

ENZYME STEREOSELECTIVITY: THE REVERSIBLE REACTION CATALYZED BY
2-KETO-4-HYDROXYGLUTARATE ALDOLASE OF ESCHERICHIA COLI

H. Paul Meloche, Claire T. Monti, and Eugene E. Dekker

The Institute for Cancer Research, Fox Chase Cancer Center,
Philadelphia, Pennsylvania 19111

and

The Department of Biological Chemistry, The University of Michigan,
Ann Arbor, Michigan 48104

Received June 16, 1975

Summary - The condensation of [$3\text{-}^3\text{H}_3$]-pyruvate with glyoxylate in ^3HOH to form [$3\text{-}^3\text{H}_2$]-ketohydroxyglutarate was catalyzed by ketohydroxyglutarate aldolase of Escherichia coli to study the stereochemical behavior of the enzyme. It was found that, a) very early in the reaction the (4S) predominates over the (4R) isomer in a ratio of about 10:1, b) during the course of the reaction the ratio of isomers decrease in parallel with the increase in product formation, c) at equilibrium the ratio of isomer distribution approaches 1:1. These results are consistent with the aldolase being stereoselective and catalyzing a reversible reaction. Consequently, although kinetic restrictions demand that initially one isomer be turned-over with greater facility than its enantiomer, with the approach to thermodynamic equilibrium the rate ratio of isomer turnover approaches unity. The separation of kinetic from thermodynamic control in this case may enable further tests to be undertaken as to how Nature directs the stereochemistry of product synthesis in an aldolase catalyzed reaction.

INTRODUCTION

2-Keto-4-hydroxyglutarate aldolase (2-oxo-4-hydroxyglutarate glyoxylate lyase: 2-oxo-4-hydroxyglutarate \rightleftharpoons pyruvate + glyoxylate) of Escherichia coli has been purified to homogeneity (1). A unique property of the enzyme is that it turns over (4S)ketohydroxyglutarate¹ approximately 10 times faster than the (4R)isomer in both the cleavage and, initially at least, the condensation reaction (1). Both substrate isomers appear to be specific for the same

¹ Abbreviation: ketohydroxyglutarate, 2-keto-4-hydroxyglutarate; TOH, ^3HOH .

catalytic site (1). This enzyme, therefore, is stereoselective in the sense that although both optical isomers are substrates, the enzyme exhibits stereopreference for (4S)ketohydroxyglutarate. In this regard, the E. coli enzyme apparently differs from ketohydroxyglutarate aldolases of both rat (2) and bovine (3) liver as well as the α -keto- β -deoxyhexarate aldolase induced by growth of E. coli on mucic acid (4). Each of these last three enzymes can turn over either optical isomer of ketohydroxyglutarate with equal facility and are thus designated nonstereospecific.

The stereoselectivity of ketohydroxyglutarate aldolase of E. coli may be viewed as an example of the specialized behavior of a nonstereospecific enzyme. This argument is based upon the following assumptions. First, there is no thermodynamic advantage in enriching for one optical isomer of ketohydroxyglutarate in a reversible reaction producing both isomers. This point is confirmed by those aldolases which catalyze the analogous reaction but produce a random isomer distribution throughout the reaction (2-4). Secondly, it follows that at equilibrium, K_{eqR} must equal K_{eqS} . Thirdly, a priori, stereopreference is the expression of a kinetic rate phenomenon. Hence, although kinetic characteristics of the E. coli aldolase may allow the (4S)isomer to be turned-over ten times more efficiently than the (4R)isomer, thermodynamics demand a random isomer distribution at equilibrium. This being the case, regardless of relative initial rates of isomer formation, the accumulation of the (4R)isomer at the expense of the (4S)isomer must be an equilibrium phenomenon occurring throughout the course of the reaction. With this model in mind, the distinction between the three nonstereospecific aldolases mentioned before and this stereoselective aldolase rests in the latter enzyme having a disproportionate kinetic competence for one of the two optical isomers of ketohydroxyglutarate. It should be noted that if an irreversible step were introduced, the turnover of ketohydroxyglutarate in a (4S) to (4R)isomer ratio of 10:1 would occur throughout the course of the reaction.

The experiments reported in this paper were undertaken to show that the

condensation of [3-³H₃]-pyruvate with glyoxylate forms [3-³H₂]-ketohydroxyglutarate whose (4S) and (4R) isomer distribution, throughout the reaction course, conforms to the above model for a reversible system. These results further refine the kinetic and stereochemical description of the enzyme, properly relate this enzyme's stereomechanistic behavior to that of the nonstereospecific aldolases catalyzing the analogous reaction, and provide an experimental example of reversible, stereoselective isomer synthesis at the enzyme level.

METHODS AND RESULTS

2-Keto-4-hydroxyglutarate aldolase was purified to homogeneity from extracts of *E. coli*, as previously described (1). The sodium salt of tritiated pyruvate (5) (50,000 cpm/ μ mole; 10 mM) was incubated at 25° with sodium glyoxylate (20 mM) at pH 7 in a total volume of 1.0 ml containing 20 mCi TOH and 2.15 units (1) of ketohydroxyglutarate aldolase. The specific activity of the water in the incubation mixture was 60,000 cpm/ μ atom H. At given time intervals, 0.1 ml aliquots were removed from the incubation mixture and reacted with 0.05 ml of 30% H₂O₂ in a distillation flask. In this process, tritiated pyruvate is oxidatively decarboxylated with formation of tritiated acetate while tritiated ketohydroxyglutarate is converted to tritiated malate. After 15 min, the bulk of the tritiated water was removed by lyophilization; the residue was dissolved in 1 ml of H₂O and re-lyophilized. The residue was then dissolved and chromatographed on a column (0.75 x 15 cm) of Dowex-1-C1 using a linear gradient of HCl (0 → 0.1 N), which procedure separates tritiated acetate from tritiated malate. The pooled malate peak was dried (from acid) in vacuo which completely excludes any contaminating acetate. By measuring the ratio of acetate to malate observed upon ion exchange chromatography, one can demonstrate the approach to equilibrium for ketohydroxyglutarate synthesis. This result is shown in Fig. 1. It is evident that after an incubation period of 1200 min, the aldol condensation reaction is near or at equilibrium.

The distribution of (4R) and (4S) ketohydroxyglutarate at any given time

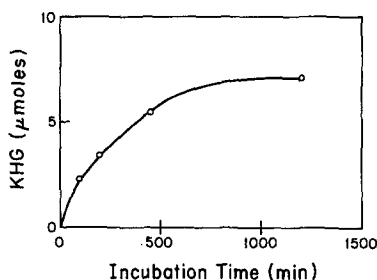


FIG. 1. Time-course of ketohydroxyglutarate formation. Ketohydroxyglutarate is designated as KHG.

during the catalytic process can be estimated by determining the percent of the total malate as the L-isomer. The method used for this purpose is presented schematically in Fig. 2. It can be seen that the condensation of tritiated pyruvate with glyoxylate yields $[3-^3\text{H}_2]-(4\text{R})$ and $-(4\text{S})$ ketohydroxyglutarate which are converted by oxidative decarboxylation to $[3-^3\text{H}_2]-\underline{\text{D}}$ - and L-malate, respectively. The convention for assigning D and L-configurations of ketohydroxyglutarate has been previously discussed (1). By this convention, L-ketohydroxyglutarate decarboxylates with formation of L-malate so that L- is (4S) and correspondingly D- is (4R). By the action of malate dehydrogenase and acetylpyridine-DPN, the $[3-^3\text{H}_2]-\underline{\text{L}}$ -malate is specifically converted to $[3-^3\text{H}_2]-\underline{\text{L}}$ -oxalacetate. In the presence of excess 2-keto-3-deoxygluconate-6-P aldolase, the labeled oxalacetate is converted in turn to tritiated pyruvate (6) which then completely exchanges its tritium with protons of water, a process also catalyzed by the aldolase (4,5). The D-isomer of $[3-^3\text{H}_2]-\underline{\text{D}}$ -malate is inert in this reaction sequence. Thus, the fraction of total tritium in malate which is released as TOH is a direct measure of the percent of L-malate in the sample as well as of the fraction of (4S) ketohydroxyglutarate formed in the aldolase-catalyzed condensation reaction. Results obtained in such experiments are shown in Table I. It can be seen that 84.7% of the condensation product exists as the (4S) isomer after incubating the reaction mixture for 100

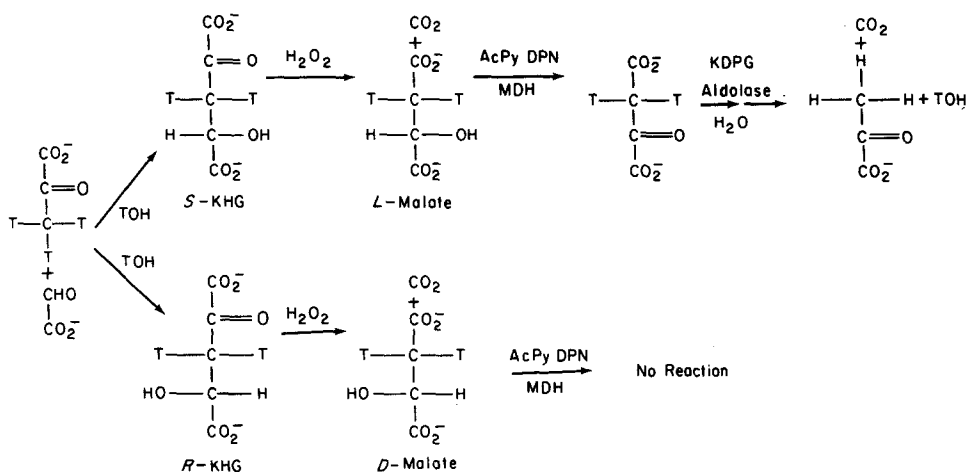


FIG. 2. Determination of the fraction of (4S)ketohydroxyglutarate in (4R,S)ketohydroxyglutarate resulting from the condensation of tritiated pyruvate with glyoxylate.

TABLE I. Fraction of [3-³H₂]-L-malate in [3-³H₂]-D,L-malate derived from [3-³H₂]- (4R,S)ketohydroxyglutarate. Tritium of [3-³H₂]-L-malate was converted to ³HOH as described in the text. The ³HOH was determined by passing an aliquot of the reaction mixture through a bed (0.75 x 4 cm) of Dowex-1(Cl), removing non-covalently bound tritium by washing with water, and then counting the wash. This process was repeated to demonstrate that detritiation had equilibrated.

Incubation Time (min)	Total tritiated malate (cpm/ml)	³ HOH released (cpm/ml)	Fraction of malate (ketohydroxyglutarate) as the (4S) isomer (%)
100	7650	6480	84.7
200	7850	6345	80.8
450	21,830	14,850	68.0
1200	26,900	14,060	52.3
Control [3- ³ H ₂]-D,L-malate	22,000	10,750	48.9

min. After 200 and 450 min of incubation, the fraction of (4S) isomer in the condensation product progressively decreases, reaching 52.3% after 1200 min when C-C bond synthesis appears to be at equilibrium (Fig. 1). A plot of the data would show the loss of (4S) isomer as a function of time to be linear up to 450 min, extrapolating to 90% (4S) isomer very early in the condensation reaction. Thus, the accumulation of the (4R) isomer at the expense of the (4S) isomer occurs throughout the course of the reaction in parallel with overall product synthesis, equilibrating to a 50-50 distribution of optical isomers. A control experiment showed that the assay system released one half the label of [3-³H₂]-D,L-malate to tritiated water as expected. The data, therefore, are consistent with ketohydroxyglutarate aldolase of *E. coli* being a reversible, stereoselective enzyme.

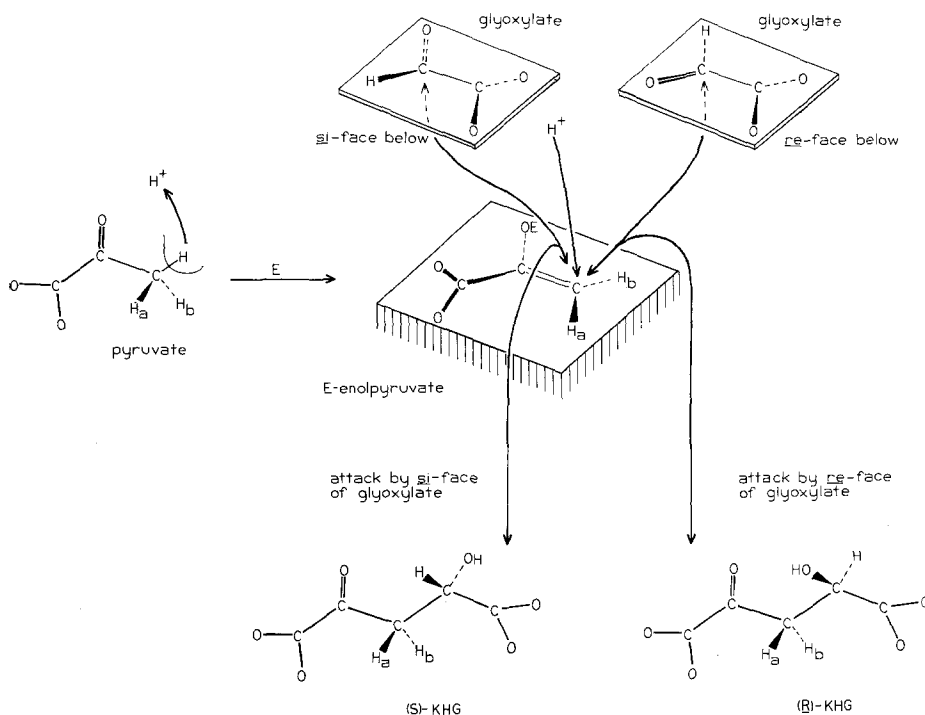


FIG. 3. Stereochemical mechanism of ketohydroxyglutarate synthesis.

Ketohydroxyglutarate is designated as KHG.

Selection for the optical isomer of ketohydroxyglutarate generated can be viewed as being controlled by which face of the carbonyl carbon atom of glyoxylate attacks enzyme-bound enolpyruvate as shown in Fig. 3. The mechanism assumed is that elucidated for bovine liver ketohydroxyglutarate aldolase and α -keto- β -deoxyhexarate aldolase of *E. coli*, in which both the exchanging proton and glyoxylate attack the same face of bound enolpyruvate (4). With these two enzymes, it was concluded that either the si² or the re face of the carbonyl carbon atom of glyoxylate could attack bound enolpyruvate with equal facility (4). An a priori difference, then, between these two enzymes and ketohydroxyglutarate aldolase of *E. coli* would be that the latter enzyme handles the carbonyl carbon of glyoxylate oriented for si face attack with much greater facility than when oriented for re face attack. It would be of interest to determine whether differences in rate limiting steps in the turnover of (4S) vs (4R)ketohydroxyglutarate consistent with differences in dissociation of the E-glyoxylate complex could be detected in future experiments with this enzyme.

ACKNOWLEDGMENTS

Supported in part by NIH Grants GM-18326, CA-06927, RR-05539, AM-03718; by NSF Grant BO-42019; and by an appropriation from the Commonwealth of Pennsylvania. The authors are indebted to Dr. Jenny P. Glusker of The Institute for Cancer Research for her aid in constructing Fig. 3.

REFERENCES

1. Nishihara, H. and Dekker, E. E. (1972) *J. Biol. Chem.*, 247, 5079-5087.
2. Rosso, R. G. and Adams, E. (1967) *J. Biol. Chem.*, 242, 5524-5534.
3. Kobes, R. D. and Dekker, E. E. (1969) *J. Biol. Chem.*, 244, 1919-1925.
4. Meloche, H. P. and Mehler, L. (1973) *J. Biol. Chem.*, 248, 6333-6338.
5. Meloche, H. P. (1970) *Anal. Biochem.*, 38, 389-392.
6. Ingram, J. M. and Wood, W. A. (1965) *J. Biol. Chem.*, 240, 4146-4151.

² The si face of the aldehyde carbon of glyoxylate is that described by the anti-clockwise orientation of ligands substituted by carbonyl oxygen, carboxyl group and hydrogen.