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COMPARISON OF THE MECHANISMS OF TWO DISTINCT ALDOLASES FROM *ESCHERICHIA COLI* GROWN ON GLUCONEOGENIC SUBSTRATES *

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Summary

Escherichia coli grown on gluconeogenic compounds as carbon sources produced two chemically and physically distinct types of fructose-1,6-biphosphate aldolases (D-fructose-1,6-bisphosphate D-glyceraldehyde-3-phosphate-lyase, EC 4.1.2.13), while these bacteria produced only a single enzyme when grown on glucose or fructose. We have investigated this enzyme in several strains of *Escherichia coli* (Crookes, K-12, and B) grown on glucose, fructose lactate, pyruvate, alanine and glycerol by comparing chemical properties and mechanisms of action. Comparison of these mechanisms was accomplished by following the fate of ^{18}O in the keto position of fructose 1,6-bisphosphate during the aldolase catalyzed cleavage reaction. The results show that the two enzymes have different mechanisms of action and are consistent with a Schiff-base mechanism for the one which was induced by gluconeogenic substrates and metal-chelate mechanism for the constitutive enzyme.

Fructose-1,6-bisphosphate aldolase (D-fructose-1,6-bisphosphate D-glyceraldehyde-3-phosphate-lyase, EC 4.1.2.13) catalyzes the reversible cleavage of fructose 1,6-bisphosphate to dihydroxyacetone phosphate and glyceraldehyde-3-*P*. From experiments demonstrating the strict requirements of a divalent metal cation for activity for the yeast enzyme [1], EPR measurements [2] and

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Abbreviation: BDMS, *t*-butyl dimethylsilyl.

^{18}O tracer studies [3], it has been concluded that Class II enzymes operate through a metal-chelate intermediate. In this latter work, [2-^{18}O]fructose-1,6-P_2 was cleaved enzymically and it was found that nearly all of the ^{18}O was retained in the product. For rabbit muscle aldolase, no ^{18}O was found in the dihydroxyacetone phosphate after the cleavage reaction. A Schiff-base mechanism necessitates an obligatory exchange of the keto oxygen of fructose-1,6-P_2 with that of the water medium, whilst a metal-chelate mechanism does not require the loss of the keto oxygen. Experiments utilizing ^{18}O as a probe of the mechanism of action of aldolase isolated from other sources showed that Class I and Class II fructose-1,6-P_2 aldolases may be distinguished by measuring the retention or loss of the keto oxygen of dihydroxyacetone phosphate derived from [2-^{18}O]fructose-1,6-P_2 [4].

In 1973, Stribling and Perham [5] reported the presence of both types of aldolases in *E. coli* when these organism were grown on lactate or pyruvate. In later work, Baldwin et al. [6] purified and characterized the class II enzyme and found that it had a molecular weight of approx. 80 000 and resembled the aldolase derived from yeast in its amino acid composition. However, its kinetic behavior as well as some chemical properties was not typical of Class II aldolases. Characterization of the purified Class I enzyme by Baldwin and Perham [7] showed it to have a molecular weight of approx. 340 000 and in comparison with physical and chemical properties of other Class I enzymes, these authors noted that it had unique kinetic properties, unlike other enzymes of this type.

In the work reported in this paper, [2-^{18}O]fructose-1,6-P_2 was used to probe the mechanism of action of fructose-1,6-P_2 aldolases isolated from *E. coli* grown on gluconeogenic substrates. We have studied the enzymes from *E. coli* strains K-12 and B as well as those from Crookes strain as reported by Perham and his associates. In addition, some physical and chemical properties of the aldolases of the several strains were compared with each other as well as with the properties of yeast and rabbit muscle.

Materials and Methods

Preparation of diazomethane. Diazomethane was prepared by utilizing the precursor *N*-methyl-*N*-nitroso-*p*-toluenesulfonamide (Aldrich Chemical Co.) according to instructions accompanying the reagent.

Enzyme preparation. Glycerol-1-phosphate dehydrogenase and triosephosphate isomerase from rabbit muscle were obtained commercially (Sigma Chemical Co.) and were used directly as their suspensions in ammonium sulfate.

Rabbit muscle aldolase (Sigma Chemical Co.) was dialyzed against 0.05 M glycylglycine containing 0.1 M potassium acetate (pH 7.0) prior to use. *Saccharomyces cerevisiae* aldolase was isolated from dried active baker's yeast (Nutritional Biochemical Co.) by the method of Mildvan et al. [2].

E. coli was grown and harvested according to the procedures of Roberts et al. [8]. The media consisted of a minimal salts solution plus a carbon source, either glucose, fructose, pyruvate, lactate or glycerol. The cells were broken using a French Pressure Press and the aldolase was precipitated at 0.4—0.8%

saturation $(NH_4)_2SO_4$ and was resuspended in a minimal volume of cold 0.5 M Tris-HCl, pH 7.0, and dialyzed against this same buffer prior to gel filtration.

Gel filtration. The enzyme preparation was concentrated to 10 ml via ultrafiltration and then dialyzed against 50 mM Tris-HCl (pH 7.0) for 6 h prior to application to a G-150 Sephadex column (2.6 × 180 cm). The column was eluted with the same buffer, and 4-ml fractions were collected. The fractions showing aldolase activity were pooled and concentrated by ultrafiltration. In the case where two peaks showing aldolase activity emerged from the column, the fractions were pooled separately.

X-Ray fluorescence. Aldolases from *E. coli* (strains B, Crookes, and K-12) grown on glucose were prepared for X-ray fluorescence analysis to determine the divalent metal associated with this Class II enzyme. The concentrated enzyme solution obtained after gel filtration was dialyzed against 0.05 M glycylglycine/0.1 M potassium acetate (pH 7.0). A protein determination (using the method of Lowry) was performed, and a volume of solution representing 5 mg of protein was twice dialyzed against 200 ml of glass-distilled water for 2 h. The enzymes retained about 80% of their fructose-1,6-P_2 cleavage activity. An acetone precipitate was prepared and carefully dried for each enzyme sample. The last 200 ml of glass-distilled water used in the dialysis was concentrated and used as a blank. Analyses were performed using a Kevex Model 600 Ultra Trace X-ray Fluorescence Spectrometer with a silver X-ray source in the direct target mode. Approx. 0.5 mg of each sample was placed on a polypropylene film for analysis.

Effect of divalent metal cations. Aldolase solutions were dialyzed against 0.05 M glycylglycine/0.1 M potassium acetate/0.1 M EDTA (pH 7.0) for 30 min to remove metal ions. The enzymes were then dialyzed against the same buffer without EDTA for 12 h. Assays for aldolase activity showed minimal or no activity. The enzyme (2.5 units) was then placed in each of several buffer solutions containing divalent metal ions at concentrations of 1, 10, and 100 mM and allowed to equilibrate for 5 min at room temperature prior to assay.

Preparation of [2-^{18}O]fructose-1,6-P_2. [2-^{18}O]Fructose-1,6-P_2 was prepared by dissolving 3 mg of the tetrasodium salt in 10 μl of $H_2{}^{18}O$ (97 atom%) in a 3 ml conical reaction vessel, and the exchange reaction was allowed to proceed overnight at room temperature.

Enzymic cleavage of [2-^{18}O]fructose-1,6-P_2. The cleavage of [2-^{18}O]fructose-1,6-P_2 by aldolase was carried out by combining separately 25, 65, 125 and 200 units of glycerol-1-phosphate dehydrogenase, and 1 μl of triosephosphate isomerase, 10 μmol of NADH, and 2.5 units of the aldolase being studied in sufficient buffer (0.05 M glycylglycine/0.1 M potassium acetate, pH 7.0) to make a total volume of 2.5 ml. (The varying amounts of dehydrogenase were added to insure that maximum retention of ^{18}O occurred as a result of rapid conversion of dihydroxyacetone phosphate to glycerol-1-phosphate. The excess triosephosphate isomerase was added to insure complete conversion of unlabeled glyceraldehyde-3-*P* first to dihydroxyacetone phosphate and then to glycerol 1-phosphate. There was thus no need to estimate the contribution of residual triosephosphate isomerase in the aldolase preparation or add inhibitors; however, an isotope dilution factor of 2 was introduced.) The solution was allowed to equilibrate at 37°C together with the reaction vessel con-

taining 3 mg of [2-^{18}O]fructose-1,6-P_2 in 10 μl of $H_2{}^{18}O$ for 30 min. The equilibrated solution was rapidly added to the reaction vessel containing the [2-^{18}O]fructose-1,6-P_2, permitted to react at 37° C for 90 s, and then these were frozen in a solid CO_2/ethanol bath. Upon thawing, the solution (still at 0° C) was passed through a 1.5 × 20 cm Dowex 50(H^+) column and eluted with water. The fractions containing glycerol 1-phosphate (fractions 2—4 of 4-ml fractions) were collected, pooled and taken to dryness on a rotary evaporator.

Stable isotope analysis of glycerol 1-phosphate. Glycerol 1-phosphate was converted to bis(BDMS) glycerol 1-phosphate dimethyl ester and was then analyzed by combined gas chromatography-mass spectrometry (GC-MS) for ^{18}O content as described previously [4]. Analyses were performed in a Finnigan 3200 GC/MS equipped with a Finnigan 6000 data system. The gas-liquid chromatograph column (0.2 mm × 2 m glass) was packed with 3% Dexsil 300 on 80/100 mesh Chromasorb Q, and helium was used as the carrier gas. The injector temperature was maintained at 150° C and the column oven was held isothermally for 2 min at 170° C followed by programming to 230° C at 6 K/min. The source and analyzer were maintained at 100° C and the separator at 275° C. An ionizing energy of 70 eV was used. The selected ion monitoring mode of the mass spectrometer was used to continuously monitor specific ions of the bis(BDMS) glycerol 1-phosphate dimethyl ester to determine the abundance of ^{18}O. Details of this technique have been published previously by Heron and Caprioli [10]. Each sample analysis represents the average of at least three isotope determinations.

Results

Gel filtration

The Class I and Class II aldolase prototypes, from rabbit muscle and yeast, respectively, were readily separable on G-150 Sephadex. Gel filtration of the aldolases from *E. coli* grown on glucose and fructose resulted in the elution of a single peak eluting in the region of yeast aldolase. However, aldolases from *E. coli* grown on lactate, pyruvate, alanine and glycerol resulted in the elution of two peaks possessing aldolase activity; one eluting in the region of rabbit muscle aldolase, the other in the region of the yeast enzyme. Generally, the first peak (proposed Class I) represented 5—10% of the total aldolase activity.

X-Ray fluorescence analysis

An analysis of a purified preparation of Class II aldolase from *E. coli* K-12 grown on glucose shows peaks representative of the Kα and Kβ lines of zinc, making up about 90% of the total metal present. Small amounts of iron, nickel, copper and manganese were also detected, although these were also detected in buffer blanks. The data for the aldolases from *E. coli* B and Crookes grown on glucose are similar, indicating zinc as the metal found in vivo in Class II *E. coli* fructose-1,6-P_2 aldolases.

Effect of divalent metal ions

After dialysis against EDTA, each aldolase solution containing 2.5 U was assayed for fructose-1,6-P_2 cleavage activity in the presence of various con-

TABLE I

EFFECT OF DIVALENT METAL IONS ON DIALYZED EDTA TREATED ALDOLASES

Metal ion concentration was 1 mM with 5 U of aldolase used in each assay. Activation is expressed relative to that of 1 mM $ZnCl_2$, taken as unity.

Source	Strain	Carbon source	Relative effect of activation			
			Co	Cu	Mn	Fe
E. coli	Crookes	Glucose	21	0.3	5	3
		Fructose	17	0.7	4	3
		Lactate	13	1	5	1
		Pyruvate	10	3.5	5	6
		Alanine	27	0.5	5	3
	B	Glucose	5	0.4	2	3
		Fructose	35	0.7	7	6
		Lactate	27	0.6	7	1
		Pyruvate	12	0.5	6	1
		Alanine	11	2	7	3
	K-12	Glucose	22	0.5	8	6
		Fructose	22	2	7	3
		Lactate	6	2	3	2
		Pyruvate	33	0.5	5	6
		Alanine	30	0.4	6	4
Yeast	*S. cerevisiae*	Nutrient broth	1	0	2	0.7

centrations of Co^{2+}, Zn^{2+}, Fe^{2+}, Cu^{2+} and Mn^{2+}. The results are shown in Table I. The reactivation of the enzyme by various metal ions is given relative to that obtained using 1 mM Zn^{2+}, the concentration of this ion giving maximum enzyme activity. The optimal concentration ranges for the various metal ions were generally 2—10 mM. Co^{2+} was the most efficient activator in all cases except yeast, where Mn^{2+} gave greatest activation, and rabbit muscle, where no activation was found. Cu^{2+}, on the other hand, partially inhibited the bacterial enzyme. The data confirm the importance of divalent metal ions with regard to bacterial enzyme activity, and with the exception of copper, all of the divalent metals tested by this in vitro assay appear to be better activators than zinc, the metal found in the native enzyme.

Enzymic cleavage of [2-^{18}O]fructose-1,6-P_2

The mechanism of action of various aldolases purified by gel filtration was investigated by measuring the ^{18}O content of the glycerol 1-phosphate resulting from the reduction of dihydroxyacetone phosphate produced by the cleavage of [2-^{18}O]fructose-1,6-P_2 by each aldolase. In this assay, the number of units of aldolase was constant at 2.5 while the number of units of glycerol-1-phosphate dehydrogenase was varied to achieve maximum ^{18}O retention in the final product.

Although a 10 : 1 ratio of dehydrogenase to aldolase generally gave this maximum, several other ratios were also used. Insufficient dehydrogenase would allow ^{18}O exchange from dihydroxyacetone phosphate prior to conversion to glycerol 1-phosphate. Excess dehydrogenase would lead to loss of ^{18}O due to back-exchange, i.e., conversion of [2-^{18}O]glycerol 1-phosphate to

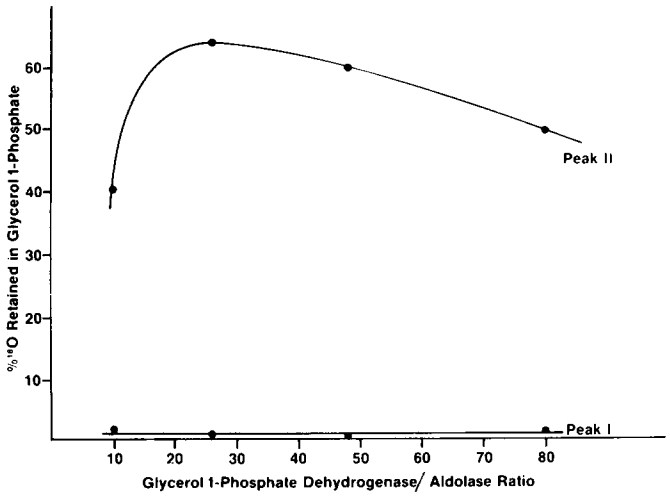

Fig. 1. Percent ^{18}O retained in α-phosphoglycerol isolated from reaction mixtures containing α-phosphoglycerol dehydrogenase, NADH, [2-^{18}O]fructose-1,6-P_2, and aldolases isolated from *E. coli* grown on lactate as the sole carbon source; Peak I is the enzyme proposed to be a Class I aldolase and Peak II, that for the Class II aldolase.

[2-^{18}O]dihydroxyacetone phosphate with subsequent exchange of the keto group. As an example, the data for aldolases isolated from *E. coli* (Crookes) grown on lactate is given in Fig. 1. Similar types of curves were generated for each type of aldolase studied. Table II shows the maximum percentage of ^{18}O retained by the fructose-1,6-P_2 aldolases from *E. coli*, yeast and rabbit muscle.

TABLE II

DETERMINATION OF ^{18}O IN PHOSPHOGLYCEROL DERIVED FROM ALDOLASE CLEAVAGE OF [2-^{18}O]FRUCTOSE-1,6-P_2

Strains of *E. coli* from which aldolase was isolated were B, Crookes and K-12.

Organism	Carbon source	% ^{18}O in phosphoglycerol		
		B	Crookes	K-12
E. coli	Glucose	64.4	71.1	60.3
	Fructose	67.3	57.9	60.2
	Lactate			
	Peak 1	4.9	0.1	1.1
	Peak 2	68.7	69.4	84.0
	Pyruvate			
	Peak 1	0.9	1.9	0.1
	Peak 2	75.2	73.9	54.7
	Alanine			
	Peak 1	3.1	2.7	2.3
	Peak 2	57.3	68.6	77.1
	Glycerol			
	Peak 1	0.1		
	Peak 2	67.9		
Rabbit muscle		2.2		
Yeast	Nutrient broth	75.9		

The data for the various *E. coli* strains grown on glucose show that these aldolases proceed with retention of ^{18}O, and are mechanistically comparable to the yeast enzyme. The aldolases obtained from *E. coli* grown on fructose give similar results. The data obtained when the various strains of *E. coli* were grown on C_3 carbon sources show no significant ^{18}O retention by any of the enzymes which eluted from the Sephadex column in a volume similar to that of rabbit muscle aldolase (designated peak 1 in Table II) and compare quite closely with the ^{18}O results from rabbit muscle aldolase. The aldolase designated peak 2 (in Table II) proceeds with retention of ^{18}O of the same general magnitude as that of the yeast enzyme.

Discussion

Fructose-1,6-P_2 aldolases from *E. coli* grown on fructose and glucose cleaved [2-^{18}O]fructose-1,6-P_2 with a major portion of the label appearing in the glycerol 1-phosphate. The amounts of ^{18}O retained were very similar to the amounts retained in the reactions when *S. cerevisiae* aldolase was used for cleavage , showing that there are mechanistic similarities between the fructose-1,6-P_2 aldolases of *S. cerevisiae* and *E. coli* grown on hexoses. This is consistent with the concept that these are Class II enzymes which operate mechanistically through a metal chelate intermediate. X-ray fluorescence analyses of the bacterial Class II enzymes show zinc to be the associated metal.

Aldolases from *E. coli* grown on C_3 carbon sources were found to be a mixture of both classes of enzymes and, as reported by Perham and his associates [5—7], could be separated by gel chromatography. One component resembled the rabbit muscle enzyme and cleaved [2-^{18}O]fructose-1,6-P_2 with no significant retention of label, consistent with an enzyme proceeding by a Schiff-base mechanism. The other component resembled the yeast aldolase in that it cleaved [2-^{18}O]fructose-1,6-P_2 with retention of label. The percentage retention of ^{18}O for the Class II enzymes is generally between 65—75%. The loss of approx. 25% of the isotope has been shown to occur through exchange of the intermediate [2-^{18}O]dihydroxyacetone phosphate prior to conversion to glycerol 1-phosphate. The half-life of exchange of [2-^{18}O]dihydroxyacetone phosphate is of the order of several seconds and these results are consistent with control experiments which show that, in the coupled aldolase-glycerol-1-phosphate dehydrogenase system used here, approx. 25% of the label will be lost as a result of non-aldolase induced exchange. Thus, Class II aldolases appear to give complete retention of the C-2 oxygen atom of fructose-1,6-P_2 in the cleavage reaction.

These data support the work of Perham and his associates in classifying the enzymes isolated from Crookes strain as Class I and II and demonstrate that despite the unique and unusual characteristics of the enzymes from *E. coli*, their mechanisms of action appear to be identical to those of other enzymes in their classes. The current work further shows that the production of two mechanistically distinct fructose-1,6-bisphosphate aldolases is not unique to Crookes strain, but can also demonstrated in strains B and K-12 when these were grown on lactate, pyruvate, alanine and glycerol. Since a Class I enzyme can be demonstrated only when C_3 carbon sources are used, it is probable that

this enzyme is involved in the process of gluconeogenesis, while the constitutive Class II aldolase is the glycolytic enzyme associated with the metabolism of hexoses.

Although it is generally assumed that bacteria produce a Class II aldolase, several exceptions have been reported. Lebherz and Rutter [11] have shown that *Micrococcus aerogenes* contains a Class I aldolase, although quite different in its physical properties to other Class I enzymes studied. Class I enzymes have also been reported in *Lactobacillus casei* [3,12,13] and *Mycobacterium smegmatis* [14]. Using *Chlamydomonas reinhardii* [15], *Chlamydomonas mundana* [16] and *Euglena gracilis* [17], it has been shown that either a Class I or a Class II aldolase can be made to predominate under certain growth conditions. When CO_2 was used as the carbon source, only a Class I aldolase could be demonstrated, but when acetate was used as the carbon source, a Class II enzyme was mainly produced. It was further suggested from these results that the Class I enzyme had a biosynthetic function and the Class II a glycolytic function.

The current work has demonstrated mechanistically distinct fructose-1,6-P_2 aldolases produced by *E. coli* when grown on C_3 carbon sources. It is not known if these two enzymes arose by an independent evolutionary mechanism, although other work showing quite different amino acid compositions and kinetic properties for these enzymes makes this possibility unlikely. Further insight into this aspect must await a detailed analysis of peptide sequences of the two enzymes.

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