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Enzymatic Conversion of α-Keto Aldehydes to Optically Active α-Hydroxy Acids Using Glyoxalase I and II[1]

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α-Keto aldehydes (RCOCHO, R = CH_3, $CH_3CH_2CH_2$, Ph, *p*-CH_3OPh, *p*-ClPh) have been converted to optically active α-hydroxy acids ($RCHOHCO_2H$) by the combined action of glutathione and the (immobilized) enzymes glyoxalase I (GX-I, EC 4.4.1.5) and glyoxalase II (GX-II, EC 3.1.2.6). The reaction seems to provide a practical if specialized method for synthesizing 1–10-g quantities of product with enantiomeric excesses (ee) in the range 75–99%. Efforts to increase the scale of the reacton are accompanied by decreases in the ee of the product and in the turnover number reached by the enzymes.

Introduction

This paper describes the conversion of α-keto aldehydes to optically active acids, using a catalytic system comprised of two enzymes, glyoxalase I[4-13] (GX-I, EC 4.4.1.5) and glyoxalase II[14,15] (GX-II, EC 3.1.2.6), and the cysteine-containing cofactor glutathione (GSH, γ-L-Glu-L-Cys-Gly) (eq 1). GX-I accepts a number of sterically unhindered α-keto aldehydes as substrates but does not accept substances that have large substituents close to the keto

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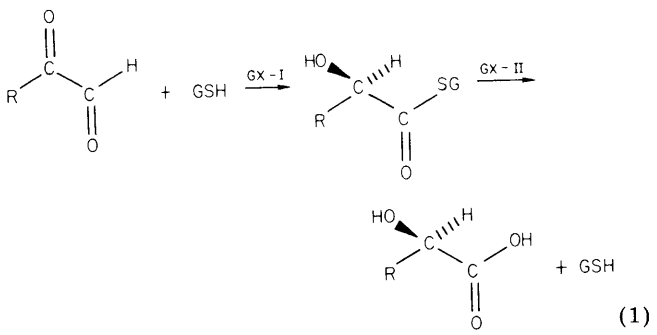

(1)

group.[5,8,10,11] Its action appears to involve an enediol-proton transfer mechanism,[12] with the hemithioacetal formed between GSH and α-keto aldehydes as substrate.[6,10] GX-II has been studied in less detail but seems to function primarily as a catalyst for the hydrolysis of thioesters of GSH.[14,15] The combination of GX-I and GX-II has been reported to convert methylglyoxal to lactic acid, having >99% the D(−) configuration,[4,7] and phenylglyoxal to mandelic acid (>95% D(−)).[10] GX-I and GX-II are commerically available.

Results

GX-I and GX-II were immobilized in a cross-linked gel derived from poly(acrylamide-*co*-*N*-(acryloxy)succinimide) (PAN)[16] in yields of ~51% and 31%, respectively. Immobilized GX-I and GX-II are stable at pH 7 and 25 °C for periods of several months in the absence of reactants. In the presence of α-keto aldehydes, both lose activity, presumably as a result of modification of arginine residues.[15,17-19] This instability of the enzymes (especially GX-II)[15] in the presence of α-keto aldehydes is the most severe limitation to the practical utility of this catalytic system.

Reactions were carried out by using two procedures. In one (called the "simultaneous" procedure here), α-keto aldehyde was added slowly to an aqueous solution of GSH containing both gel-immobilized enzymes. In the second (called the "sequential" procedure), immobilized GX-I was used to convert α-keto aldehyde to the intermediate thioester. The GX-I was then removed, and immobilized GX-II was used in a separate step to hydrolyze the thioester to product. Removal of the GX-II and readdition of GX-I and additional α-keto aldehyde permitted limited reuse of GSH, howbeit with a decrease in the enantiomeric excess of the product α-hydroxy acid.

Table I summarizes representative results obtained with these two procedures. These results illustrate several features of our experience with this reaction. First, reactions run with the "simultaneous" procedure give better efficiencies for use of GSH (as reflected in TN_{GSH}) than those with the "sequential" procedure. Particularly on large scale, however, the TN's for the enzymes (especially GX-II) are lower in the "simultaneous" procedure, probably as a result of deactivation of the enzymes by reaction with α-keto aldehyde. Since the enzymes are the greatest contributor to the cost of these syntheses, in most cases it is more practical simply to accept GSH as a stoichiometric reagent, use the "sequential" procedure, and recover as much GSH as possible (typically ~50%) for reuse by isolation at the conclusion of the reaction. Second, the enantiomeric excess in the product is variable. It appears that use of large quantities of GX-II (reflected in low values of TN_{GX-II} in Table I) leads to relatively high values of ee. We conclude from this observation, and from others,[20] that the intermediate α-hydroxy thiol esters (RCHOHCOSG) epimerize under the reaction conditions: the highest values of ee are observed under conditions in which these species would be expected to have relatively short lifetimes.

Conclusions

The enzymatic conversion of α-keto aldehydes to α-hydroxy acids represents a practical if specialized method for the small-scale (<10 g) preparation of these latter species.[21] The advantage of this method is that the product is obtained in an enantiomerically enriched form. Further, if relatively large quantities of GX-II are used, the enantiomeric excess of the product may be high. The enzymes are commerically available, and the experimental procedure is straightforward. Although most of our work has been carried out with immobilized enzymes, these reactions may also be conducted with soluble enzymes. The stabilities of the soluble enzymes are lower than those of the immobilized enzymes, but the procedure is simplified by omission of the immobilization step and the lower stability is compensated for by avoiding the significant loss in enzymatic activity (50–69% of initial activity) that accompanies the immobilization step. The enzymes appear to accept a useful range of substrates,[5,10,14] although their activity is very low when R is a group that introduces steric bulk close to the keto group.

The deficiencies of this method are also several. First, yields of α-hydroxy acids are not high, and enzyme costs are such that the reaction is expensive to scale up.[22] Efforts to increase the yield or to produce larger quantities of product seem to be frustrated by spontaneous epimerization of the intermediate RCHOHCOSG in the reaction and by deactivation of the glyoxalase enzymes (especially GX-II) by reaction with α-keto aldehyde (and perhaps also with thioester). Second, only one enantiomer of the α-hydroxy acids is accessible by this route.

Experimental Section

General Procedures. Glyoxalase I (EC 4.4.1.5, from yeast), glyoxalase II (EC 3.1.2.6, from beef liver), D-lactic dehydrogenase (EC 1.1.1.28), L-lactic dehydrogenase (EC 1.1.1.27), and reduced glutathione were obtained from Sigma Chemical Co. (*R*)-(+)-α-Methoxy-α-(trifluoromethyl)benzeneacetic acid was from Aldrich. All other materials were reagent grade. pH measurements were made on a Radiometer PH M62 standard pH meter. Enzymatic assays and absorbance measurements were performed on a Gilford 240 spectrophotometer equipped with a constant temperature bath and a strip-chart recorder. A Varian T-60 was used to record 60-MHz ^1H NMR spectra, and a Bruker WH-250 was used for

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(20) The use of multiple cycles in the "sequential" reactions leads to a reduced ee. This result can be explained by an α-hydroxy carboxylate catalyzed epimerization of the α-hydroxy thiol ester. In addition, phenylglyoxals having electron-withdrawing substituents seem to give mandelic acids with lower enantiomeric excesses than with electron-donating substituents.
(21) Optically active α-hydroxy acids have been obtained by resolution (Collet, A.; Jacques, J. *Bull. Soc. Chim. Fr.* **1973**, 3330–3334) or by the conversion of optically active α-bromo acids (Levene, P. A.; Mori, T.; Mikeska, L. A. *J. Biol. Chem.* **1927**, *75*, 337–365) or α-amino acids (Baker, C. G.; Meister, A. *J. Am. Chem. Soc.* **1951**, *73*, 1336–1338). In addition, they can be prepared by asymmetric reactions (Meyers, A. I.; Slade, J. *J. Org. Chem.* **1980**, *45*, 2785–2791 and references contained therein).
(22) Costs: GX-I: $7.10/100 U; GX-II: $43.00/100 U; GSH: $305/mol. The major contributor to the cost of the reactions is the price of GX-II. Attempts to substitute less expensive esterases, such as acylase I, acetylesterase, carboxylesterase, and *Candida cylindracea* lipase for the GX-II were unsuccessful. With extensive screening it might, however, be possible to develop a better (i.e., less expensive) esterase for these glutathione thiol esters.

Table I. Synthesis of Chiral α-Hydroxy Carboxylic Acids from α-Keto Aldehydes Using Glyoxalase I (GX-I), Glyoxalase II (GX-II), and Glutathione (GSH)

R	RCOCHO, mmol	GSH, mmol	product ($RCHOHCO_2M$)[a] M	g	%	ee R, %	reaction type[b]	TN[c] GX-I	GX-II	GSH	cycles[d]
p-ClPh	278	139	Ca/2	14	26	76[e]	simul	5×10^7	1×10^6	2	
Ph	65	7.5	H	7	75	91[e]		3×10^7	1×10^5	9	
p-MeOPh	11	2	Ca/2	2	84	94[e]		4×10^6	2×10^4	6	
CH_3	324	7	Zn/2	8	17	97[f]		4×10^6	9×10^4	46	
$CH_3CH_2CH_2$	28	0.6	Ba/2	4	79	>98[e]		2×10^6	5×10^4	47	
	20	25		2	54	>99[e]	seq	2×10^6	6×10^5	0.8	1
	29	36		2	30	>99[e]		4×10^6	4×10^5	0.8	1
	67	50		4	30	85[e,g]		2×10^7	9×10^5	1.3	3
	98	68		12	65	80[e,g,h]		3×10^7	1×10^6	1.4	3

[a] Yields (in grams and percent based on RCOCHO) are for isolated products. The yield before isolation was 73% for the lactic acid producing system, as measured by enzymatic assay. Suitable assays did not exist for the measurement of other products in solution. [b] Reaction type is discussed in the text. [c] TN = turnover number = moles of $RCHOHCO_2H$ generated per mole of enzyme or glutathione originally present in the solution. These turnover numbers are not corrected by taking into account enzyme or glutathione present at the conclusion of the reaction. [d] "Cycle" refers to the number of times that the sample in a sequential procedure was used in a sequence of formation of RCHOHCOSG and hydrolysis of this species. [e] Enantiomeric excess determined by NMR analysis, using diastereomeric esters (see the text). [f] Enantiomeric excess determined by enzymatic analysis. [g] Insignificant quantities of active enzymes were recovered upon completion of the reactions except for these cases. An average of 55% GX-I and 35% of GX-II initial activities were recovered at the end of each cycle. [h] Enantiomeric excess was 89% after two cycles.

250-MHz spectra. Distilled water was deionized with a Barnstead ion-exchange column and then redistilled, using a Corning AG-1b type still; this water was used to prepare all solutions. Peristaltic pumps from LKB were used for the addition of substrates and base, while the pH was maintained at a constant value with a Chemtrix pH controller.

Methylglyoxal was steam-distilled under argon before use. (p-Chlorophenyl)glyoxal,[23] (p-methoxyphenyl)glyoxal,[23] propylglyoxal,[24] S-(p-bromobenzyl)glutathione,[25] and PAN[16] were synthesized following literature procedures. Glyoxalase I activity was measured in buffered solutions (pH 6.0–8.0) that were 10 mM in $MgCl_2$, 3.0 mM in methylglyoxal, and 3.0 mM in GSH at 30 °C. The increase in absorbance at 240 nm (ϵ 3370 M^{-1} cm^{-1})[26] was directly related to enzymic activity. Glyoxalase II activity was determined in buffered solutions (pH 6.0–8.0) containing 0.6 mM S-lactoylglutathione at 30 °C, with the decrease in absorbance at 240 nm being measured. The concentrations of methylglyoxal,[26] propylglyoxal,[26] phenylglyoxal,[5] (p-methoxyphenyl)glyoxal,[5] (p-chlorophenyl)glyoxal,[5] glutathione,[26] and L-lactic acid[26] were measured enzymatically by using literature procedures. D(−)-Chloromandelic acid was prepared by resolution.[21]

Immobilization of Enzymes. The glyoxalase enzymes were immobilized following the procedure of Pollak et al.[16] GX-I was immobilized in a 0.3 M Hepes, pH 7.5, solution containing 10 mM $MgCl_2$, 5 mM 1,3-dithiopropan-2-ol (DTP), and 0.8 mM S-(p-bromobenzyl)glutathione.[25] Optimal yields were obtained when a ratio of 0.5 mg of enzyme to 100 mg of PAN-500 (contains 500 μmol of active ester groups per g of solid) was used. The average yield of immobilized enzyme was 51%, with 14% of the initial activity present in the wash. GX-II was immobilized in a 0.2 M P_i, pH 7.0, solution containing 5 mM $MgCl_2$, 5 mM DTP, and 8 mM GSH with an enzyme to PAN-500 ratio of 2 mg/100 mg. An average of 31% of the initial activity was present in the gel, with an additional 8% in the wash.

α-Hydroxypentanoate: "Simultaneous" Procedure. The pH of 150 mL of a solution containing MOPS (7.5 mmol), $MgCl_2$ (1.5 mmol), GSH (0.6 mmol), and 1,3-dithiopropan-2-ol (DTP, 0.9 mmol) was adjusted to 6.8 with 2 N NaOH. The solution was deoxygenated with a stream of argon and soluble GX-I (160 U) and GX-II (100 U) added. Over an 8-day period 69 mL of an aqueous solution of propylglyoxal (28 mmol) was added, together with additional GX-I (180 U) and GX-II (75 U). The pH was maintained at 6.8 with 2 N NaOH by using a pH controller. Upon completion of the reaction, the mixture was acidified to pH 1.5 with concentrated HCl and extracted continuously for 40 h with ether (0.7 L). The ether was concentrated under reduced pressure, and the residue was diluted with H_2O (40 mL), heated briefly, and neutralized with $BaCO_3$. The mixture was boiled and filtered while hot, and the residue was washed with boiling H_2O (10 mL). The filtrate volume was reduced to 25 mL by gentle boiling, the solution cooled (70 °C), and ethanol (15 mL) added. After cooling to 4 °C, the precipitate was filtered, washed with ether, and dried, yielding 4.1 g of barium α-hydroxypentanoate (>98% ee R; 79% based on propylglyoxal). The activities of GX-I and GX-II at the end of the reaction were only 6% and 3%, respectively, of the original values.

α-Hydroxypentanoate: "Sequential" Procedure. A 100-mL aqueous solution of propylglyoxal (27 mmol) containing GSH (34 mmol), $MgCl_2$ (1.0 mmol), and DTP (1.0 mmol) was adjusted to pH 7.0 with 10 N NaOH and then deoxygenated with argon. Immobilized GX-I (22 U) was added. After 47 h the GX-I was removed by gentle centrifugation (~3000 rpm) and immobilized GX-II (6.6 U) added to the supernatant. The pH of the suspension was maintained between 6.7 and 7.0 with 1 N NaOH and a pH controller. After 67 h the mixture was centrifuged to remove the GX-II and additional propylglyoxal (31 mmol) and GSH (9 mmol) was added to the supernatant. The pH of the solution was adjusted to 6.9 with 10 N NaOH, the solution deoxygenated, and immobilized GX-I (22 U) added. Fifty hours were required for completion of the reaction, at which time the GX-I was removed and immobilized GX-II (6.5 U) added to the supernatant. Seventy hours later the suspension was centrifuged to remove the GX-II, and to the resulting solution was added propylglyoxal (40 mmol) and GSH (25 mmol). Following adjustment of pH 6.8 with 10 N NaOH, immobilized GX-I (40 U) was added. The suspension was centrifuged after 48 h to isolate the GX-I, and immobilized GX-II (5.4 U) was added. Forty-three hours later an additional portion of GX-II (5.1 U) was added. After 71 h the reaction was complete, the GX-II was removed by centrifugation, and the supernatant was acidified to pH 1.5 with concentrated HCl. The acidified solution was extracted with ether and barium α-hydroxypentanoate (12.0 g, 32 mmol; 80% ee R; 65% yield based on propylglyoxal) isolated as described above. The average amount of initial activity recovered at the end of each step was 56% for GX-I and 37% for GX-II.

p-Chloromandelate. A "simultaneous" procedure, utilizing immobilized GX-I and II, converted a solution of (p-chlorophenyl)glyoxal (279 mmol, dissolved in Me_2SO) to p-chloromandelic acid (3.5 g, 19 mmol) and calcium p-chloromandelate

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(11 g, 27 mmol). The final reaction mixture was 14% (v/v) Me_2SO. Calcium carbonate was used in the preparation of calcium *p*-chloromandelate. The overall yield was 26% (based on (*p*-chlorophenyl)glyoxal) and the product was composed of a 76% ee of the *R* enantiomer. The recovered GX-I and GX-II had 3% and 59%, respectively, of their initial activities.

Mandelic Acid. An aqueous suspension of phenylglyoxal (65 mmol in 350 mL of H_2O, pH 6.3) was converted in a "simultaneous" process with immobilized enzymes to mandelic acid (7.4 g, 49 mmol; 91% ee *R*; 75% based on phenylglyoxal). The recovered enzymes were not active.

***p*-Methoxymandelate.** A "simultaneous" procedure, using soluble GX-I and GX-II, converted 256 mL of an aqueous solution of (*p*-methoxyphenyl)glyoxal (11 mmol) to the corresponding α-hydroxy acid. Calcium *p*-methoxymandelate (1.9 g, 4.6 mmol; 94% ee *R*; 84% based on (*p*-methoxyphenyl)glyoxal) was isolated with calcium carbonate. No enzymatic activity remained upon completion of the reaction.

Lactate. Methylglyoxal solutions are known to contain significant amounts of acid, persumably lactic acid.[4] A steam-distilled solution (450 mL of 2.97 M methylglyoxal, 0.48 M acid) was treated with 16 portions of Dowex-1 × 8/50 (OH^-) (15-mL portions, 20 min each) to give a solution (380 mL) of 1.62 M methylglyoxal and 8.7 mM acid. A "simultaneous" procedure, using immobilized enzymes, converted 200 mL of this solution (324 mmol methylglyoxal) to lactic acid, which was isolated as the zinc salt (7.9 g, 28 mmol; 97% ee *R*; 17% based on methylglyoxal), using zinc carbonate. No enzymatic activity was recovered.

Determination of Enantiomeric Compositions. D- and L-lactate were measured by using enzymatic assays.[26] The same value of enantiomeric excess was found for the lactate present in the reaction mixture before workup and for the zinc lactate isolated as product. This observation indicates that no enantiomeric enrichment occurs during workup. Assays for other materials were based on Mosher's method,[27,28] chemical shifts for the OCH_3 protons of the diasteromeric forms of $RCH(OCOC^*(CF_3)(OCH_3)Ph)CO_2CH_3$ differed by ~0.1 ppm, and those for the CO_2CH_3 group by ~0.03 ppm. At 250 MHz, the latter gave base line separations and were used to determine enantiomeric excess. The absolute configurations of the compounds were assigned by comparison with spectra of compounds derived from resolved or enriched α-hydroxy methyl esters. The methyl esters used were prepared by using a procedure described below for methyl 2-hydroxypentanoate. Control experiments carried out with the methyl esters of mandelic and *p*-chloromandelic acids showed that the procedure for the determination of the enantiomeric compositions does not result in enantiomeric enrichment.

Methyl 2-Hydroxypentanoate. The ester was prepared by placing the barium salt (0.5 g) of 2-hydroxypentanoic acid in MeOH (20 mL), adding acetyl chloride (0.5 mL), and heating the mixture under reflux for 30 min. The mixture was cooled, filtered to remove $BaCl_2$, and concentrated under reduced pressure. Ether (10 mL) was added, residual $BaCl_2$ removed by filtration, and solvent removed under vacuum. The resulting liquid was used directly in determination of enantiomeric excess.

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Registry No. (*p*-Chlorophenyl)glyoxal, 4998-15-6; phenylglyoxal, 1074-12-0; (*p*-methoxyphenyl)glyoxal, 1076-95-5; methylglyoxal, 78-98-8; propylglyoxal, 7332-93-6; (*R*)-4-chloro-α-hydroxybenzeneacetic acid 1/2 Ca, 79083-97-9; (*R*)-α-hydroxybenzeneacetic acid, 611-71-2; (*R*)-4-methoxy-α-hydroxybenzeneacetic acid 1/2 Ca, 79083-98-0; (*R*)-α-hydroxypropanoic acid 1/2 Zn, 18975-98-9; (*R*)-α-hydroxypentanoic acid 1/2 Ba, 79083-99-1.

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