TAGATOSE-1,6-DIPHOSPHATE ACTIVATION OF LACTATE DEHYDROGENASE FROM STREPTOCOCCUS CREMORIS

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SUMMARY: Tagatose-1,6-diphosphate was an effective substitute for fructose-1,6-diphosphate in the activation of lactate dehydrogenase (EC 1.1.1.27) from Streptococcus cremoris AM2. The K_m for pyruvate, V_{max} and 0.5 values (activator concentration at half-maximal velocity) were similar with each activator. Of the other sugar phosphates and glycolytic intermediates tested only glucose-1,6-diphosphate activated the enzyme although the 0.5 value was 200 times that for the ketohexose diphosphates. Lactate dehydrogenases from several other organisms belonging to the Lactobacillaceae were equally stimulated by fructose-1,6-diphosphate and tagatose-1,6-diphosphate.

INTRODUCTION

Lactose transport in streptococci possessing the group N antigen (Streptococcus cremoris, S. lactis and S. diacetilactis) involves a phosphoenolpyruvate phosphotransferase system (1) similar to that in Staphylococcus aureus (2). The resulting lactose phosphate is hydrolysed by phospho-β-galactosidase (3) giving D-glucose and D-galactose-6-P. A new pathway has been elucidated recently in S. aureus by which D-galactose-6-P is further metabolized through tagatose derivatives (4,5):D-galactose-6-P ⟶ D-tagatose-6-P ⟶ D-tagatose-1,6-P_2 ⟶ D-glyceraldehyde-3-P + dihydroxyacetone-P. This pathway, named the D-tagatose-6-P pathway, has also been demonstrated in group N streptococci (6).

Fructose-1,6-P_2 markedly activates both pyruvate kinase (7) and lactate dehydrogenase (8) from these organisms and these control mechanisms may regulate the glycolytic pathway and determine the balance of end products. Fructose-1,6-P_2 activation of lactate dehydrogenases from several other organisms belonging to the Lactobacillaceae has been reported and a high degree of fructose-1,6-P_2 specificity has been

claimed since none of the other sugar phosphates or glycolytic intermediates tested could substitute for fructose-1,6-P_2 (9-13). Lactic acid bacteria are usually homofermentative when grown on glucose but become heterofermentative when grown on galactose (14). An explanation for this change in fermentation pattern, could be that galactose is metabolized through the tagatose-6-P pathway leaving no intracellular fructose-1,6-P_2. However this communication shows that tagatose-1,6-P_2 activates lactate dehydrogenase as effectively as fructose-1,6-P_2.

MATERIALS AND METHODS

Streptococcus cremoris AM2, S. lactis ML3 and S. diacetilactis DRC1 were grown at 32°C and S. faecalis NCDO 581 at 37°C in T5 broth (15). S. faecalis ATCC 8043, Lactobacillus casei ATCC 7469 and Bifidobacterium bifidum ATCC 11863 were grown at 37°C in MRS broth (16). B. bifidum was grown anaerobically. Cells were disrupted (15) and lactate dehydrogenases were partially purified by treatment of cell extracts with streptomycin sulphate followed by ammonium sulphate precipitation and gel filtration. The procedures used were similar to those described for pyruvate kinase purification (7).

Lactate dehydrogenase assays were carried out spectrophotometrically at 25°C by measuring the initial rate of NADH oxidation at 340 nm. The standard assay system consisted of 50 mM triethanolamine-HCl buffer (pH 7.5), 10 mM sodium pyruvate, 0.2 mM fructose-1,6-P_2, 0.1 mM NADH and enzyme sample in a final volume of 1.0 ml. Reactions were initiated by addition of 5 µl lactate dehydrogenase solution (0.15 µg protein). The specific activity of lactate dehydrogenase is defined as µmoles of NADH oxidized per min per mg protein (units per mg protein). Protein was estimated by the method of Lowry et al. (17).

Sugar phosphates and glycolytic intermediates were obtained in the most stable salt form and as the grades with highest analytical purity from Sigma Chemical Co., St Louis, Mo. Where necessary, salts were converted to the appropriate form using standard procedures and all solutions were adjusted to pH

TABLE 1. ANALYSIS OF TAGATOSE-1,6-P_2 (Ba SALT)

Enzymatic assay	tagatose-1,6-P_2	70%
	tagatose-1,6-P_2.Ba	98%
	tagatose-6-P	<1%
For $C_6 H_{12} O_{12} P_2 Ba$		
Calculated	C15.1, H2.5, P13.0, Ba 28.9	
Found	C15.7, H2.7, P12.8, Ba 28.7	
Total P/acid-labile P	= 1.94	
Total P/ketohexose	= 1.97	

Enzymatic analyses were performed in 50 mM triethanolamine-HCl buffer (pH 7.5, 4 mM $MgCl_2$, 0.12 mM NADH) using tagatose-1,6-P_2 aldolase coupled with triose-P isomerase and α-glycerol-P dehydrogenase (23). The decrease in absorbance at 340 nm was recorded with limiting tagatose-1,6-P_2. Tagatose-6-P was estimated by addition of phosphofructokinase and ATP to the above assay system.

Total and acid-labile P were determined by the method of Fiske and SubbaRow (24). Complete release of acid-labile P required heating at 100°C in 1N HCl for 20 min. Ketohexose was determined by the method of Roe using fructose-1,6-P_2 as the standard (25).

7.5. Fructose-6-P kinase (type III), fructose-1,6-P_2 aldolase (grade I) and α-glycerophosphate dehydrogenase/triosephosphate isomerase (type III) were obtained from Sigma. Tagatose-1,6-P_2 aldolase was partially purified from cell extracts of galactose-grown S. cremoris AM2 using procedures similar to those described for lactate dehydrogenase.

Tagatose-6-P was synthesized from D-galacturonic acid (18-20) and converted to tagatose-1,6-P_2 using fructose-6-P kinase and a slight excess of ATP (21). The reaction was monitored and ATP maintained at rate-limiting concentrations by gradual addition to the reaction mixture. When all the tagatose-6-P had been phosphorylated, adenine nucleotides were removed with mercuric nitrate. After removal of excess mercury with hydrogen sulphide, tagatose-1,6-P_2 was purified by barium salt fractionation (22). The acid barium salt was washed with absolute ethanol and anhydrous diethyl ether and dried over phosphorus pentoxide under high vacuum.

RESULTS AND DISCUSSION

Analysis of tagatose-1,6-P_2. Enzymatic analysis showed that the barium salt of tagatose-1,6-P_2 was 98% pure (Table 1). Tagatose-6-P was not detectable. The ratios of total P to acid-labile P and ketohexose were 1.94 and 1.97 respectively, the theoretical value being 2 (Table 1). Tagatose-1,6-P_2 was not cleaved by fructose-1,6-P_2 aldolase from rabbit muscle. With 0.8 mM ketohexose diphosphate the rate of tagatose-1,6-P_2 cleavage was less than 0.05% the rate of fructose-1,6-P_2 cleavage, in agreement with the results of Bissett and Anderson (4) but in contrast to the data of Tung et al. (21). Our results indicated that tagatose-1,6-P_2 contained less than 0.001% fructose-1,6-P_2.

Activation of lactate dehydrogenase by tagatose-1,6-P_2. Lactate dehydrogenase from S. cremoris AM2 was purified 22-fold to a specific activity of 310 units per mg protein. Fructose-1,6-P_2 and tagatose-1,6-P_2 aldolase impurities were less than 0.1% of the lactate dehydrogenase activity, while NADH oxidase impurity was less than 0.01%. In 0.05 M triethanolamine-HCl buffer the pH optimum for lactate dehydrogenase changed from a relatively sharp peak at pH 7.8, in the absence of fructose-1,6-P_2, to a broad plateau of maximal activity between pH 6.2 and 7.6 in the presence of saturating fructose-1,6-P_2. In the absence of fructose-1,6-P_2 no activity was detectable at pH values below 6.9.

Activator saturation curves for lactate dehydrogenase are shown in Figure 1. Tagatose-1,6-P_2 activated lactate dehydrogenase as effectively as fructose-1,6-P_2 since the 0.5 values and the V_{max} values for the two activators were not significantly different (Table 2). The low 0.5 values indicate a high sensitivity of the enzyme for the activators in vitro.

Pyruvate saturation curves for lactate dehydrogenase are shown in Figure 2. The K_m (pyruvate) and V_{max} values for lactate dehydrogenase were not significantly different with either tagatose-1,6-P_2 or fructose-1,6-P_2 as the saturating activator.

Specificity for the activating ligand. The following sugar phosphates and glycolytic intermediates were tested as potential activators of lactate dehydrogenase at final

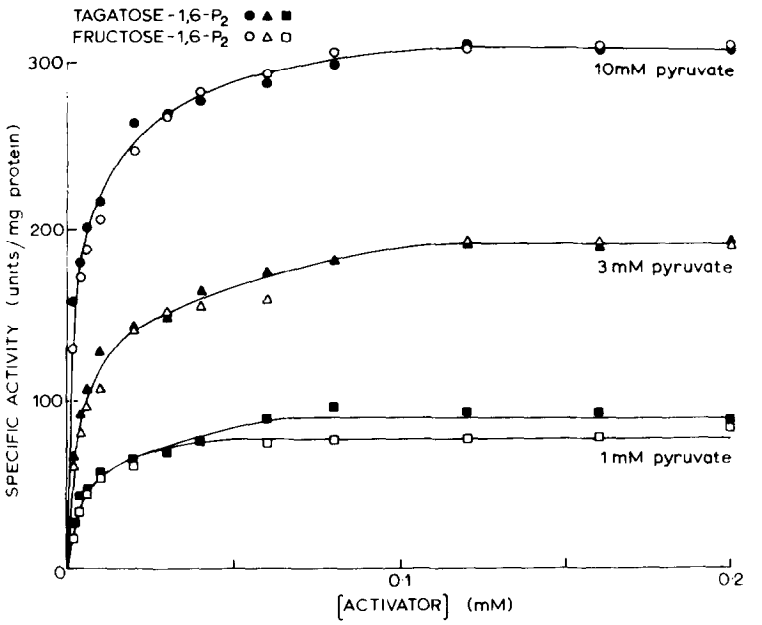

Figure 1. Activator saturation of lactate dehydrogenase from S. cremoris AM2. The standard assay was used except that activator and pyruvate concentrations were varied as indicated.

TABLE 2. ACTIVATION OF LACTATE DEHYDROGENASE FROM S. cremoris AM2 BY KETOHEXOSE DIPHOSPHATES. V_{max} and 0.5 values were derived by the method of Wilkinson (26) from data plotted in Figure 1.

Pyruvate concentration (mM)	Tagatose-1,6-P_2		Fructose-1,6-P_2	
	0.5 value (SE)*	V_{max} (SE)+	0.5 value (SE)*	V_{max} (SE)+
10	2.5 (0.3)	305 (6)	3.4 (0.3)	309 (5)
3	4.7 (0.5)	189 (4)	6.2 (0.7)	187 (6)
1	6.4 (0.8)	95 (3)	6.1 (0.4)	84 (1)

* Micromolar (standard error).

+ Units/mg protein (standard error).

concentrations of 1 mM in the standard assay system without fructose-1,6-P_2:dihydroxyacetone-P, fructose-1-P, fructose-6-P, galactose-1-P, galactose-6-P, glucose, glucose-1-P, glucose-6-P, DL-glyceraldehyde-3-P, P-enolpyruvate, 6-P-gluconate, 2-P-glycerate, 3-P-glycerate, ribose-5-P, ribulose-5-P,

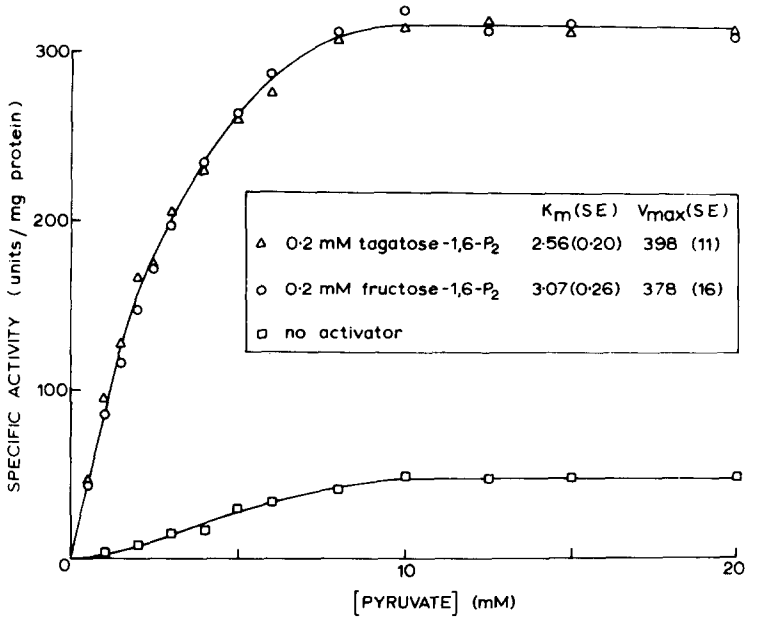

Figure 2. Pyruvate saturation of lactate dehydrogenase from S. cremoris AM2. The standard assay was used except that pyruvate concentration was varied and activators were present at saturating concentrations as indicated. The K_m for pyruvate (mM) and V_{max} (units per mg protein) values with their standard errors (SE) were derived by the method of Wilkinson (26).

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ribulose-1,5-P_2, sedoheptulose-7-P, tagatose-6-P and xylulose-5-P. These compounds neither activated nor inhibited the enzyme and did not change the velocity of the enzyme reaction when either tagatose-1,6-P_2 or fructose-1,6-P_2 were present at concentrations giving half-maximal velocity. Apparent stimulation by erythrose-4-P was due to fructose-1,6-P_2 contamination. Besides the ketohexose diphosphates, glucose-1,6-P_2 was the only effective activator found. However, while the V_{max} with glucose-1,6-P_2 was 90% of that using saturating fructose-1,6-P_2 the 0.5 value for glucose-1,6-P_2 was 0.5 mM or about 200 times the values for the ketohexose diphosphates. Enzymatic analysis indicated that glucose-1,6-P_2 contained less than 0.001% fructose-1,6-P_2. The lactate dehydrogenase from S. lactis ML3 gave similar data with the above compounds.

Distribution among the Lactobacillaceae of tagatose-1,6-P_2 activation of lactate dehydrogenase. Fructose-1,6-P_2-dependent lactate dehydrogenases were partially purified from various

TABLE 3. TAGATOSE-1,6-P_2 ACTIVATION OF LACTATE DEHYDROGENASE FROM VARIOUS BACTERIA BELONGING TO THE LACTOBACILLACEAE

Organism	Specific activity (units.mg protein^{-1})		
	Activator		
	None	Tagatose-1,6-P_2*	Fructose-1,6-P_2*
S. cremoris AM2	0.0	260	243
S. lactis ML3	3.7	105	111
S. diacetilactis DRC1	1.3	232	221
S. faecalis NCDO 581	4.6	295	257
S. faecalis ATCC 8043	2.4	38	39
B. bifidum ATCC 11863	0.6	13	12
L. casei ATCC 7469	0.8	129	114

* 1 mM final concentration.

Lactate dehydrogenases were purified 6-19 fold by treatment of crude extracts with streptomycin sulphate followed by ammonium sulphate precipitation and gel filtration (see Methods). NADH oxidase activity was not detectable in these preparations. Lactate dehydrogenase assays (1.0 ml, 25°C) were performed in 0.1 M imidazole-HCl buffer (pH 6.2) containing 10 mM sodium pyruvate, 0.1 mM NADH and 4 mM $MnCl_2$. Reactions were initiated by adding lactate dehydrogenase (0.2-3.5 µg protein).

organisms belonging to the Lactobacillaceae. The activation produced by 1 mM fructose-1,6-P_2 was compared with that given by 1 mM tagatose-1,6-P_2 at pH 6.2 in the presence of Mn^{2+} (Table 3). Each enzyme was equally stimulated by either ketohexose diphosphate, indicating that fructose-1,6-P_2 is not the only effective activator of lactate dehydrogenase as has been suggested (9-13).

The group N streptococci can metabolize D-galactose through both the D-tagatose-6-P and Leloir pathways (6) although the relative participation of the two pathways is unknown. The present data suggest that whichever pathway is used for carbohydrate fermentation there is an intermediate present capable of lactate dehydrogenase activation. We have found that when these organisms are grown on limiting glucose or lactose, lactic acid accounts for about 95% of the carbohydrate fermented. However with limiting galactose, lactic acid accounts for only about 60% of the sugar fermented. The present results do not explain this change in fermentation pattern on substitution of galactose for glucose.

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