SUBSTRATE SPECIFICITY OF L-RHAMNULOSE 1-PHOSPHATE ALDOLASE*

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Hexose diphosphate aldolase (HDP aldolase) catalyzes the reversible splitting of D-fructose 1,6-diphosphate into dihydroxyacetone phosphate (DHAP) and D-glyceraldehyde 3-phosphate. While the enzyme is absolutely specific for DHAP, a number of aldehydes can be substituted for D-glyceraldehyde 3-phosphate. Only one of the two possible trans isomers of the ketose product is formed (or split) in the reaction catalyzed by HDP aldolase; namely, the isomer with the configuration L at C-3 and D at C-4 in the Fischer projection formula (Rutter, 1961). Ghalambour and Heath (1962) have determined the specificity of L-fuculose 1-phosphate aldolase from Escherichia coli. This aldolase catalyzes the reversible splitting of L-fuculose 1-phosphate and of certain other cis ketose phosphates having the configuration D at C-3 and C-4. It has been shown by Takagi and Sawada (1964a, 1964b) and Sawada and Takagi (1964) that L-rhamnose is utilized by E. coli according to the following pathway: L-rhamnose ⟶ L-rhamnulose ⟶ L-rhamnulose 1-phosphate ⟶ DHAP + L-lactic aldehyde. These studies were carried out with partially-purified enzymes of low specific activity and enzyme specificity was not extensively investigated. Recently we described the purification of L-rhamnulokinase from extracts of E. coli as well as the preparation and characterization of L-rhamnulose 1-phosphate (Chiu and Feingold, 1964). We have now purified L-rhamnulose

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1-phosphate aldolase (RP aldolase) and investigated its specificity. These results are reported in this paper.

Materials and Methods: L-rhamnulose 1-phosphate was prepared as previously described (Chiu and Feingold, 1964). L-fuculose 1-phosphate and L-lactic aldehyde were gifts from Dr. E. C. Heath, Johns Hopkins University. D-glyceraldehyde 3-phosphate was generously given by Dr. C. E. Ballou, University of California, Berkeley. Dr. H. Lardy, University of Wisconsin, kindly provided L-sorbose 1-phosphate, L-sorbose 6-phosphate, and L-sorbose 1,6-diphosphate. HDP aldolase was used to synthesize 6-deoxy-L-sorbose 1-phosphate from DHAP and L-lactic aldehyde. D-lactic aldehyde was prepared by ninhydrin degradation of L-threonine (Huff and Rudney, 1959). All other compounds and enzymes were commercial products.

RP aldolase activity was determined as follows. Reaction mixtures contained: (μmoles) NADH, 0.2; L-rhamnulose 1-phosphate, 2; glycyl-glycine buffer, pH 7.5, 45; α-glycerophosphate dehydrogenase, 50 μg; in a total volume of 1 ml. Ten μl of enzyme were added to start the reaction and the decrease in absorbance was followed at 340 mμ, using an automatic recording spectrophotometer equipped with a device to hold the temperature at 37 C. A unit of enzyme activity is defined as the amount of enzyme required to release one μmole of DHAP per min.

Protein concentration was determined by the method of Waddel (1951). The method of Dische and Devi (1960) was used to determine 6-deoxyketohexose; ketohexose was qualitatively demonstrated by the same method. Ketopentose was demonstrated by the method of Meijbaum (1939), and ketotetrose by the method of Dische and Dische (1958).

Results: Cell-free extracts of L-rhamnose-grown E. coli K40 were prepared as previously described (Chiu and Feingold, 1964). Enzyme fractionation was carried out in Na and K phosphate buffer, pH 7.0, containing mercaptoethanol. Nucleoproteins were precipitated from the crude extract with $MnCl_2$. The resultant supernatant fluid was fractionated with solid

$(NH_4)_2SO_4$, and the fraction which precipitated between 35-45% saturation was collected and dissolved in buffer. This solution was fractionated with acetone at -20 C; the fraction which precipitated between 52-60% (v/v) acetone concentration was dissolved in buffer and freed from acetone by passage through a column of Sephadex G-100. Further fractionation was effected by elution from a column of DEAE Sephadex A-50 with an increasing gradient of NaCl. Active protein in the column effluent was precipitated by addition of solid $(NH_4)_2SO_4$ to 60% saturation. The precipitate was then extracted with a small volume of buffer 42% saturated in $(NH_4)_2SO_4$. The extract was discarded and the remaining precipitate was dissolved in a small volume of buffer. The purification is summarized in Table 1.

TABLE 1

Purification of L-rhamnulose 1-phosphate Aldolase

Fraction	Specific Activity*	Purification (-fold)	% Recovery
$MnCl_2$	0.06	1	100
1st $(NH_4)_2SO_4$	0.30	5	89
Acetone	1.70	29	54
DEAE Sephadex	2.30	39	54
2nd $(NH_4)_2SO_4$	5.80	98	32

*Units per mg protein at 37 C.

The purified enzyme yielded one band, comprising over 90% of the protein present, upon acrylamide gel electrophoresis. The optimum pH was 7.5. K_m for L-rhamnulose 1-phosphate was 6.4×10^{-3} M. The apparent equilibrium constant, K_{eq}, for the reaction L-rhamnulose 1-phosphate $\rightleftharpoons$ DHAP + L-lactic aldehyde was 0.083 at 37 C.

The substrate specificity of RP aldolase for different ketose phosphates was determined by substituting the compounds for L-rhamnulose 1-phosphate in the RP aldolase assay. The results are summarized in Table 2,

in which the relative activity of RP aldolase with a number of different substrates is compared. The reaction rate with L-rhamnulose 1-phosphate is taken as 100.

TABLE 2

Specificity for Ketose Phosphates of L-rhamnulose 1-phosphate Aldolase

Substrate	Relative Rate	C-3,4 Configuration
L-rhamnulose 1-phosphate	100.0	trans
D-fructose 1,6-diphosphate	1.8	trans
L-fuculose 1-phosphate	1.7	cis
D-ribulose 1,5-diphosphate	1.3	cis
6-deoxy-L-sorbose 1-phosphate	0.8	trans
L-sorbose 1-phosphate	0.3	trans
L-sorbose 1,6-diphosphate	0.0	trans
D-fructose 1-phosphate	0.0	trans
L-sorbose 6-phosphate	0.0	trans
D-fructose 6-phosphate	0.0	trans

The specificity of RP aldolase for aldehydes in the direction of condensation was determined as follows. Incubation mixtures at 37 C contained: (μmoles) DHAP, 2.0; aldehyde, 2.5; glycyl-glycine buffer, pH 7.0, 40; in a total volume of 1.0 ml. The reaction was started by addition of 2.5 μg of enzyme in 10 μl of solution. At times 0, 1, 3, 5, 10 and 20 min, 0.15 ml samples were removed and inactivated at 100 C for 1 min. Residual DHAP in the samples was determined with α-glycerophosphate dehydrogenase and NADH. Disappearance of DHAP equalled 6-deoxyketose appearance in mixtures containing L-lactic aldehyde. Formation of ketoses was qualitatively demonstrated by chromogen formation in other reaction mixtures in which activity was observed. In addition, each of these reaction mixtures contained an organic phosphate with the expected paper-chromatographic

(n-propanol-conc. NH_4OH-H_2O; 7:3:1) and paper-electrophoretic (pH 5.8) behavior. Treatment of the reaction products with acid phosphatase released carbohydrates with the relative paper-chromatographic (butanone-acetic acid-H_2O; 75:25:10) mobilities expected for the free ketoses. These results are summarized in Table 3.

TABLE 3

Aldehyde Specificity of L-rhamnulose 1-phosphate Aldolase

Aldehyde	Relative Rate*	Presumed Product**
L-Lactic Aldehyde	100	L-rhamnulose 1-phosphate
D-Glyceraldehyde	50	D-sorbose 1-phosphate
Glycolaldehyde	40	L-xylulose 1-phosphate
Acetaldehyde	15	5-deoxy-L-xylulose 1-phosphate
Formaldehyde	10	D-erythrulose 1-phosphate
D-Lactic Aldehyde	10	6-deoxy-D-sorbose 1-phosphate
L-Glyceraldehyde	0	---
D-Glyceraldehyde 3-phosphate	0	---
Propionaldehyde	0	---

*The reaction rate with L-lactic aldehyde is taken as 100.
**In assigning structures it is assumed that RP aldolase catalyzes formation of only the isomer with the configuration D at C-3 and L at C-4.

Discussion: These results establish the validity of the aldolase-catalyzed step in the proposed pathway of L-rhamnose utilization in E coli. As pointed out by Eagon (1961), this may not be the only pathway of L-rhamnose utilization in bacteria. However, details concerning other pathways have not been reported.

RP aldolase is highly specific for ketose 1-phosphates with the configuration D at C-3 and L at C-4 (Table 2), in contrast to HDP aldolase, which is specific for the diastereoisomeric trans configuration. The data

of Table 3 show that aldehydes other than L-lactic aldehyde can react with DHAP to form condensation products. By analogy with HDP aldolase and L-fuculose 1-phosphate aldolase it is assumed that specificity for DHAP is absolute and also that only one of the four possible diastereoisomers is formed. These aspects are under investigation.

This is the first reported study of the specificity of an aldolase specific for ketoses such as L-xylulose 1-phosphate (Table 3). Aldolases for three of the four possible diastereoisomeric ketose 1-phosphates have now been demonstrated. The remaining, as yet undemonstrated, aldolase would be expected to be specific for compounds like L-ribulose 1-phosphate and L-psicose 1-phosphate.

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