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Methylglyoxal Synthetase, Enol-Pyruvaldehyde, Glutathione and the Glyoxalase System

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Abstract: enol-Pyruvaldehyde (ePY or 2-hydroxypropenal, O=C(H)−C(OH)=CH_2) a transient intermediate in the alkaline decomposition of the triosephosphates to methylglyoxal is now observed by UV and ^{1}H NMR spectroscopy as the immediate product of the methylglyoxal synthetase (MGS) reaction: dihydroxyacetone-P → P_i + ePY → methylglyoxal (MG). Analysis of ePY formed from 1-^{13}C- and (1*R*, 3*S*) -[1,3-^{2}H]-DHAP establishes the stereochemical course of its formation by MGS. Its rate of ketonization is much too slow to be in the sequence required for the assay of MGS by coupling of the MG produced to glyoxalase I (Glx I): MG + glutathione (GSH) → (*S*)-lactylglutathione (D-LG). Instead, ketonization occurs by way of the hemithioacetal (HTA) formed between ePY and GSH, and could be either an enzymatic function of Glx I or occur nonenzymatically at an activated rate. Enzymatic ketonization was ruled out because the methyl group of D-LG formed from specifically labeled ePY is achiral. Chemical ketonization of ePY is activated by general bases, such as acetate, and by thiols such as GSH and 2-mercaptoethanol, which disrupt its stabilizing double bond conjugation as hemithioacetal (HTA) adducts. 2-Mercaptoacetate combines both functions, acting as the HTA adduct of ePY with the appended carboxylate group presumably positioned to promote abstraction of the enol proton and protonation of the enolate carbon at an accelerated rate. In the MGS-Glx I system (dihydroxyacetone-P → ePY, ePY + GSH → GS-ePY, GS-ePY → GS-MG, GS-MG → D-LG), the nonenzymatic 2nd and 3rd steps describe the catalytic role of GSH in the critical ketonization process and set the stage for its participation in the glyoxalase system.

Introduction

The "glyoxalase system" refers to the two enzymes, glyoxalase I and II, that together with glutathione remove the cytotoxic keto aldehyde methylglyoxal from most cells: MG + GSH → D-LG → D-lactate + GSH. MG is a readily permeable product of unicellular organisms that contain MGS, but is formed in all cells under physiological conditions due to the inherent instability of their triose-P metabolites,[1] imperfections in enzymes that modify them,[2,3] and from sources yet to be identified.[4] Most of the MG of cells resides in long-lived amine complexes of proteins[4] and possibly nucleic acids,[5] which may explain its toxicity,[6] which in *E. coli* depends on the growth conditions even in high MGS expressing cells.[7] That inhibitors of glyoxalase I inhibit tumor growth[8] is evidence for MG toxicity in cells not known to contain MGS. The toxicity of MG from carbohydrate sources in *E. coli* depends on the content of glyoxalase I, notwithstanding the presence of a glutathione-independent glyoxalase III and NADH-dependent aldehyde-reducing enzymes.[9]

The nonenzymatic and the MGS dependent decomposition of dihydroxyacetone-P (DHAP) have a common chemistry (Scheme 1), with the exception that the enzyme is stereospecific for the *pro-S* proton of C-1 of DHAP, whereas both are nonstereospecific in the formation of the methyl group of MG.[10] This latter observation suggested that the immediate product of the MGS reaction was the enol form of MG, which then ketonized in solution. Harrison and co-workers[11−13] have provided extensive kinetic and structural studies of *E. coli* MGS, complexed with analogues of the proposed enediol-P intermediate, showing Asp-71, the proton-abstracting base, at the 1*si* face of the intermediate and the bridge oxygen to PO_3 to be out of the plane, a geometry required for the β-elimination of $-OPO_3$ in the MGS reaction sequence (Scheme 1). On the basis of the geometry of the bound inhibitors and the positions of neighboring protein residues the enediol-P intermediate is best interpreted

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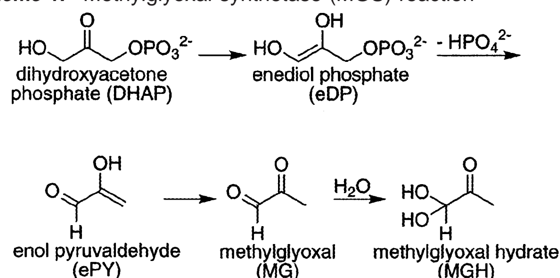
Scheme 1. Methylglyoxal synthetase (MGS) reaction



as a cis-enediol.[13] Structural comparisons with trioseP isomerase, which has a minor MG synthetase side reaction[2,3] are instructive.

Surprisingly, the properties of 2-hydroxypropenal seem not to have been described.[14] Because many enols are known to ketonize at measurable rates,[15,16] the availability of recombinant MGS suggested a simple method for its possible preparation. Of interest would be the following: (1) a direct demonstration of ePY as the product of MGS, as would be shown by its accumulation in a solution of the enzyme and DHAP; (2) a study of the stereochemistry of its formation by MGS; (3) a study of the mechanisms of its ketonization; and (4) the possibility that the introduction of GSH into the glyoxalase system evolved from a role in the ketonization step.

Materials and Methods

Materials. The enzymes, MGS from *E. coli*, the wild-type recombinant form described by Saadat and Harrison[11] was generously provided by Dr. David Harrison. The enzyme is a homohexamer, with $k_{cat} = 220\ s^{-1}$, K_m of DHAP = 0.2 mM, when assayed at pH 7 with 15 mM GSH and excess Glx I by following the increase in absorbance due to the final product, D-LG. Methylmalonyl-CoA:pyruvate transcarboxylase, was a generous gift of Prof. Harland Wood. The fumarase of E. coli, prepared in this lab, was made available as a plasmid by Dr. Mark Donnelly, Argonne National Laboratory. Other enzymes and chemicals were purchased from Sigma.

Methods. (1*S*)-^{2}H,^{3}H−Fructose-1,6-P_2 (FDP) was prepared from 1-^{3}H-glucose (Sigma), hexokinase, phosphoglucose isomerase, phosphofructokinase, and pyruvate kinase in D_2O with slightly less than 2 equiv. of P-enolpyruvate. The formation of pyruvate was followed in periodic samples using lactate dehydrogenase and NADH.[17] (1*S*)-H−FDP-d_6 from glucose-d_7 (Merck of Canada) and 3-^{13}C−FDP from 3-^{13}C−glucose (Cambridge Isotopes) were prepared in the same way in H_2O. In the former case, addition of phosphofructokinase was delayed to allow the deuterium at C-2 of the glucose-6-P to fully exchange with solvent.[18] FDP, pyruvate, and malate were purified on Dowex-1-Cl^- columns using 0.2, 0.02, and 0.01 N HCl, respectively, for elution in about 3, 5, and 4 column volumes, respectively. DHAP was assayed with glycerol-P dehydrogenase and NADH. FDP was assayed by the further addition of FDP aldolase. When the isolated FDP was used to generate the ePY for NMR studies in D_2O it was treated with activated charcoal to remove contaminating ATP, reabsorbed on a short Dowex 1-Cl^- column which was next exchanged with D_2O before the FDP was eluted with 0.2 N DCl. The pH was carefully adjusted to ~6 with dilute NaOH in D_2O for storage at −10 °C until conversion to ePY. Samples of ePY for ^{1}H NMR studies were generated by reaction of DHAP with MGS in D_2O, or of labeled FDP with aldolase and MGS in D_2O and stored in dry ice or liquid nitrogen, if necessary, prior to use. 3-^{13}C FDP was used to generate 1-^{13}C ePY. (1*S*)-[1,3-^{2}H] FDP, used to generate [1,3-^{2}H]-ePY, was prepared from glucose-d_7. NMR spectra were recorded at 500 MHz on a Bruker instrument at 5°. DSS (0.03 mM) was included as an internal standard.

To determine the stereochemical course of the formation of lactoyl glutathione, the D-lactate, derived by treatment with Glx II, was converted to pyruvate in good yield at pH 7.5 by incubation with NADH (5 mM), D-lactate dehydrogenase (1 mg), phenazine methosulfate (0.2 mM) and catalase for 30 min at 37° with shaking in the dark.[19] Catalase was included to minimize the accumulation of H_2O_2 with loss of pyruvate. The isolated pyruvate was converted to malate using transcarboxylase, methylmalonyl-CoA, NADH, and malate dehydrogenase as described previously.[20]

All enzymatic studies were done at 25 °C.

Abbreviations. (Bis-Tris) 2-bis {hydroxyethyl}amino-2-{hydroxymethyl}1,3-propanediol, (DHAP) dihydroxyacetone-P, (D-LG) D-lactoyl glutathione, (DSS) sodium 2,2,-dimethyl-1-silapentane-5-sulfonate, (ePY) enol-pyruvaldehyde, (eDP) cis-enediol-3-P, (FDP) fructose-1,6 bisphosphate, (Glx) glyoxalase, (GSH) glutathione, (GS-ePY) HTA-adduct of GSH and ePY, (GS-MG) HTA-adduct of GSH and MG, (HTA-) hemithioacetal, (MG) methylglyoxal, (MGH) methylglyoxal hydrate, (MGS) MG synthetase, (PEP) phosphoenolpyruvate, (2PG) 2-phosphoglycolate.

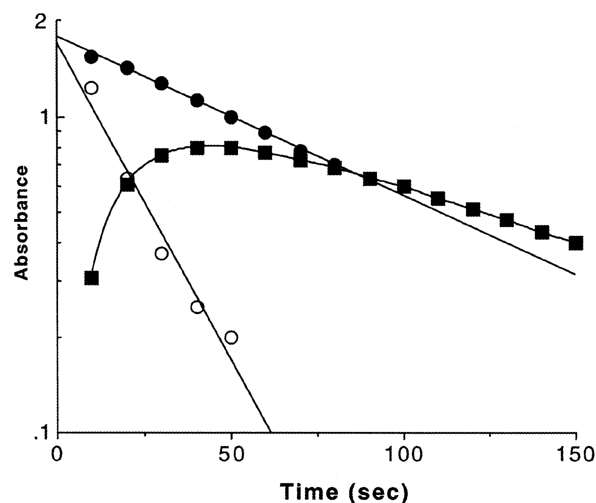


Figure 1. Absorbance changes upon mixing DHAP and MGS. MGS (36 U) was mixed into 1 mM DHAP, pH 7.5 in H_2O (open circles), D_2O (filled circles), or D_2O with 0.6 mM P_i (filled squares). The 245 nm absorbance was recorded at 10 s. intervals.

Results

Demonstration of an Unstable Product of the MGS Reaction. To search for an enol product, the MGS reaction was monitored in the UV, at 245 nm.[21] A large amount of enzyme was used so that a transient product with a signal intensity expected of an enol could be detected if its decay rate were not much greater than 20% per sec. Indeed, a rapid increase in absorbance was observed followed by its decay with $t_{1/2} = 10$ s in H_2O and 60 s in D_2O, Figure 1. In the presence of P_i, a strong inhibitor of MGS,[22] the synthetic phase was greatly slowed. A prolonged period during which synthesis and decay of the intermediate are equal in rate was followed by net decay

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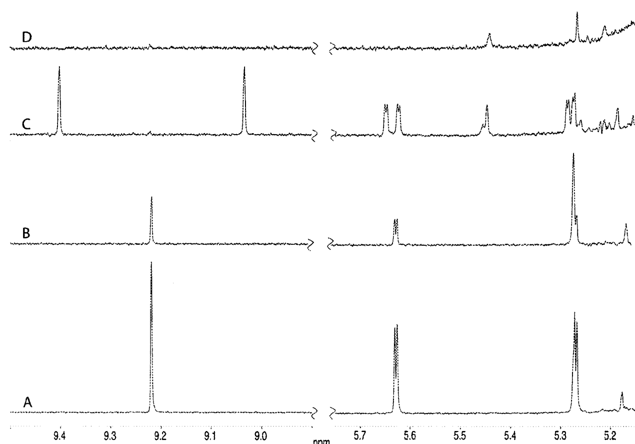


Figure 2. ^{1}H NMR spectra of enol-pyruvaldehyde (ePY), methylglyoxal hydrate (MGH) and labeled ePY. (A) ePY generated from the reaction of DHAP and MGS; (B) MGH and ePY, formed after incubation of the (above) sample of ePY at ca. 40 °C for several minutes; (C) 1-^{13}C ePY generated from the reaction of 1-^{13}C DHAP and MGS; (D) [1,3-^{2}H]-ePY generated from (1*R*),(3*S*)-[1,3-^{2}H]-DHAP and MGS.

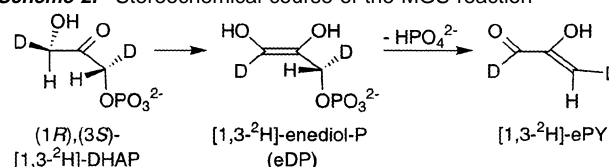
as the substrate is largely consumed,[23] presumably by its ketonization to MG, which does not absorb significantly at this wavelength.

Spectral Characterization of the Transient Product. To determine its spectrum in the ultraviolet, the transient product of MGS was generated under conditions of slow ketonization ($t_{1/2} \approx 27$ min in 50 mM Bis Tris buffer, pH 5.8, in D_2O) with a known amount of DHAP. 2-Phosphoglycolate (2PG), 0.3 mM, a strong inhibitor of MGS,[11] was added to terminate synthesis within 15 s. The absorbance from 225 to 275 nm was recorded, a sample taken for DHAP determination, and the absorbance was allowed to decay completely. From the changes in absorbance and the measured decrease in DHAP, a peak molar absorbance of 5000−5400 was determined at pH 5.8 and ~6400 at pH 7.4. The λ_{max} found, 245 nm, agrees with prediction based on empirical rules for related α,*β*-unsaturated carbonyl compounds.[21]

The transient intermediate formed from DHAP was characterized by proton NMR under conditions of relative stability (pH 5.8−6.0 and 5° C, $t_{1/2} = 90$ min in D_2O), Figure 2A. A peak for the aldehyde proton at 9.22 ppm and two doublets ($^2J_{HH} = 2.3$ Hz) for the geminal hydrogens were observed centered at 5.63 and 5.27 ppm. Coupling of the aldehyde proton to the vinyl protons is too weak to be seen. A shoulder at the upfield doublet that grows with time, Figure 2B, is derived from the C1 proton of MG aldehyde hydrate.[24] The transient proton spectrum formed from 1-^{13}C DHAP generated from 3-^{13}C FDP (Figure 2C) is also consistent with ePy. The aldehyde proton is split by the aldehyde ^{13}C nucleus ($^1J_{CH} = 183.9$ Hz). The 2-fold greater coupling constant ($^3J_{CH} = 12.5$ Hz vs 6.1 Hz) establishes the downfield proton as trans to the aldehyde group.[25,26]

To examine the stereochemistry of the MGS reaction, the DHAP was labeled with ^{1}H at the C3 *pro-R* position (by virtue of the *pro-R* specificity of phosphoglucose isomerase in the

Scheme 2. Stereochemical course of the MGS reaction



synthesis starting with perdeutero-glucose-6-P in H_2O).[18,27] If the specifically labeled 3-^{1}H enediol-P intermediate (Scheme 1) were the immediate product of MGS and rapidly decomposed to the enolaldehyde ($k = 10^6$ s^{-1} in solution)[1], one would expect the C3 ^{1}H of ePY to be randomly located and the pairs of doublets (Figure 2A) would appear as two singlets. If the *β*-elimination step occurs on the enzyme, only one of the singlets should be ^{1}H labeled. As observed (Figure 2D) the downfield doublet has disappeared, is ^{2}H, and the upfield doublet is seen as a singlet. With further incubation at 25° in D_2O, this peak decreased in intensity. This decrease establishes that this peak does not derive from the C1-hydrogen of the MGH that would form; this hydrogen would not give a signal, having been derived from the C3-deuterium of glucose-*d*$_7$. By using glucose-*d*$_7$ for the synthesis of DHAP the formation of protio-glyceraldehyde-3-P in the aldolase reaction is avoided. The chemical decomposition of glyceraldehyde-3-P to MG in D_2O leads to perdeutero-MGH. Therefore, the shoulder at the upfield doublet position, shown in Figure 2B, would not be expected to increase with the formation of MGH.

The combination of experiments C and D establishes that the hydrogen that is cis to the aldehyde of ePY is derived from the *pro-S* hydrogen of DHAP, as shown in Scheme 2. This requires that in the conversion of the cis-enediol-P to ePY, expulsion of monophosphate must occur from the (1*si,2re)*-face of the molecule (Scheme 2). This is the same face from which Asp 71 must abstract the C1-proton of DHAP.

pH and Buffer Effects on the Ketonization Rate of ePY. To examine the influence of pH and buffer on the ketonization rate, a small volume of 2PG was added to quench the enzyme after a brief synthetic period. The reaction conditions were then adjusted as desired, and the subsequent decline in absorbance was monitored in the absence of enzymatic synthesis. Rates of absorbance change, 0.69/ $t_{1/2}$, were linear for at least 3 half-lives. A low buffer-independent rate, $\sim 3 \times 10^{-4}$s^{-1}, is observed from pH 2−7. Higher rates are seen at high pH indicating specific OH^- catalysis: $k_{OH^-} = 0.05$ s^{-1} at pH 10. Carboxylates, piperizine alkylsufonates, and P_i provide a linear Brønsted plot (Figure 3) consistent with general base catalysis with $\beta \approx 0.8$, similar to the value for ketonization of vinyl alcohol, 0.77.[29] However, ketonization of ePY is about 75-fold slower than that of vinyl alcohol under the same conditions, probably due to the double bond conjugation of the ground state of the former. HCO_3^- and Bis-Tris are significantly less effective catalysts than predicted from their basicity and are therefore preferred for use as buffers.

Coupling of MGS and Glyoxalase I. MGS is usually assayed by coupling with Glx I and GSH by following the increase at 240 nm due to D-LG. The D_2O effect of the coupled

(23) The MGS rate becomes more strongly inhibited as P_i increases and DHAP decreases as a consequence of sigmoidal kinetics,[10,22] to such an extent that it becomes very difficult to run the reaction to completion.

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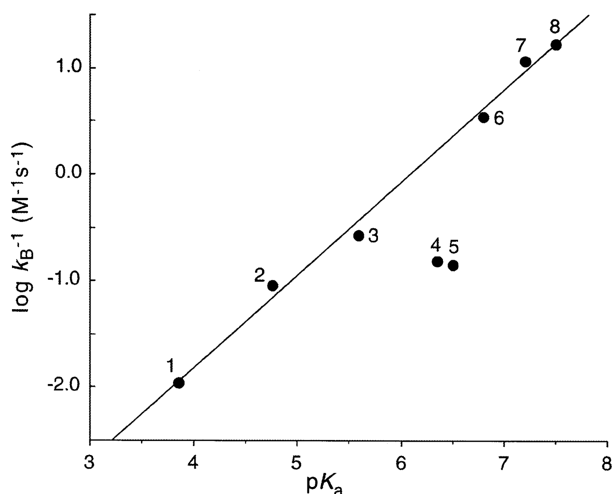


Figure 3. Brønsted plot. **1.** lactate pK_a 3.86, **2.** acetate pK_a 4.76, **3.** succinate pK_a 5.59, **4.** HCO_3^- pK_a 6.35, **5.** Bis-Tris pK_a 6.5, **6.** PIPES pK_a 6.8, **7.** P_i pK_a 7.2, and **8.** HEPES pK 7.5, where $k_{B^-} = 0.69/t_{1/2} \cdot x$(buffer concentration) $x \cdot f_{B^-}$ and f_{B^-} is the fraction of buffer in the basic form.

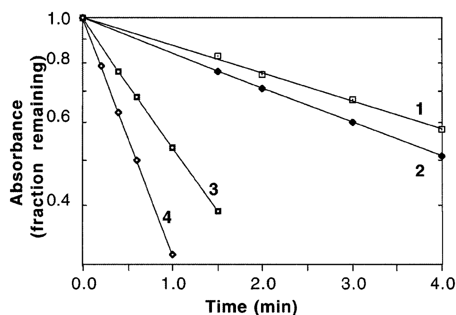


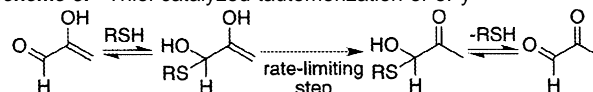
Figure 4. Activation of ketonization by thiols. ePY was generated in Bis-Tris (50 mM, pH 5.9) and quenched. Additions were **1.** none, **2.** oxidized **GSH** (2 mM), **3.** 2-mercaptoacetate (2 mM), **4.** GSH (2 mM).

system with limiting MGS was only 1.5−2, compared with ∼6 for the spontaneous loss of the absorbance due to ePY, Figure 1. No D_2O effect was observed in the rate of production of ePY by MGS assayed by the initial increase in absorbance at 245 nm, nor was a primary effect seen with 1-^2H-DHAP in the Glx I-coupled reaction.[10] Therefore, the rate-limiting step of the MGS reaction does not involve proton transfer. No lag was seen in the Glx-coupled system in low buffer at pH 6 with sufficient Glx I and GSH to complete the assay within 60 s although $t_{1/2}$ of ePY by the spectral decay assay was ∼300 s. Therefore, activation of the ketonization of ePY by GSH, Glx I or the combination of GSH and Glx I is required.

It was noted that GSH (pK_a 3.59, 8.75, and 9.65),[30] leads to an increased rate of fall in absorbance of ePY. Using 2 mM GSH at pH 5.9, $t_{1/2}$ was 36 s (Figure 4). A comparable amount of oxidized GSH showed only a small acceleration relative to the background rate, $t_{1/2} \approx 380$ s. 2-Mercaptoethanol increased the rate of loss of absorbance ∼5-fold, $t_{1/2} \approx 69$ s at 2 mM.

Is the loss of absorbance at 245 nm in Figure 4 a measure of the ketonization of ePY? Because thiols are not reported to be catalysts of ketonization, two alternative explanations were considered: a Michael (vinyl) addition of the thiol to the ePY or the formation of a hemithioacetal adduct. Immediately after

Scheme 3. Thiol-catalyzed tautomerization of ePy



the loss of the 245 nm absorbance following the addition of GSH to newly generated ePY the Glx I reaction was detected without a lag and to the same extent found if time were allowed for MG to be formed spontaneously, before addition of the GSH and Glx I. Therefore, the loss of absorbance found upon addition of GSH to ePY cannot be the result of a Michael addition. For the formation of a hemithioacetal adduct of ePY, per se, to be responsible for the loss of the ultraviolet absorbance, the assay without a lag suggests that either Glx I catalyzes the ketonization of the adduct as well as its well-known isomerization reaction, i.e., GS-ePY → GS-MG → D-LG, or else the ketonization of the adduct is nonenzymatic and sufficiently fast for coupling to Glx I.

If the HTA adduct of GSH with stereospecifically formed ePY were ketonized by action of Glx I, then one might expect to be able to generate D-LG with an isotopically chiral CH_3 group. To examine this possibility, specifically labeled $3\text{-}^2H,^3H$-DHAP was generated from labeled FDP[10] and aldolase, and converted to D-LG by coupling MGS and excess Glx I in H_2O. Chirality in the Glx I reaction should result in unequal ^3H labeling of the geminal protons of malate, a consequence of isotope discrimination of the transcarboxylase reaction.[20,31] The results, based on labilization of ^3H by reaction with fumarase, were identical to those obtained with the noncoupled system where chirality is lost in the formation of MG.[10] Thus, 49 and 50 ± 1% of the ^3H of isolated malate was labilized by fumarase compared with 37 or 64% found previously[20] with pyruvate formed from Z and E ^3H−PEP, respectively. Therefore, ketonization would seem to occur prior to the Glx I reaction, and be nonenzymatic:[32] GS-ePY → GS-MG.

Ketonization of Hemithioacetals of ePY. Formation of HTA adducts of simple aldehydes is known to be very rapid,[33] much faster than the rates seen in Figure 4. Thus, if an HTA-ePY adduct is an intermediate in ketonization it would be present in small amount in a preequilibrium leading to the loss of the slower change of the absorbance noted, Scheme 3.

According to this model, the rate of apparent ketonization should become nonlinear with respect to thiol concentration when the concentration of thiol is comparable to or greater than the dissociation constant of the complex. No departure from linear dependence on mercaptoethanol concentration was observed up to 40 mM, the limit for reliable measurements at 5° C. Thus, K_{diss} of the mercaptoethanol-ePY adduct will have to be considerably greater than 40 mM. This should not be unexpected given that the dissociation constant of adducts of acetaldehyde with simple thiols is about 40 mM,[33] and that the value for the conjugation-stabilized enolaldehyde should be much greater. This implies that only a small fraction of ePY will be present in the HTA-activated forms to obtain the effects seen in Figure 4. This small fraction will ketonize more rapidly than the same amount of ePY, if for no other reason than its loss of conjugate-stabilization.

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Table 1. Functional and Bifunctional Activators[a]

activator	concentration ($M \times 10^{-3}$)	$k_{activator}$ ($M^{-1}s^{-1}$)[b]	succinate effect[d]	D_2O effect[f]
GSH	2	8.4	2.4	2.04
succinate	100	0.14		6.4
$HSCH_2CH_2OH$	2	4.3	9.7	1.9
$HSCH_2CO_2^-$	0.20	37.0	1.3[e]	1.24[g]
$HSCH_2CH_2NH_3^+$	0.08	97.0[c]	3.0	3.7

[a] enolPyruvaldehyde was generated in 50 mM Bis-Tris, pH 5.8 in H_2O, D_2O, or 100 mM succinate in H_2O at 25 °C. MGS activity was quenched with 2PG. Activators were then added to the noted final concentration and the absorbance change recorded. [b] The ketonization rate in H_2O, corrected for the rate with BisTris buffer alone (2.2×10^{-3} s^{-1}), was measured with the concentration of activator noted. "$k_{activator}$" is the corrected rate divided by the activator concentration. [c] Compared with $HOCH_2CH_2NH_3^+$, 0.006 $M^{-1}s^{-1}$. [d] Calculated with correction for the rates with and without 100 mM succinate (0.014 and 0.0022 s^{-1}). [e] The succinate effect with 3-mercaptopropionate was 1.2-fold. [f] Corrected for D_2O effect in the absence of activators. [g] 1.0, determined for 3-mercaptopropionate

Bifunctional Catalysis. If thiols, such as mercaptoethanol, activate ketonization as the HTA adduct of ePY and buffers, such as acetate, do so by promoting the enol to keto conversion one might expect an even greater activation by 2-mercaptoacetate, which has the possibility of intramolecular buffer catalysis. Indeed, mercaptoacetate and 2-mercaptoethylamine are much better catalysts than mercaptoethanol (Table 1). Using 2 mM mercaptoethanol, it would require ~300 mM lactate, a base with the same pK_a as mercaptoacetate, to obtain the rate found with 2 mM mercaptoacetate. It would require 650 mM ethanolamine with 2 mM mercaptoethanol to achieve the rate found with 2 mM mercaptoethylamine. The smaller enhancements by 100 mM succinate seen with these activators compared with mercaptoethanol, Table 1, may indicate shielding of the enol site by the carboxylate and amino groups of these HTA adducts. The greater rate with GSH and lower succinate effect seen with GSH than with mercaptoethanol suggest that the *C*-terminal glycine carboxyl of GSH may also play a catalytic role.

The much lower D_2O effects seen in Table 1 for the ketonization rate with all HTA-adducts compared with succinate may not be the result of a decreased kinetic role of proton transfer. HTA adducts are known to reach ~2.9-fold *greater* concentration at equilibrium in D_2O due to inverse fractionation factor for the HTA adduct (Ø ≈ 1.25) and the unfavorable value for thiols (Ø ≈ 0.43).[34] Therefore, a solvent kinetic isotope effect in the ketonization of ePY of ~5.9 should be decreased to ~2.0, as found with GSH and mercaptoethanol (Table 1). The larger effects seen with the mercaptoethylamine adduct, 3.9, may indicate a decrease in the fractionation factor contribution as the ketonization rate increases relative to the decomposition of the adduct.

Discussion

The use of enzymes to synthesize enols is not new. Both as an enzymatic end product,[35,36] and as a bound intermediate liberated by acid denaturation,[37] these enols have been useful for enzymatic and chemical mechanism studies. The source enzyme can be inactivated, as in the present case, and conditions altered for further study within the lifetime of the enol product. By lowering the temperature to 5°, after its synthesis from DHAP in D_2O at 25°, ePy was sufficiently stable for its NMR spectrum to be recorded.

The original proposal that enolpyuvaldehyde and not methylglyoxal is the product of methylglyoxal synthetase was based on the generalization that enzymatic products should not be stereospecifically random as was found to be the case in forming the methyl group of MG.[10] An exception to this rule, subsequently found with acetoacetate decarboxylase,[32] required a reexamination of the assumption that MG was not the immediate product of the MGS reaction. This conclusion has now been verified by identification of a transient intermediate in great excess of enzyme equivalence (Figure 1), with spectral properties of ePY (Figure 2). The observation that ePY is stereospecifically formed in the MGS reaction (Figure 2D) made it possible to evaluate whether its ketonization might be enzyme catalyzed in the formation of the methyl group of the MGS/GSH/Glx I system. Because stereospecificity was not observed in the CH_3 group of the D-LG product, it was concluded that ketonization of ePY is not enzyme catalyzed, again with the application of the enzyme stereochemical rule. That free ePY is formed stereospecifically establishes ePy as the primary, and with P_i, the only product of methylglyoxal synthetase, or more accurately DHAPase.

Our conclusion that both the general base, Asp 71, and the eliminated OPO_3 group occur from the same face of the enediol-3-P intermediate (Scheme 2) depends on the stereochemistry of the proton abstraction of the first step (ref 10) and the structure of the intermediate. On the basis of X-ray diffraction studies of the Harrison group, the −NOH and COH groups of phosphoglycolohydroxamic acid, a very high affinity inhibitor, are hydrogen bonded to the active site in a cis-geometry.[13] The contribution of the present work, identifying the methylene protons in relation to the aldehyde group of the enolpyruvaldehyde product, Figure 2C and D, establishes the stereochemical course of the enzymatic reaction, shown in Scheme 2.

The uncatalyzed ketonization of ePY, ~0.25% s^{-1} at pH 7, is much too slow to be a step in the MGS:Glx I couple. The enhanced stability of ePY compared with other enols, and its very unfavorable association with thiols and H_2O compared to other aldehydes are undoubtedly due to conjugation of the ene- and aldehyde groups. The absence of evidence for hydration of the carbonyl of ePY in the proton NMR spectra is consistent with 0.8% hydration of benzaldehyde.[40]

In support of the HTA adduct as the form in which GSH activates ketonization of ePY are the following factors: (1) a similar effect with mercaptoethanol, (2) oxidized glutathione is inactive, (3) 2-mercaptoacetate and mercaptoethylamine are manyfold more active than equivalent amounts of mercaptoethanol and the corresponding base, appearing to act as bifunctional activators. An eight-membered ring structure would provide a linear alignment of donor-proton-acceptor that should be favorable for proton transfer.[38,39] (4) The low solvent isotope effect found with GSH and mercaptoethanol, ~2 compared with that of the uncatalyzed or general base-catalyzed reactions, 5−6, is readily explained by the inverse solvent effect expected for the HTA-substrate equilibrium and is not inconsistent with slow proton transfer.

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The following published evidence may be cited to support the kinetic model in which the rate-limiting step for HTA catalyzed ketonization is the ketonization step (Scheme 3). Rate-limiting formation of HTA adducts would require the observation of an inverse solvent isotope effect, a reflection of a 2-fold higher concentration of RS^- at equilibrium in D_2O.[33] However, inverse effects have not been found either in the Glx I coupled assay or in the direct assays, Table 1. With respect to the last step of Scheme 3, it has been shown that the decomposition of HTA adducts of keto aldehydes[24] and of acetaldehyde with weakly acidic thiols,[33,41] are specific base, not general base, catalyzed. The effects of succinate shown in Table 1, as well as those of the bifunctional activators, are therefore not consistent with rate-limiting dissociation of the HTA-MG adduct. It will be useful to determine the relative equilibria for formation of the several bifunctional HTA adducts of ePY before their kinetic differences can be attributed to mechanistic factors. Direct measurement of adduct formation at higher concentrations of the several mercaptans, by following the loss of 245 nm absorbance by rapid flow kinetic methods, will be necessary to establish the rates and equilibria for formation of the adducts. It should then be possible to compare the intrinsic rates of ketonization of simple HTA-ePY adducts with that of other enols and determine if the HTA function of simple thiols plays a role other than the loss of double bond conjugation. One such role (as suggested by a reviewer of this paper), might be for the HTA-hydroxyl group to activate a bridging water molecule for proton transfer to the enolate-carbon.

enolPyruvaldehyde will not be a major metabolite in modern nucleated cells, given their lack of MGS. However, in some microorganisms, as much as 40% of the glycolytic flux may utilize the MGS/glyoxalase pathway without exceeding the capacity of MGS, the ketonization of ePY and the glyoxalase system.[42] With 1−5 mM GSH,[43,44] a 5−25% per sec. rate of ketonization ensures that GS-MG, not MG will be available for the Glx I reaction. In bypassing free MG, primarily as MGH, the slow dehydration step, ∼1% per sec,[45] is avoided as a rate-limiting step in reacting with GSH. When MG is observed in cell growth media[9] it is probably due to insufficient Glx I activity with which to trap the generated GS-MG.

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One is left to wonder whether the use of GSH in activating the otherwise slow ketonization of ePY and in bypassing the slow dehydration of MGH may have played an evolutionary role in the selection of GS-MG to be the substrate form of the glyoxalase system. If so, other glyoxalase systems may exist in nature. For example, most actinomycetes lack GSH, but contain mycothiol (made up of linked *N*-acetylcysteine, glucosamine, and myoinositol) as their major low molecular weight reducing agent[44] . It will be of interest if these organisms evolved a glyoxalase system that uses mycothiol, which should be about as active as glutathione in catalyzing the ketonization of enolpyruvaldehyde.

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