Methylglyoxal Synthase Uses the Trans Isomer or Triose-1,2-enediol 3-Phosphate

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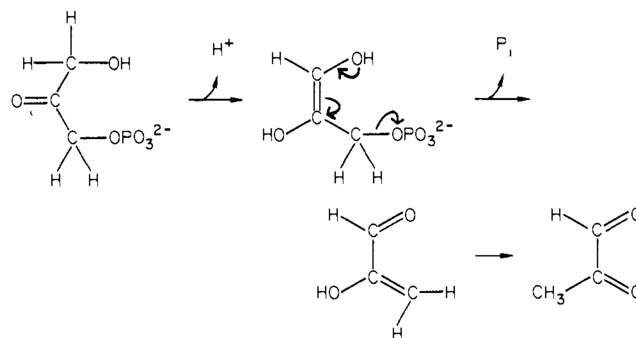
Abstract: The conversion of L-glyceraldehyde-3-P to either dihydroxyacetone-P or to P_i plus methylglyoxal under alkaline conditions is completely blocked by $K_3Fe(CN)_6$. This confirms the role of enediols, known to be readily oxidized by ferricyanide, as intermediates in these models of the triose-P isomerase and methylglyoxal synthase reactions. Triose-P isomerase, which does not react with L-glyceraldehyde-P directly, is able to convert 60% of it to dihydroxyacetone-P by reacting with the *cis*-enediol-P that is formed during hydroxide catalyzed enolization at pH 9.5. Methylglyoxal synthase prevents the oxidation by added ferricyanide of the remaining trans isomer, converting it to P_i at a diffusion-controlled rate. The synthease enzyme previously had been shown to be inactive toward the *cis*-enediol-P that is released upon the denaturation of triose-P isomerase during catalysis. Since methylglyoxal synthase accepts the *trans*-enediol-P as a substrate, it is likely that it or the *trans*-enediolate is an intermediate in the catalytic reaction with dihydroxyacetone-P.

The mechanism proposed[1] for the methylglyoxal synthase reaction (also dihydroxyacetone-phosphate phospho-lyase, EC 4.2.99.11), Scheme I, includes abstraction of the *pro-S* hydrogen of the hydroxymethyl group of the specific substrate, dihydroxyacetone-P (DHAP), followed by β-elimination of P_i from the resulting enediol-P. This mechanism is also thought to explain the conversion of triose-P's to P_i and methylglyoxal (MG) in alkali. In the enzyme-catalyzed reaction proton addition to form methylglyoxal was shown to be nonstereospecific so that it was considered likely that the product released from the enzyme was either the enoladehyde which would then ketonize in solution or the enediol-P itself, followed by uncatalyzed decomposition. A similar mechanism is generally accepted for the alkali-catalyzed reaction.

We have shown[2] that an enediol-P liberated by acid treatment of triose-P isomerase during catalysis is sufficiently stable to be tested for utilization by other enzymes. In this way fructose bisphosphate aldolase, the Zn^{2+}-dependent enzymes of yeast, and triose-P isomerase were found to use the same enediol-P species. Based on a least-motion model for the intramolecular hydrogen transfer, all isomerases are believed to use *cis*-enediol intermediates.[3] Methylglyoxal synthase was found not to utilize the triose-P isomerase intermediate. This would be expected if the enediol-P were the true product of the synthase reaction or if the active site of the synthase was inaccessible to the isomerase-derived enediol-P. Another and testable possibility is that the isomerase and the synthease utilize enediol-P intermediates of opposite stereochemistry. It was therefore necessary to test the synthase with the trans isomer of tri-1,2,3-hydroxypropene-3-P (X).

Scheme I



Experimental Section

Methylglyoxal synthase was isolated from *Proteus vulgaris* cells (ATCC 13315) by the procedure of Tsai and Gracy[4] and had a specific activity of 8 units/mg. Synthase activity was determined by the glyoxalase I coupled spectrophotometric method.[5] The highest reported specific activity of 10 units/mg and molecular weight of 66000[4] were used to calculate the molar concentration of active enzyme in solution.

L-Glyceraldehyde [^{32}P]phosphate (or [^{32}P]-L-G3P) was prepared enzymatically by phosphorylation of L-glyceraldehyde (Aldrich) with *Escherichia coli* glycerol kinase (Calbiochem): 0.5 mL containing $\sim 10^8$ cpm of [γ-^{32}P]ATP (2.2×10^{11} cpm/μmol at the time of preparation), L-glyceraldehyde (54 mM), $MgCl_2$ (5 mM), and triethanolamine (TEA) hydrochloride (88 mM TEA·HCl, pH 7.6) was incubated with glycerol kinase (0.04 unit) for 15 min at 25 °C. About 85% conversion of radioactivity to an acid-stable form occurred. The reaction was quenched with Cl_3CCO_2H (0.2 M, final), and any unreacted ATP was removed by adsorption on charcoal. This solution was used as the source of labeled G3P without further purification to avoid raising the [^{32}P]P_i content of the product. ^{32}P in all triose phosphates was assayed by liquid scintillation after treatment with alkali (15 min, 0.5 N NaOH, 37 °C) and extraction of [^{32}P]P_i into 2-butanol as the acid molybdate complex.[6] The preparation was free of DHAP and D-G3P but contained 4–5% [^{32}P]P_i.

Unlabeled L-G3P was prepared from a DL mixture as follows: DL-G3P (200 μmol) was prepared from the acetal according to Calbiochem and incubated in 1.5 mL at pH 7.6 (0.1 M TEA·HCl) with triose-P isomerase (TIM, 20 units), α-glycerol-P dehydrogenase (α-GPD, 30 units), NADH (0.3 mM), yeast alcohol dehydrogenase (40 units, Sigma), ethanol (340 μmol), and semicarbazide (70 mM). Disappearance of alkali-labile phosphate and of D-G3P were followed. The reaction was complete within 4 h. CCl_3CO_2H was added to precipitate protein and the supernatant was used as a source of L-G3P (95% yield). P_i was determined with malachite green and acid molybdate.[7,8] DHAP was determined by the glycerol-P dehydrogenase reaction measuring NADH oxidation. D-G3P was then measured after further addition of triose-P isomerase. The remaining alkali-labile [^{32}P]P was ascribed to L-G3P.

Results

Hall and Knowles[9] have studied the slow interconversion of DHAP and G3P in neutral solution as a possible model for the isomerase reaction. The enediol character of the model reaction was assumed and if correct would suggest conditions for preparing a mixture, however transient, of the enediols. However, evidence[10,12] that both acid- and base-catalyzed isomerase models occur

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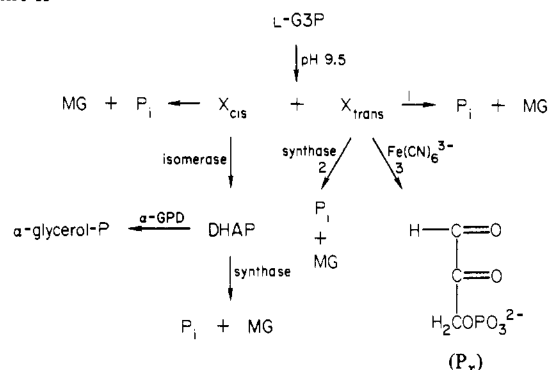
Table I. Conversion of L-G3P to DHAP Proceeds via an Enediol-P Intermediate[a]

additions	P_i, mM	DHAP, mM	D-G3P, mM
	2.92	0.083	0.128[b]
$K_3Fe(CN)_6$, 1 mM	0.25	0.002	0.002

[a] An incubation mixture (0.4 mL, 25 °C) containing [^{32}P]-L-G3P (11.7 mM), NADH (0.25 mM), α-glycerol-P dehydrogenase (20 units), and buffer (80 mM glycine–NaOH, pH 9.5) was monitored spectrophotometrically at 340 nm. After 15 min the decrease in the NADH absorbance measured the DHAP formed, and further NADH oxidation after the addition of excess triose-P isomerase represented the amount of D-G3P. The presence of $K_3Fe(CN)_6$ did not interfere with either the dehydrogenase or isomerase activities. The [^{32}P]-P_i and [^{32}P]-L-G3P remaining were determined as described under Experimental Section. [b] Corrected to ~0.14 mM based on 25% breakdown of L-G3P during the incubation.

Scheme II[a]



[a] X = triose-enediol-P.

by hydrogen conserving pathways, i.e., presumed hydride transfer mechanisms and the marked instability found[2] for the *cis*-enediol-P, $t^1/_2$ ~7 ms, necessitate verification of the enediol character of this model for triose-P interconversion in need of verification. This was done, Table I, by showing that $K_3Fe(CN)_6$, an efficient oxidant of enediols, is able to prevent the conversion of L-G3P to DHAP and D-G3P as well as prevent the formation of P_i and methylglyoxal. A solution of L-G3P at pH 9.5 therefore provides a model for both the isomerase and synthase reactions acting through triose-enediol-P's. The partition of the mixed enediol-P's to DHAP, DL-G3P, and P_i plus methylglyoxal was on the order of 1/3.4/34 at pH 9.5 both in 80 mM glycine buffer (Table I) and in 20 mM buffer (data not shown).

The ability of triose-P isomerase to efficiently trap the *cis*-enediol-P was next used to specifically free the cis/trans mixture of that species and hopefully leave behind sufficient trans isomer to test as a substrate for the synthase. Since action of the synthase would *not* be expected to alter the formation of P_i greatly from the 90% predicted from the above nonenzymatic partition study, a test of its reactivity was devised which required that the synthase compete with ferricyanide for the enediol-P not removed by the isomerase, Scheme II.

As shown in Table II, addition of 2 μM rabbit muscle triose-P isomerase, an amount 30-fold greater than required for half-maximum trapping of the cis isomer,[2] decreased the P_i formed under standard conditions to ~38%. Therefore, one may conclude that enolization of G3P at pH 9.5 results in formation of the trans isomer 38% of the time. With sufficient ferricyanide this remaining source of [^{32}P]P_i can be diverted completely to the alkali-stable keto–aldehyde form (Table I). By use of 0.8 mM $K_3Fe(CN)_6$ in addition to the isomerase, the trans isomer was further lowered by 65% as shown by the further decrease in [^{32}P]P_i formed. When 2 units of synthase was added, the effect of the ferricyanide was almost completely reversed as would be expected if the synthase acted on the *trans*-enediol that would otherwise

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Table II. Methylglyoxal Synthase Uses the *trans*-Enediol-P[a]

glycerol-P dehydrogenase, units	additions	[^{32}P]P_i, % of total ^{32}P
7	none	62
7	TIM	24
7	TIM + $K_3Fe(CN)_6$	8.4
7	TIM + $K_3Fe(CN)_6$ + MGS	22.6
21	TIM + $K_3Fe(CN)_6$ + MGS	23.6
14	TIM + $K_3Fe(CN)_6$ + MGS	22.8
3.1	TIM + $K_3Fe(CN)_6$ + MGS	32.6
1.8	TIM + $K_3Fe(CN)_6$ + MGS	39.3

[a] The incubation mixture (0.5 mL, 0.08 M Gly–NaOH, pH 9.5, 25 °C) contained [^{32}P]-L-G3P (4.5 × 10^5 cpm), NADH (0.4 mM), and the noted amount of rabbit muscle glycerol-P dehydrogenase (Sigma). Triose-P isomerase or TIM (2 μM), $K_3Fe(CN)_6$ (0.8 mM), and methylglyoxal synthase or MGS (2 units) were added as indicated. After 1 h the [^{32}P]P_i formed was assyed directly as the radioactivity extracted as the molybdate complex with 2-butanol.

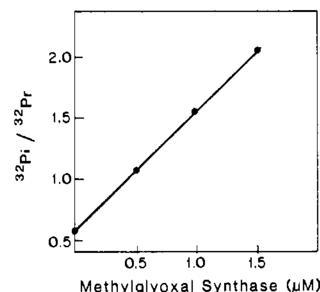


Figure 1. Effect of methylglyoxal synthase on the trapping of *trans*-enediol-P. The incubation mixture of 0.5 mL contained Gly–NaOH (80 mM, pH 9.5), [^{32}P]-L-G3P (5 × 10^5 cpm), NADH (0.4 mM), α-glycerol-P dehydrogenase (7 units), triose-P isomerase (100 units), $K_3Fe(CN)_6$ (0.8 mM), and methylglyoxal synthase (0–1.5 μM). [^{32}P]P_i was determined at zero time and after 30 min at 25 °C when about half of the L-G3P had reacted. Incubations without ferricyanide were used to determine the total formed trans isomer, the partition of which ([^{32}P]P_i/[^{32}P]P_r) could then be calculated from the [^{32}P]P_i values of incubations containing ferricyanide and varying synthase levels.

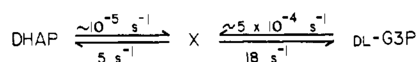
have been oxidized. The experiment was repeated with 3-fold more glycerol-P dehydrogenase to rule out DHAP as the source of the [^{32}P]P_i increment caused by the synthase. Lowering glycerol-P dehydrogenase to 3 units apparently allowed sufficient DHAP to accumulate in the steady state and provide a significant target for the 2 units of synthase present. In similar experiments it was shown that addition of 5 mM P_i, a strong inhibitor of synthase, almost completely erased the action of the synthase in competing with ferricyanide for the *trans*-enediol-P.

The effect of synthase concentration on reversing the ferricyanide trap was determined, Figure 1. Linear dependence of the partition ratio between P_i and the ferricyanide oxidized product, P_r (2–ketoglyceraldehyde–3–P), was observed as a function of synthase concentration at pH 9.5 with 7 units of glycerol-P dehydrogenase. When the symbols of Scheme II are used, the data can be interpreted to estimate k_2, the interaction rate constant of synthase with the *trans*-enediol-P, as

$$\frac{[P_i]}{[P_r]} = \frac{k_1[X] + k_2[E][X']}{k_3[K_3Fe(CN)_6][X'']}$$

where different symbols are used for the *trans*-enediol-P to avoid specifying whether the diol or diolate form is reacting. Use of the ratio of slope/intercept gives an expression independent of the ferricyanide concentration term since slope = $k_2[X']/(k_3[K_3Fe(CN)_6][X''])$ and intercept = $k_1[X]/(k_3[K_3Fe(CN)_6][X''])$. Assuming a k_1 value for the trans isomer to be equal to that obtained for the *cis*-enediol-P, 180 s^{-1}, at pH 9.5 by established procedures,[2] one obtains the relation $k_2([X']/[X]) = 2 \times 10^8$ M^{-1} s^{-1}. The high order of this value coupled with the observation that the species X is most certainly the *enediol*, as shown by the virtually constant size of k_1 from pH 1 to 9.5, suggests that the

Scheme III



species with which the synthase interacts must also be the enediol or at least cannot be the enediolate exclusively. In support of this a similar experiment requiring 16 h at pH 8.5 gave about the same value for $k_{2(\mathrm{apparent})}$.

Discussion

The high efficiency of methylglyoxal synthase in its action on the *trans*-enediol-P corresponds to a k_{cat}/K_m value of $\sim 10^8$ M^{-1} s^{-1}. Without knowing the K_m value, one cannot compare the catalytic capacity of the enzyme with the rate constant for β-elimination in solution. It seems likely that the K_m for the *trans*-enediol-P would be less than 10^{-5} M, which is the K_i of phosphoglycolate[4], so that the rate of enzyme-catalyzed β-elimination may be $<10^3$ s^{-1} and therefore possibly no greater than the solution rate of decomposition of the intermediate. Therefore, it follows that the synthase can be viewed primarily as a catalyst of the proton abstraction step for which it is many orders of magnitude superior to the neutral solution process. That the enzyme does catalyze the β-elimination step, as shown here, probably stereospecifically, indicates that the enolaldehyde, not the enediol-P, is the true enzyme product.

The reaction of methylglyoxal synthase with the presumed *trans*-enediol-P proceeds entirely in the β-elimination direction and not significantly to free DHAP which, if formed, would have been trapped by the dehydrogenase and, therefore, could not have appeared as P_i in Table II. Consistent with this, DHAP with tritium in the position for enolization is transformed in the methylglyoxal synthase reaction without isotopic discrimination or isotope exchange[1], a combination of properties best explained if a step following substrate binding and prior to enolization is rate limiting. This may also explain the low value of $k_{cat}/K_m = 10$ $s^{-1}/10^{-4}$ M = 10^5 M^{-1} s^{-1}. A slow conformational change preceding enolization has also been proposed in the triose-P isomerase reaction to explain differences between steady-state and partition kinetics.[13]

Ferricyanide must not be able to react with the enediol-P after it binds to the synthase; otherwise, the action of the enzyme could not fully overcome the oxidation that is observed in solution. As with triose-P isomerase,[14] $K_3Fe(CN)_6$ present when the enzyme acts catalytically with DHAP does not prevent formation of the normal synthase product, again consistent with the enediol-P not being the enzyme product. Therefore, both enzymes have high, diffusion-limited rates of combination with their enediol-P intermediates which show no evidence of dissociating during catalysis.

One can estimate absolute rate constants for the solution model of triose-P isomerase at pH 9.5 from the partition values of Table I, the measured half-life of L-G3P, ~35 min, the ratio at equilibrium DHAP/DL-G3P = 11, and by assuming the same rate constant, ~180 s^{-1}, for both the *cis*-enediol-P and the *trans*-isomer. When the two isomers are symbolized as X, the values are shown in Scheme III. The calculated equilibria serve to emphasize the central accomplishment of triose-P isomerase in shifting the solution equilibrium between DHAP and enediol-P from $\sim 2 \times 10^{-6}$ to $\sim 10^{-1}$ on the enzyme. It is important to ask what aspects of these two similar molecules are being differentially recognized by the enzyme.

The "advantage" of a *cis*-enediol intermediate for the aldose–ketose isomerases has been assumed to be that it allows a single electrophilic center, an acid or metal, to polarize the carbonyl group of both substrate and product.[3] The synthase reaction has no such need. However, there could be a further role for the proton that is abstracted in the enolization step. The phosphate bridge oxygen of the enediol-P should leave from out of plane of the intermediate and may receive help from the protonated base if the geometry is correct. This would regenerate the base for another cycle of reaction with the formation of the true product, the enolaldehyde. In the case of triose-P isomerase, the regeneration of the base occurs with formation of product as shown by intramolecular proton transfer. The very low rate of β-elimination of $-OPO_3^{2-}$ suggests that the phosphate bridge oxygen may be effectively restricted to the plane of the *cis*-enediol-P when bound to isomerase.[17]

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Registry No. L-Glyceraldehyde-3-P, 20283-52-7; dihydroxyacetone-P, 57-04-5; *trans*-X, 85202-45-5; *cis*-X, 85202-46-6; TIM, 9023-78-3; methylglyoxal synthase, 37279-01-9.

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