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TRITIUM EXCHANGE REACTIONS CATALYZED BY 2-OXO-4-HYDROXYGLUTARATE ALDOLASE FROM *ESCHERICHIA COLI* K-12

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Summary

Tritiated water and tritiated substrates have been used to study exchange reactions catalyzed by *Escherichia coli* 2-oxo-4-hydroxyglutarate aldolase (4-hydroxy-2-oxoglutarate glyoxylate-lyase, EC 4.1.3.16, 2-oxo-4-hydroxyglutarate ⇌ pyruvate + glyoxylate). With pyruvate, the enzyme catalyzes a rapid first-order exchange of all three methyl hydrogens in the absence of added acceptor aldehyde (i.e. glyoxylate). This reaction is not rate limiting for aldol condensation or cleavage; quite different pH-activity profiles for the exchange reaction versus aldol cleavage and also comparative effects that pH changes have on K_m and V values for the two processes favor this conclusion. The exchange reaction with 2-oxobutyrate, a substrate analog, is stereoselective; one methylene hydrogen is removed at a 6-fold faster rate than the other but eventually both are exchanged. No tritium exchange occurs with glyoxylate.

Introduction

The aldol-type reaction is an important method for lengthening or shortening carbon chains of molecules. In addition to catalyzing aldol condensation and cleavage reactions, most aldolases also catalyze exchange reactions involving the proton or protons on the carbon atom adjacent to the carbonyl group of a sub-

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Abbreviation: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

strate. The stereospecific exchange of one proton of dihydroxyacetone phosphate, as catalyzed by fructose-1,6-bisphosphate aldolase [1—6], was the first such reaction studied. Exchange reactions have also been demonstrated with 2-oxo-3-deoxy-6-phosphogluconate aldolase [7—9], 2-deoxy-D-ribose-5-phosphate aldolase [10], and a pyruvate-aspartate semialdehyde condensing enzyme [11]. Acetoacetate decarboxylase, which functions via a mechanism analogous to Schiff base forming aldolases, catalyzes proton exchange between acetone and solvent [12]. Transaldolase catalyzes the exchange of one proton of dihydroxyacetone only as a step in a concerted process (i.e. of Schiff base formation and aldol cleavage, or of condensation and Schiff base hydrolysis) [13,14]. Isocitrate lyase, malate synthase, and citrate synthase, which catalyze reactions similar to aldolases, all require addition of the second substrate for catalysis of tritium exchange [15—19]. Such enzyme-catalyzed exchange reactions are normally stereospecific for one proton unless several protons happen to be equivalent. 2-Oxo-3-deoxy-6-phosphogluconate aldolase catalyzes the exchange of all three protons of pyruvate but only one of 2-oxobutyrate [7—9]. Likewise, 2-deoxy-D-ribose-5-phosphate aldolase catalyzes the exchange of the three methyl protons of acetaldehyde, but only one equivalent of tritium is incorporated into propionaldehyde [10].

The terminal step in the mammalian catabolism of L-hydroxyproline, whereby the carbon chain is degraded to smaller fragments, is catalyzed by 2-oxo-4-hydroxyglutarate aldolase (4-hydroxy-2-oxoglutarate glyoxylate-lyase, EC 4.1.3.16, 2-oxo-4-hydroxyglutarate $\rightleftharpoons$ pyruvate + glyoxylate) [20]. The protein which catalyzes this reaction has been obtained in homogeneous form from extracts of *Escherichia coli* K-12 [21,22]. Although it functions via a typical Schiff base mechanism, *E. coli* 2-oxo-4-hydroxyglutarate aldolase has been found to have many atypical properties when compared with other aldolases [21]. With the goal of studying the mechanism of the reaction catalyzed, we report here the results of experiments on hydrogen/tritium exchange reactions catalyzed by this enzyme.

Materials and Methods

Materials. 2-Oxo-4-hydroxyglutaric acid (DL- and L-) was synthesized by non-enzymic transamination of either erythro-4-hydroxy-DL-glutamic acid or *threo*-4-hydroxy-L-glutamic acid, respectively [20]. The racemic mixture of erythro-4-hydroxyglutamic acid was synthesized as described before [23] whereas the *threo*-L-isomer was purchased (as L-*allo*-γ-hydroxyglutamic acid) from Calbiochem (La Jolla, CA, U.S.A.). Sodium glyoxylate, sodium pyruvate, and sodium 2-oxobutyrate were products of Sigma (St. Louis, MO, U.S.A.). 2,4-Dinitrophenylhydrazine was obtained from Matheson, Coleman and Bell (Norwood, OH, U.S.A.), *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes) from Calbiochem (La Jolla, CA, U.S.A.), Triton X-100 from Research Products International (Elk Grove Village, IL, U.S.A.), and suspensions of crystalline catalase and rabbit muscle lactate dehydrogenase from Sigma (St. Louis, MO, U.S.A.).

Tritiated water (5 Ci/mmol) was purchased from Amersham Corp. (Arlington Heights, IL, U.S.A.). The primary and secondary scintillators used in scintilla-

tor solutions I and II were products of either Packard Instrument Co. (Downers Grove, IL, U.S.A.) or New England Nuclear (Boston, MA, U.S.A.); a tritiated water standard ($2.59 \cdot 10^6$ dpm/g) was from Packard Instrument Co. (Downers Grove, IL, U.S.A.). Sodium [3-$^3H_3$]pyruvate was generously provided by Dr. H.P. Meloche (Papanicolaou Cancer Research Institute, Miami, FL, U.S.A.); this product was established as being 97—99% pure by paper chromatographic and high voltage paper electrophoretic methods. Crystalline 2,4-dinitrophenylhydrazone derivatives were synthesized by adding a solution of the appropriate carbonyl compound to 0.1% 2,4-dinitrophenylhydrazine in 2 N HCl and recrystallizing the product from ethanol/water mixtures.

2-Oxo-4-hydroxyglutarate aldolase was purified from extracts of *E. coli* cells as described by Dekker et al. [22]. By this procedure, the enzyme is purified over 2000-fold and migrates as a single protein band in polyacrylamide gel electrophoresis. The specific activity of preparations used was approximately 50 μmol glyoxylate formed/20 min per mg protein at 37°C under standard assay conditions [24].

Methods. Two methods were used for estimating tritium exchange activity Exchange assay I involved incubating the aldolase with substrate in buffered solution containing ^3H_2O; incubation volume = 0.5 ml. A solution (10 ml) of 0.1% 2,4-dinitrophenylhydrazine in 2 N HCl was then added. The acidic dinitrophenylhydrazone derivatives were extracted, separated by paper chromatography, eluted, and quantitated spectrophotometrically [25]. The following extinction coefficients, obtained by preparing solutions of the respective crystalline 2,4-dinitrophenylhydrazone derivatives, were used: $2.14 \cdot 10^4$ $M^{-1} \cdot cm^{-1}$ (367 nm) for the pyruvate derivative; $2.09 \cdot 10^4$ $M^{-1} \cdot cm^{-1}$ (385 nm) for the derivative of 2-oxobutyrate; $2.18 \cdot 10^4$ $M^{-1} \cdot cm^{-1}$ (367 nm) for the glyoxylate derivative. Levels of radioactivity in both the original incubation mixture and the eluted dinitrophenylhydrazone derivatives were determined by liquid scintillation counting in either scintillator solution I (seven parts of toluene containing 0.4% 2,5-diphenyloxazole and 0.1% 1,4-bis[2-(4-methyl-5-phenyloxazolyl]benzene with three parts of absolute ethanol) or solution II (seven parts of toluene containing 0.58% 2,5-diphenyloxazole and 0.01% *p*-bis-(*o*-methylstyryl)benzene with six parts of Triton X-100). Quench corrections were estimated with internal standards or from an experimentally determined quench curve. Radioactive measurements were made with a Packard Model 3320 scintillation spectrometer.

Exchange assay II measured the formation of ^3H_2O from [3-$^3H_3$]pyruvate; the method is essentially that which Rose and Rieder used [5]. Before use, solutions of tritiated pyruvate were quantitated with lactate dehydrogenase. Aliquots (50 μl) of incubation mixtures containing tritiated pyruvate, buffer, and 2-oxo-4-hydroxyglutarate aldolase were removed with a Hamilton syringe at timed intervals, applied to individual columns (0.5 × 4.0 cm) of Dowex-1 (Cl^-) ion-exchange resin, and the columns then washed with two 1 ml volumes of water. Samples of the effluent fluid were used for radioactivity measurements in scintillator solution II.

The aldol cleavage activity of 2-oxo-4-hydroxyglutarate aldolase was followed by colorimetric determination of glyoxylate [23]. Two methods were used to assay the aldolase-catalyzed condensation of pyruvate with glyoxylate.

Condensation assay I is the same as exchange assay I except that ^3H_2O was omitted and the 2,4-dinitrophenylhydrazone of 2-oxo-4-hydroxyglutarate was quantitated using an extinction coefficient of $1.97 \cdot 10^4\ M^{-1} \cdot cm^{-1}$ (380 nm). Condensation assay II measured the malate formed after peroxide-catalyzed decarboxylation of 2-oxo-4-hydroxyglutarate. Buffered incubation mixtures (0.5 ml) contained aldolase, pyruvate, plus glyoxylate. The reaction was stopped and 2-oxo-4-hydroxyglutarate (as well as substrates) decarboxylated by adding 0.2 ml of 0.75 M acetate buffer (pH 5.0) containing 3% H_2O_2. Although virtually quantitative decarboxylation occurs in less than 1 min, the mixtures were incubated at 37° C for 7 min to insure complete reaction. The pH of the mixture was then adjusted to 8.5 with NaOH solution and 1 µl of a catalase suspension (160 units) subsequently added. After this mixture had been incubated for 5 min at 37° C, an aliquot was removed for the determination of L-malate by method I of Lowry and Passonneau [26]. Corrections were not necessary for the small amount of D-malate formed from D-2-oxo-4-hydroxyglutarate [21].

The concentrations of proteins in solution were determined by the method of Lowry et al. [27] with crystalline bovine serum albumin as a standard.

Results

When 0.02 of unit 2-oxo-4-hydroxyglutarate aldolase from *E. coli* is incubated at 37° C with 0.8 mM sodium [3-$^3H_3$]pyruvate in a solution containing 50 mM sodium phosphate + 5 mM sodium pyrophosphate buffer (pH 6.7), 5 mM glutathione, and 5 mM sodium pyruvate (final vol. = 0.2 ml), an exchange reaction with formation of ^3H_2O is readily observed. This exchange takes place in the absence of glyoxylate. The reaction is linear with time (0—10 min) and with enzyme concentration (0—0.3 unit); no reaction occurs in appropriate controls. With sodium [3-$^3H_3$]pyruvate as substrate, a limit of three µatoms of tritium are released as ^3H_2O when the reaction is allowed to go to completion. The reaction flows first-order kinetics.

The possibility of an unusual tritium exchange occurring with glyoxylate was examined; this was done since glyoxylate forms an 'abortive' Schiff base intermediate with apparently the same active-site lysyl residue of the enzyme that interacts with pyruvate [21]. Under conditions where exchange with pyruvate is essentially complete after 10 min, no exchange occurs with glyoxylate even after 12 h.

The rate of [3-$^3H_3$]pyruvate detritiation is faster than the rate of pyruvate condensation with glyoxylate (Fig. 1). Although the experimental conditions cannot be identical for these two reactions, the highest condensation rates ever achieved are not more than double that shown.

The rates of tritium exchange and aldolytic cleavage of 2-oxo-4-hydroxyglutarate were compared as a function of pH (Fig. 2). Only slight variations are seen in the rate of exchange over the pH range tested. The rate of aldolytic cleavage, however, steadily rises over the same range; this aldolase has previously been found to have a pH optimum of 8.6 for cleavage of DL-2-oxo-4-hydroxyglutarate in Tris or glycylglycine buffer [21]. At all pH values, the process of tritium exchange is faster than the aldol cleavage reaction.

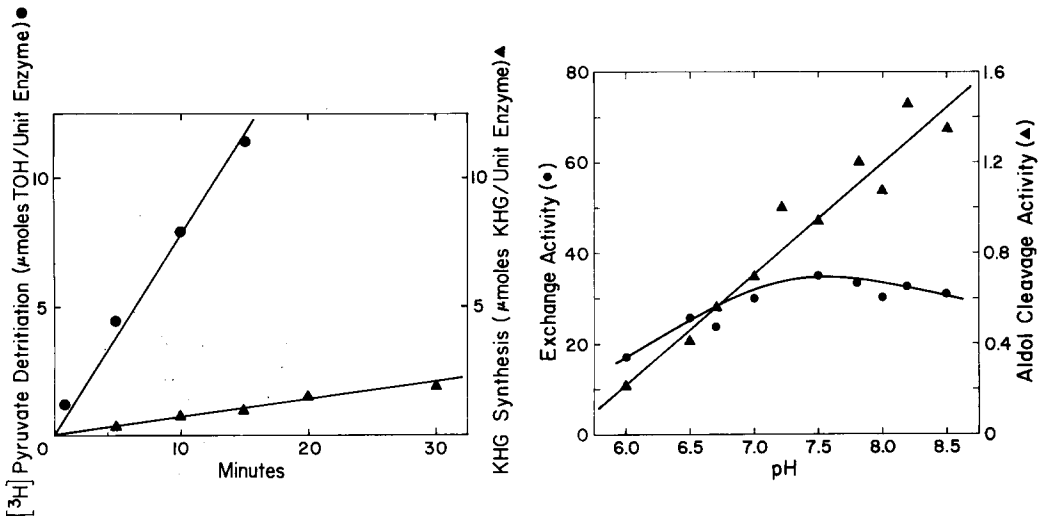

Fig. 1. Rates of aldol condensation and tritium exchange catalyzed by *E. coli* 2-oxo-4-hydroxyglutarate aldolase. The measure tritium exchange, the reaction mixture (0.3 ml) contained 50 mM Hepes buffer (pH 8.2), 5 mM glutathione, 3.3 mM sodium pyruvate, 0.5 mM sodium [3-$^3H_3$]pyruvate, and 0.02 unit of enzyme. After the mixtures were subjected to a prior incubation for 3 min at 37° C, the reaction was started by adding [3-$^3H_3$]pyruvate. Aliquots (50 μl) were removed at the times indicated and assayed for ^3H_2O by exchange assay II. To measure 2-oxo-4-hydroxyglutarate synthesis, the mixture (6.0 ml) contained 50 mM Hepes buffer (pH 8.2), 5 mM glutathione, 5 mM sodium pyruvate, 2.5 mM sodium glyoxylate, and 1.7 units of aldolase. After the mixture was subjected to a prior incubation for 5 min at 37° C, the reaction was started by adding glyoxylate. Aliquots (1.0 ml) were removed at the times indicated, added to 10 ml of a 0.1% solution of 2,4-dinitrophenylhydrazine in 2 N HCl, and product measured by condensation assay I. The data were corrected for any observed non-enzymic reaction. KHG, 2-oxo-4-hydroxyglutarate.

Fig. 2. Effect of pH on tritium exchange and on cleavage of 2-oxo-4-hydroxyglutarate as catalyzed by *E. coli* aldolase. The reaction mixtures (1.0 ml) for measuring substrate cleavage contained 50 mM Hepes buffer, 5.0 mM glutathione, 5 mM DL-2-oxo-4-hydroxyglutarate, and 0.1 unit of enzyme. The mixtures were incubated at 37° C for 20 min; glyoxylate was determined colorimetrically [23]. Aldol cleavage activity is expressed as μmol of glyoxylate formed/min per mg protein (▲). To measure tritium exchange, the reaction mixtures (0.2 ml) contained 50 mM Hepes buffer, 5 mM glutathione, 5.0 mM sodium pyruvate, 0.8 mM sodium [3-$^3H_3$]pyruvate, and 0.02 unit of aldolase. Incubations and assays were performed as indicated in the legend to Fig. 1. Exchange activity is expressed as μmol of ^3H_2O formed/min per mg protein (●).

Tritium exchange with 2-oxobutyrate was also examined since this compound is a close structural analog of pyruvate, and hydrogen atoms in the molecule (if exchangeable) are non-equivalent. Furthermore, reduction with $NaBH_4$ in the presence of 2-oxobutyrate causes some inactivation of *E. coli* 2-oxo-4-hydroxyglutarate aldolase [21], indicating that this compound binds as a Schiff base to the active-site lysyl residue, and yet it is not a substrate for aldol cleavage. Incubation of the aldolase (at 37° C in 0.5 ml containing 50 mM HEPES buffer (pH 7.0), 10 mM sodium 2-oxobutyrate, and 1 mCi ^3H_2O) showed that tritium incorporation into 2-oxobutyrate is linear with enzyme concentration (0—2 units), a good indication that the process is enzyme catalyzed. The rate of this reaction, however, is considerably slower than with pyruvate as substrate; much higher levels of enzyme and longer periods of reaction (30 min) are required. When the reaction is allowed to approach comple-

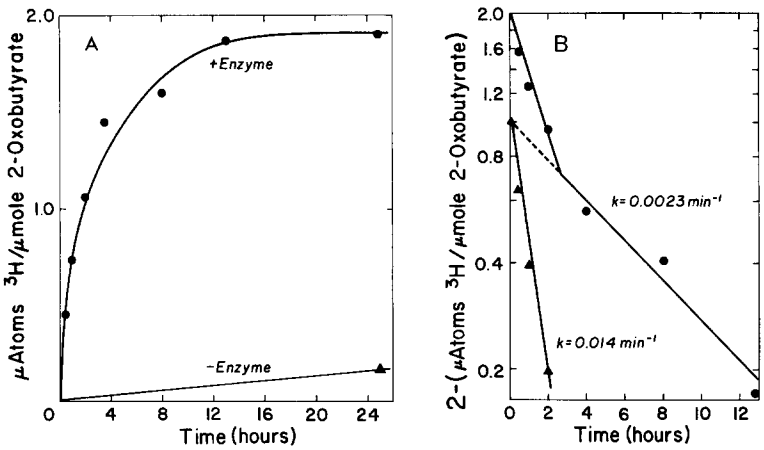

Fig. 3. Stereoselectivity of tritium exchange with 2-oxobutyrate. The reaction mixtures (0.5 ml) contained 50 mM Hepes buffer (pH 7.0), 10 mM sodium 2-oxobutyrate, 0.02% sodium azide, 1 mCi ^3H_2O, and 3.6 units of *E. coli* aldolase. After the mixtures were incubated at 37°C for the times shown, 5 μl were removed to determine the specific activity of the ^3H_2O and the balance of the solution used to measure radioactive 2-oxobutyrate by exchange assay I.

tion (Fig. 3A), 1.9 μatoms of tritium are incorporated/μmol 2-oxobutyrate. Under the same experimental conditions, but in the absence of 2-oxo-4-hydroxyglutarate aldolase, less than 0.2 μatom tritium is exchanged. As was found earlier for the methyl hydrogen atoms of pyruvate, therefore, proton exchange in this instance also is not stereospecific. When, however, the data are examine as a first-order plot of 2 minus the μatoms ^3H incorporated/μmol 2-oxobutyrate versus time, a biphasic curve is obtained (Fig. 3B). Since the slower rate extrapolates to a value of approximately one, exchange with the two methylene hydrogens of 2-oxobutyrate is stereoselective with one hydrogen exchanging considerably faster than the other. The first-order rate constants listed show that, under the experimental conditions used, the one rate exceeds the other by a factor of 6.

K_m and V values for the reactions considered in this paper are listed in Table I. All values were determined by double-reciprocal plots [28]. The K_m value for pyruvate detritiation varies only slightly and in an irregular manner over a pH range of 6.5—8.2; only slight changes in V are also seen. Pyruvate tritiation has the same K_m but a higher V; isotope effects may possibly cause this apparent difference. Tritiation of 2-oxobutyrate contrasts in having almost a 10-fold higher K_m value and a 70-fold lower V.

The kinetic constants for cleavage of L-2-oxo-4-hydroxyglutarate vary more extensively with changes in pH. The K_m for L-2-oxo-4-hydroxyglutarate and V for aldol cleavage both increase with increasing pH; these values, however, are never greater than those for pyruvate-exchange reactions. K_m values for the synthesis of 2-oxo-4-hydroxyglutarate are more difficult to compare since each value depends upon the concentration of the other substrate. A direct correlation between these two parameters was found (i.e. lower and higher K_m values at low and high substrate concentrations, respectively). A V value of 1.0,

TABLE I

COMPARATIVE K_m AND V VALUES FOR TRITIUM EXHANGE AND ALDOL CLEAVAGE-CONDENSATION REACTIONS

Reaction	pH	K_m (mM)	V
Pyruvate tritiation	7.0	2.7	7.1 *
Pyruvate detritiation	6.5	1.1	3.0 *
	6.7	3.8	3.5
	8.2	2.3	2.6
2-Oxobutyrate tritiation	7.0	19.2	0.1 *
Cleavage of	6.5	0.1	0.05 **
L-2-oxo-4-hydroxyglutarate	7.0	0.3	0.1
	8.2	1.3	0.2
Synthesis of		for pyruvate	
L-2-oxo-4-hydroxyglutarate	8.2	1.8 (at 0.5 mM glyoxylate)	
(condensation assay II)		27.0 (at 10.0 mM glyoxylate)	1.0 ***
		for glyoxylate	
		0.2 (at 0.5 mM pyruvate)	
		2.7 (at 10.0 mM pyruvate)	

* μatoms of tritium exchanged/min per unit of enzyme.
** μmol of substrate cleaved/min per unit of enzyme.
*** μmol of product formed/min per unit of enzyme. A theoretical value; see text.

representing the theoretical value at infinite pyruvate and glyoxylate concentrations, was determined by secondary plots. In practice, however, substrate inhibition occurs and the theoretical value is never approached; the highest experimental values ever obtained approximated 0.14 μmol of 2-oxo-4-hydroxyglutarate formed/min per unit of enzyme.

Discussion

The data show that *E. coli* 2-oxo-4-hydroxyglutarate aldolase catalyzes an exchange reaction with the methyl hydrogens of pyruvate in the absence of glyoxylate, the acceptor aldehyde; exchange also occurs with the next higher homolog of pyruvate, namely 2-oxobutyrate. This process of exchange is not rate limiting in the overall cleavage reaction, a conclusion substantiated by comparative maximum velocity data for pyruvate hydrogen exchange versus 2-oxo-4-hydroxyglutarate cleavage. The pH-activity profiles have also been found to be quite different for the two processes (exchange vs. aldol cleavage). Furthermore, the K_m and V values for tritium exchange with pyruvate vary only slightly over the pH range of 6.5—8.2 whereas over the same range of pH aldol cleavage of 2-oxo-4-hydroxyglutarate shows a 10-fold increase in K_m and about a 4-fold increase in V. Such results provide additional evidence that the two reactions (tritium exchange vs. aldol cleavage) have different rate-limiting parameters. Comparative V data suggest that proton exchange is not rate limiting for the condensation reaction either.

As expected, the exchange reaction catalyzed by 2-oxo-4-hydroxyglutarate aldolase is non-stereospecific with pyruvate since the three exchangeable methyl hydrogens are stereochemically equivalent. With 2-oxobutyrate, both methylene hydrogen atoms are eventually exchanged but some stereoselectivity

is shown since exchange of one hydrogen proceeds at a 6-fold faster rate than the other. Such results may not be too surprising since previous experiments showed that this aldolase is not totally stereospecific in catalyzing the aldol cleavage or formation of 2-oxo-4-hydroxyglutarate, i.e. the apparent K_m values for aldol cleavage of the L- and D-isomers of 2-oxo-4-hydroxyglutarate are 2.3 mM and 25.0 mM, respectively, and the V with the former isomer is approximately 5-fold higher than with the latter [21].

Establishing that *E. coli* 2-oxo-4-hydroxyglutarate aldolase catalyzes a proton exchange with pyruvate further supports the conclusion that this enzyme functions via a Schiff base mechanism [21]. Where, however, class I (Schiff base mechanism) fructose-1,6-bisphosphate aldolases show congruent pH optimum curves for proton exchange and aldol cleavage [29], the pH optima for these two processes differ considerably with 2-oxo-4-hydroxyglutarate aldolase from *E. coli* (Fig. 2). In this respect, 2-oxo-4-hydroxyglutarate aldolase is similar to class II fructose-1,6-bisphosphate aldolases; several bacterial aldolases of this class have pH optima of about 7.5 and 6.1 for cleavage of fructose-1,6-bisphosphate and proton exchange activity, respectively [29]. Different pH optima for these two processes are also seen with a pyruvate-aspartic semialdehyde condensing enzyme that functions via a Schiff base mechanism [11]. It is becoming increasingly evident, therefore, that the distinction between class I and class II fructose-1,6-bisphosphate aldolases on the basis of pH optima is not valid for aldolase-type enzymes in general.

The following differences and similarities have now been established for various aldolases regarding their ability to catalyze proton exchange with substrates that form enzyme-bound enolates. Fructose-1,6-bisphosphate aldolase stereospecifically removes one hydrogen atom in the formation of the dihydroxyacetone phosphate enolate anion [1,3,5]. 2-Deoxyribose-5-phosphate aldolase and 2-oxo-3-deoxy-6-phosphogluconate aldolase are similar in that both exchange any of the three methyl hydrogen atoms of acetaldehyde [10] or pyruvate [7], respectively, but stereospecifically exchange a single methylene hydrogen atom of propionaldehyde in the former case [10] and of 2-oxobutyrate in the latter [8]. *E. coli* 2-oxo-4-hydroxyglutarate aldolase, in comparison, non-stereospecifically catalyzes the exchange of all three methyl hydrogen atoms of pyruvate in forming the pyruvyl anion but will also exchange both methylene hydrogen atoms of 2-oxobutyrate (although at quite different rates).

2-Oxo-4-hydroxyglutarate aldolase of *E. coli* is similar to muscle fructose-1,6-bisphosphate, 2-oxo-3-deoxy-6-phosphogluconate, and bovine liver 2-oxo-4-hydroxyglutarate aldolases in that all function via Schiff base mechanisms and all catalyze an exchange reaction in the absence of the acceptor aldehyde. However, *E. coli* 2-oxo-4-hydroxyglutarate aldolase differs from these other enzymes since it is neither absolutely stereospecific nor totally non-stereospecific in catalyzing exchange and aldol-type reactions; rather it is stereoselective. In experiments designed to separate kinetic from thermodynamic control of the reaction catalyzed by *E. coli* 2-oxo-4-hydroxyglutarate aldolase, it has recently been shown that although kinetic restrictions demand that initially the L-isomer of 2-oxo-4-hydroxyglutarate be turned-over with greater facility than its enantiomer, the rate ratio of isomer turnover approaches unity

as thermodynamic equilibrium is attained [30]. 3-Oxo-4-hydroxyglutarate aldolase of *E. coli*, therefore, handles the carbonyl carbon of glyoxylate oriented for si face attack on the enzyme-bound enolpyruvate with much greater facility than when oriented for re face attack. It would not appear possible to conduct experiments analogous to those of Meloche and Mehler [31] to further probe the stereochemical constraints that are involved in the mechanism of the reaction catalyzed by *E. coli* 2-oxo-4-hydroxyglutarate aldolase since hydrogen exchange with pyruvate as catalyzed by this enzyme is so much more rapid than C-C synthesis. Also, so far we have not been able to detect a condensation of 2-oxobutyrate (rather than pyruvate) with glyoxylate.

Acknowledgements

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