al., 1981) and with **5** and **5′** argues for a single base being responsible for this process. To our knowledge, no other enzyme-catalyzed proton-transfer reaction displays these stereochemical properties.

Two other enzymes which display unusual stereochemical processing of their substrates are cytochrome P-450cam and NAD-dependent UDPglucose epimerase (Scheme VI, A and B, respectively). The radical enzyme, cytochrome P-450cam, displays nonstereospecific hydrogen abstraction followed by stereospecific hydroxylation (Gelb et al., 1982). Both the *endo*- and *exo*-5-deuteriocamphors generate the deuteriated and nondeuteriated *exo*-5-hydroxycamphors. The *endo*-5-hydroxycamphor is not observed, even though the enzyme is capable of removing the endo hydrogen. The oxygen is delivered only from the exo side. The presence of *exo*-5-hydroxy-*endo*-5-deuterio- and *exo*-5-hydroxycamphors can only be explained by nonstereospecific hydrogen abstraction during hydroxylation of the deuteriocamphor. NAD-dependent UDPglucose epimerase employs one face of the NAD

Scheme VI: Enzymes Displaying Nonstereospecificity[a]



[a] Cytochrome P-450cam (top panel, A) catalyzed hydroxylation of 5-*exo*- and 5-*endo*-deuteriocamphor (* represents loss of stereochemistry). NAD-dependent UDPglucose epimerase (bottom panel, B) utilizes the B-face of NAD(H) in catalyzing a nonstereospecific oxidation/reduction.

nicotinamide ring to catalyze a nonstereospecific oxidation/reduction reaction at the C-4′ alcohol/ketone of the hexose moiety of substrate. It has been argued that the 4′-keto species rotates about the β-phosphorus/C-1′ oxygen bond interchangeably presenting either side of the carbonyl for reduction by the immobile NADH (Kang et al., 1975).

The observed reduced rate of processing of the glutathiolactaldehydes does not appear to be due to poor binding. The value of the K_I determined for GHA from inhibition studies (1.46 mM) indicates that the concentrations of the glutathiolactaldehydes used in the NMR studies (≈20 mM) are at least an order of magnitude above K_I. There are a number of other possibilities which could account for the diminished rate observed for the processing of these compounds. It is possible that the insertion of a methylene unit between glutathione cysteine sulfur and the hydroxymethine proton which is abstracted and transferred has two deleterious effects. First, the thiohemiacetal functionality of normal substrate may stabilize the incipient carbanion in the transition state leading to the enediol intermediate. Divalent sulfur increases the kinetic and thermodynamic acidity of an adjacent carbon to a higher degree than other second- and third-row heteroatoms (Tagaki, 1977). For example, the ability of sulfur to stabilize α-carbanions is reflected in the increased acidity (4–7 orders of magnitude) of various thioethers with respect to their corresponding oxygen ethers (Bordwell et al., 1976). This stability may arise from electron density polarization (Steitweiser & Williams, 1975) of divalent sulfur and a hyperconjugative interaction between the lone pair on carbon and the low lying $d^*_{\sigma R}$ orbital (Epiotis et al., 1976). Second, it is quite likely the the interposition of a methylene group between the sulfur and the chiral carbon of the lactaldehyde moiety poses some steric problems to proper orientation of the methine proton for efficient abstraction. Our experience suggests that this effect may differ widely with the source of the enzyme. For example, while the results reported here are for the GX I from human erythrocytes, attempts to demonstrate isomerization of **5** and **5′** with the yeast enzyme have been unsuccessful, despite the fact that they bind to the enzyme. Our observation is contrasted by the similar kinetic and mechanistic properties measured for GX I from both mammalian sources and yeast (Marmstal et al., 1979). However, in addition to displaying distinct primary and quaternary properties, the yeast enzyme displays sensitivity to thiol-blocking agents as well as inhibition by *S*-(*p*-bromobenzyl)glutathione, while the mammalian enzymes do not. Therefore, it appears that the analogues reported here have elucidated subtle mechanistic differences in the enzyme from the two sources.

The ability of glyoxalase I to isomerize both diastereomeric glutathiolactaldehydes by catalyzing a nonstereospecific proton abstraction followed by a stereospecific proton delivery suggests it processes normal thiohemiacetal substrates in an analogous manner. The phenomenon of nonstereospecific substrate isomerization reported here supports the physiological arguments advanced by Creighton and co-workers (Creighton et al., 1988). As they have pointed out, in erythrocytes the catabolism of methylglyoxal is under kinetic control and is independent of hydration equilibria. They have shown that the level of GX I present in erythrocytes allows for the rate of enzyme-catalyzed thiohemiacetal isomerization to *S*-D-lactoylglutathione to compete with the noncatalyzed decomposition rate of the thiohemiacetals. That is, through evolution a balance has been achieved a relatively high enzymatic catalytic efficiency (k_{cat}/K_m) and the expenditure of energy for GX I synthesis such that the efficient catabolism of methylglyoxal is limited by encounter with available GX I. Furthermore, our direct demonstration of nonstereospecific substrate isomerization is supported by the arithmetic arguments that suggested a 6-fold advantage for the direct utilization of both thiohemiacetals compared to utilization of only one (Creighton et al., 1988). A more detailed understanding of GX I and its intriguing catalytic mechanism is at present limited by the lack of structural information at the atomic level.

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Structure of the Human Pancreatic Cholesterol Esterase Gene

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ABSTRACT: The gene for human pancreatic cholesterol esterase consists of 11 exons and 10 introns and is 9.2 kb in length. The last and longest exon (841 nucleotides) is unique to the human gene. Functional amino acids are encoded on separate exons. The leader sequence is encoded by a single exon which carries two additional N-terminal amino acids of the mature functional protein. A positive TATA element is identified 43 nucleotides from the start codon. Pulse-field gel electrophoresis and hybridization with various cDNA probes and direct sequence data revealed the existence of a CEase-like gene. Partial sequence analysis of this gene from a human cosmid library and human genomic DNA showed a premature stop signal in exon 10, shortly after the codon for the active-site histidine. Both the functional gene and the CEase-like gene have a polyadenylation signal in the 3′-untranslated region. Thus, the complex gene structure for this intestinally active enzyme may provide in part a potential molecular explanation for the well-known heterogeneity of the intestinal absorption of cholesterol.

Pancreatic cholesterol esterase (CEase)[1] (EC 3.1.1.13) promotes the intestinal absorption of cholesterol esters by catalyzing hydrolysis of the ester bond, an obligatory step in the uptake of this form of dietary sterol (Vahouny & Treadwell, 1964). Recent studies have shown that a serine residue and a histidine residue play a key role in the hydrolytic mechanism (Kissel et al., 1989; DiPersio et al., 1991). Moreover, Bosner et al. (1988) described a heparin binding site on pancreatic CEase that serves to bind this enzyme to the absorptive membrane of the enterocyte to facilitate cholesterol absorption. Kyger et al. (1989) reported the first cDNA cloning of the enzyme, and genomic cloning of rat

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[1] Abbreviation: CEase, cholesterol esterase.

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