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# D-Tagatose 1,6-Diphosphate Aldolase from Lactic Streptococci: Purification, Properties, and Use in Measuring Intracellular Tagatose 1,6-Diphosphate

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Two D-ketohexose 1,6-diphosphate aldolases are present in *Streptococcus cremoris* $E_8$ and *S. lactis* $C_{10}$. One aldolase, which was induced by growth on either lactose or galactose, was active with both tagatose 1,6-diphosphate (TDP) and fructose 1,6-diphosphate (FDP), having a lower $K_m$ and a higher $V_{max}$ with TDP as the substrate. This enzyme, named TDP aldolase, had properties typical of a class I aldolase, being insensitive to EDTA and showing substrate-dependent inactivation by sodium borohydride. Sodium dodecyl sulfate-gel electrophoresis indicated a subunit molecular weight of 34,500. The amino acid composition of TDP aldolase is reported. When the enzyme was incubated with either triose phosphates or FDP, the equilibrium mixture contained an FDP/TDP ratio of 6.9:1. The other aldolase, which had properties typical of a class II aldolase, showed activity with FDP but not with TDP. The intracellular TDP concentration, measured with the purified TDP aldolase, was 0.4 to 4.0 mM in cells growing on lactose or galactose and was lower (0 to 1.0 mM) in cells growing on glucose. The intracellular concentration of FDP was always higher than that of TDP. The role of ketohexose diphosphates in the regulation of end product fermentation by lactic streptococci is discussed.

Lactose transport in lactic streptococci (*Streptococcus cremoris* and *S. lactis*) involves a phosphoenolpyruvate-dependent phosphotransferase system (26, 37) similar to that in *Staphylococcus aureus* (28). The lactose phosphate formed is hydrolyzed by phospho-β-galactosidase (16, 18), giving D-glucose and D-galactose 6-phosphate (37). The D-tagatose 6-phosphate pathway, which was first demonstrated in *Staphylococcus aureus* (2, 4) and later in group N streptococci (3), is involved in the metabolism of galactose 6-phosphate to triose phosphates via the intermediate formation of D-tagatose 6-phosphate and D-tagatose 1,6-diphosphate (TDP). In *Staphylococcus aureus* the tagatose 6-phosphate pathway is required for utilization of galactose as well as lactose (4). However, lactic streptococci have the enzymatic potential to metabolize galactose via two initially separate routes, namely, the D-tagatose 6-phosphate pathway and the galactose 1-phosphate pathway (Leloir pathway) (3). Depending on the strain and on the exogenous galactose concentration, one or both pathways appear to operate (36). Galactose fermentation was heterolactic (36) and lactose fermentation was homolactic (33) with most *S. lactis* and *S. cremoris* strains. When the strains were growing on galactose, the intracellular levels of both the lactate dehydrogenase activator (fructose 1,6-diphosphate [FDP]) and pyruvate-formate lyase inhibitors (triose phosphates) were reduced, possibly accounting for the diversion of lactate to other products (36). The lactate dehydrogenase from *S. lactis* (8, 32, 33) and *S. cremoris* (17, 19, 32, 33) is dependent upon FDP for activity, whereas TDP appears to be the only other effective activator (32, 33). However, the intracellular TDP concentrations have not been estimated due to lack of the appropriate aldolase in pure form, and the total intracellular concentration of activator for lactate dehydrogenase may be greater than indicated by the intracellular concentration of FDP alone. It is therefore necessary to measure the cellular TDP concentrations as well as FDP concentrations in investigations concerning the role of ketohexose diphosphates in the regulation of end products.

The three enzymes from *Staphylococcus aureus* involved in the tagatose 6-phosphate pathway, D-galactose 6-phosphate isomerase (7), D-tagatose 6-phosphate kinase (5), and TDP aldolase (6), have been studied in some detail. The TDP aldolase is a class I aldolase as it was not inhibited by EDTA, had no divalent metal ion requirement, and was inactivated by sodium

borohydride in the presence of substrate (6). The three enzymes from lactic streptococci have not been studied.

The present study reports on (i) the purification and properties of TDP aldolase from *S. cremoris* $E_8$ and (ii) the use of this enzyme to measure the intracellular TDP concentration in lactic streptococci.

## MATERIALS AND METHODS

**Organisms and culture conditions.** All strains were from the collection held at the New Zealand Dairy Research Institute. *S. lactis* ATCC 11454 was originally obtained from the American Type Culture Collection, Rockville, Md., and is designated here as strain 133. Strain 133 $Lac^-$ is a spontaneous lactose-negative variant isolated and kindly supplied by G. P. Davey.

Static batch cultures were grown at 30°C in T5 complex broth (35) which contained 28 mM galactose or glucose or 14 mM lactose; the initial pH was 7.2. Standardized carbohydrate solutions were filter sterilized before addition to autoclaved broth. Cells were grown for at least 20 generations on the appropriate carbohydrate before use in experiments.

**Aldolase assays.** The standard assay (1 ml) contained 50 mM triethanolamine-hydrochloride buffer (pH 7.8), 0.25 mM NADH, nonlimiting amounts of the coupling enzymes α-glycerolphosphate dehydrogenase (1.2 U) and triose phosphate isomerase (11.5 U), limiting amounts of aldolase, and either 5 mM FDP or 0.16 mM TDP. Rates were corrected for NADH oxidase activity which was measured before addition of ketohexose diphosphate. The reaction was followed at 340 nm (25°C), using a Gilford model 250 spectrophotometer. The rate was proportional to aldolase concentration and was initially constant with time. One unit of aldolase activity was defined as the amount of enzyme that catalyzed the cleavage of ketohexose diphosphate at an initial rate of 1 μmol/min. The unit will be prefixed by TDP or FDP to identify the substrate used. The unsaturating TDP concentration (0.16 mM) was routinely used to conserve TDP.

**Extraction and assay of intermediates.** Cells growing exponentially were extracted with perchloric acid, and the intermediates were assayed enzymatically in neutralized extracts, using a fluorescence spectrophotometer (24, 36).

D-Glyceraldehyde 3-phosphate (GA3P), dihydroxyacetone phosphate (DHAP), and FDP were assayed by using the glyceraldehyde 3-phosphate dehydrogenase method (24). For TDP assay, purified TDP aldolase was added after assay of FDP with rabbit muscle FDP aldolase (which does not cleave TDP [32]). The reaction mixture (2.5 ml) contained 50 mM imidazole-HCl buffer (pH 7.0), 20 mM sodium arsenate, 3.3 mM cysteine-HCl, and 1 mM $NAD^+$. The sample (10 to 100 μl) was added followed by the four coupling enzymes (10 μl each) in the order: rabbit muscle glyceraldehyde 3-phosphate dehydrogenase (4 U); rabbit muscle triose phosphate isomerase (30 U); rabbit muscle FDP aldolase (1 U), and TDP aldolase (0.6 U), which was free of NADH oxidase activity and was prepared as described in this paper (specific activity, 21 TDP U per mg of protein). The intracellular concentrations of intermediates were calculated on the basis that 1 g (dry weight) of cells was equivalent to 1.67 ml of intracellular fluid (cytoplasm) (38).

The above glyceraldehyde 3-phosphate dehydrogenase method was used to measure the equilibrium products of TDP aldolase. A 0.2-ml sample from the reaction mixture was quenched by the addition of 0.1 ml of 10% trichloroacetic acid, stored on ice, and assayed after the addition of 100 μl of 1 M triethanolamine-HCl buffer (pH 7.8).

**Purification of TDP aldolase from *S. cremoris* $E_8$.** Cultures were grown at 30°C in T5 complex broth containing 28 mM galactose. Cells (~3 g, dry weight) were harvested near the end of the logarithmic phase, washed in 200 ml of 50 mM phosphate buffer (pH 6.7) containing 1 mM dithioerythritol, and suspended in 70 ml of the same buffer. All subsequent steps were carried out at 0 to 4°C. Cells were disrupted by shaking for 2 min with glass beads in a Mickle disintegrator, and debris was removed by centrifugation at 23,500 × *g* for 5 min. Nucleic acids were precipitated from the cell-free extracts by dropwise addition of streptomycin sulfate (10%, wt/vol; final concentration, 0.75%). After standing for 45 min, the precipitate was removed by centrifugation at 23,500 × *g* for 15 min. Solid ammonium sulfate was added to the supernatant (43.6 g/100 ml) over a 5-min period to bring the solution to 70% saturation. After standing for 30 min, the solution was centrifuged at 23,500 × *g* for 15 min. Ammonium sulfate (17.1 g/100 ml) was added to the supernatant to bring the solution to 95% saturation. After standing for 30 min, the precipitate was collected by centrifugation (23,500 × *g* for 15 min). The precipitate was redissolved in 5.0 to 6.0 ml of 50 mM Tris-hydrochloride buffer containing 100 mM KCl and 1 mM dithioerythritol (pH 8.2) and dialyzed against the same buffer (2 × 2 liters) for at least 15 h. The dialyzed sample was applied to a DEAE-cellulose column (16- by 2.5-cm column of DE52 Whatman resin) preequilibrated in the same dialysis buffer. The column was eluted with 200 ml of the initial buffer (50 mM Tris-hydrochloride buffer containing 100 mM KCl and 1 mM dithioerythritol, pH 8.2) before the following gradient was started: 325 ml of initial buffer mixing with 325 ml of final buffer (50 mM Tris-hydrochloride buffer containing 300 mM KCl and 1 mM dithioerythritol, pH 7.9). Fractions containing TDP activity with a specific activity of >15 U per mg of protein were pooled and concentrated to 4 to 5 mg of protein per ml by ultrafiltration, using a XM-50 Diaflo membrane. The sample was dialyzed for 15 h against 2 liters of 50 mM phosphate buffer (pH 7.2) containing 1 mM dithioerythritol. A sample (2 to 3 ml) was loaded onto a column (56 by 2.6 cm) of Sephacryl S-200 preequilibrated in dialysis buffer. The fractions containing TDP aldolase with high specific activity (>15 U per mg of protein) were pooled and concentrated by ultrafiltration to 4 to 6 mg of protein per ml.

**Borohydride treatment.** The borohydride treatment method used was that described by Grazi et al. (12). The reaction mixture (3 ml) contained 50 mM sodium acetate buffer (pH 6.0), 10 mM DHAP, and 500 μg of purified aldolase. Sodium borohydride (300 μl, 0.5 M) was added as six aliquots. Each aliquot was added over a 1-min period with a 2-min interval between additions. The pH was maintained at 6.0 by automatic addition of 2 M acetic acid. Samples were removed at intervals for assay of aldolase activity.

**Sodium dodecyl sulfate-polyacrylamide gel electro-**

TABLE 1. Specific activities of TDP aldolase in cell-free extracts from strains of *S. lactis* and *S. cremoris* grown on different sugars[a]

| Strain | Sugar in growth medium | TDP aldolase sp act[b] |
|---|---|---|
| *S. cremoris* ($AM_2$, $M_2$, $C_{13}$, $E_8$, HP, $ML_1$) | Galactose | 1.1–1.8 |
| | Glucose | 0.1–0.3 |
| | Lactose | 0.5–0.7 |
| *S. lactis* ($C_{10}$, $H_1$) | Galactose | 1.0–1.6 |
| | Glucose | 0.1–0.2 |
| | Lactose | 0.4–1.0 |
| *S. lactis* ($ML_3$, $ML_8$, 7962) | Galactose | 0.7–0.8 |
| | Glucose | 0–0.004 |
| | Lactose | 0.8–1.0 |

[a] Culture conditions and enzyme assay procedures are described in the text. The cell-free extracts were prepared as described for TDP aldolase from *S. cremoris* $E_8$.

[b] Micromoles of TDP cleaved per milligram of protein per minute.

**phoresis.** The procedure of Weber and Osborn (39) with 10% acrylamide gels was used for electrophoresis. Subunit molecular weight was determined by comparison of the mobility of the purified TDP aldolase with those of proteins having known molecular weights (39). The protein standards used were: gamma globulin (rabbit), RNase A (bovine pancreas), chymotrypsinogen A (bovine pancreas; Miles-Seravac), ovalbumin, bovine serum albumin (BDH), glyceraldehyde 3-phosphate dehydrogenase (rabbit muscle), glutamate dehydrogenase (bovine liver), aldolase (rabbit muscle), pyruvate kinase (rabbit muscle), and alcohol dehydrogenase (yeast).

**Native enzyme molecular weight.** The apparent molecular weight of the purified TDP aldolase from *S. cremoris* $E_8$ was determined on a Sephacryl S-200 column (2.5 by 75 cm), using 50 mM potassium phosphate buffer (pH 7.4) containing 0.3 mM dithiothreitol. The following protein standards were used: cytochrome *c* (horse heart), trypsinogen (bovine pancreas), ovalbumin, bovine serum albumin, aldolase (rabbit muscle), and glutamate dehydrogenase (bovine liver).

**Amino acid analysis.** The amino acid analysis of the purified TDP aldolase from *S. cremoris* $E_8$ was determined with a Locarte MK IV amino acid analyzer on samples hydrolyzed under vacuum for 24 h in 6 M HCl at 110°C. Tryptophan was determined spectrophotometrically by the method of Edelhoch (11).

**Other procedures.** Bacterial density was determined directly by using membrane filters (34). Protein was determined by a modification (15) of the Lowry method, using bovine serum albumin as the standard.

**Materials.** Unless otherwise specified, all biochemicals were obtained from Sigma Chemical Co., St. Louis, Mo., and were the grades with highest analytical purity. Sephacryl S-200 was obtained from Pharmacia Fine Chemicals, Uppsala, Sweden. TDP was prepared as described by Thomas (32) and used as the sodium salt. L-GA3P was prepared and assayed as described by Crow and Wittenberger (9).

## RESULTS

**Specific activities of TDP aldolase from strains of *S. lactis* and *S. cremoris*.** The specific activity and affinity for TDP of TDP aldolase was measured in cell-free extracts from a number of *S. lactis* and *S. cremoris* strains (Table 1) to determine the best source of the enzyme. In the five *S. cremoris* strains the specific activity of TDP aldolase was at least twofold higher in galactose-grown cells than in lactose-grown cells, whereas glucose-grown cells possessed little activity. The sugar in the growth medium had a similar effect on enzyme specific activity with two *S. lactis* strains ($C_{10}$ and $H_1$), whereas the other three strains had little or no activity when grown on glucose and there was little difference between the activities with galactose- and lactose-grown cells. The $K_m$ values for TDP, determined from Lineweaver-Burk plots and using crude extracts from all of the strains listed in Table 1, ranged from 0.1 to 0.4 mM. The TDP aldolase in *S. cremoris* $E_8$, grown on galactose, had a high specific activity (1.7 U per mg of protein) and a low $K_m$ (0.1 mM), making it a suitable enzyme for purification.

**Purification.** Results of a typical purification of TDP aldolase from *S. cremoris* $E_8$ are summarized in Table 2. A 13.5-fold purification of TDP aldolase was achieved with a yield of 30%. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the purified TDP aldolase (10 to 80 μg of protein loaded) indicated the presence of one band correpsonding to a subunit molecular weight of 34,500. The single protein peak and

TABLE 2. Purification of TDP aldolase from galactose-grown *S. cremoris* $E_8$[a]

| Treatment | Sp act (U/mg of protein) | | Total activity (U) | |
|---|---|---|---|---|
| | FDP | TDP | FDP | TDP |
| Cell-free extract | 3.15 | 1.72 | 1,158 | 632 |
| Streptomycin sulfate | 3.10 | 1.60 | 1,130 | 583 |
| $(NH_4)_2SO_4$ (70–95%) dialyzed precipitate | 10.7 | 7.34 | 610 | 418 |
| DEAE-cellulose[b] | | | | |
| Peak I | 14.3 | 19.92 | 165 | 222 |
| Peak II | 16.8 | 0.80 | 156 | 7.4 |
| Sephacryl S-200 filtration of peak I | 16.8 | 23.2 | 137 | 190 |

[a] Data from a typical purification of enzyme from ~3 g (dry weight) of cells.

[b] See Fig. 1.

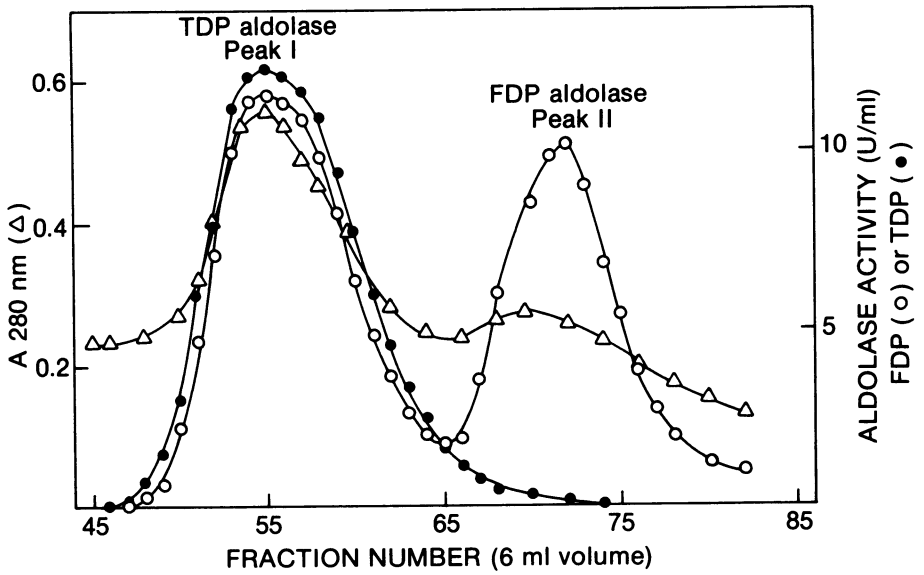


FIG. 1. Separation of TDP aldolase (●) from FDP aldolase (○) by DEAE-cellulose column chromatography (KCl gradient). A, Absorbance.

associated aldolase activity (with TDP or FDP) had an estimated molecular weight of 105,000, using the calibrated Sephacryl S-200 column.

In cell-free extracts the specific activity of aldolase with FDP as the substrate was 1.8-fold higher than with TDP. At the end of the purification process this ratio had decreased to 0.7:1. Evidence for two different aldolase activities was first observed during ammonium sulfate fractionation in which the 0 to 70% and the 70 to 95% precipitates contained ratios of FDP to TDP aldolase activity of 4:1 and 1.5:1, respectively. The DE-52 gradient separated the two different ketohexose diphosphate aldolase activities (Fig. 1). Peak I had aldolase activity for both TDP and FDP, whereas peak II had mainly FDP aldolase activity. Fractions of high activity in peak I were pooled, concentrated, and further purified as described in Materials and Methods. The FDP and TDP aldolase activities from peak I were not further separated from each other when rerun through an identical DE-52 column or when purified by gel filtration. The concentrate from the Sephacryl S-200 column was designated TDP aldolase and was used in all subsequent studies. This preparation was also used for measuring the intracellular concentration of TDP. It is unlikely that interfering enzymes are present in this preparation as only one subunit band was present on sodium dodecyl sulfate gels. The specific activity of the TDP aldolase preparations was 20 to 24 U per mg of protein, which compares favorably with the enzyme purified to electrophoretic homogeneity from *Staphylococcus aureus* (6) (specific activity, 19.5 U per mg of protein). The 13.5-fold purification of the *S. cremoris* enzyme is low compared with the 155-fold purification achieved for the *Staphylococcus aureus* enzyme and reflects the higher initial specific activity found in crude extracts from *S. cremoris*.

Peak II (Fig. 1) contained a high ratio of FDP/TDP aldolase activity (21:1). The low amount of TDP aldolase activity was due to trailing of peak I, evident when fractions (69 to 80) of peak II (Fig. 1) were pooled, concentrated, and rerun through an identical DE-52 column. The aldolase activity of fractions from this rerun showed a small FDP/TDP aldolase peak followed by a large FDP peak containing ~95% of the FDP aldolase activity present in the total fractions. The fractions corresponding to the latter half of this second peak were concentrated to 2 to 4 mg of protein per ml. This concentrate was designated FDP aldolase (no activity detected with 1.6 mM TDP) and used in comparative studies with the purified TDP aldolase.

**Properties of TDP aldolase.** The properties of TDP aldolase were studied with both FDP and TDP as substrates to further investigate the apparent involvement of one enzyme in both activities (Table 3). The pH optimum for both substrates was 7.0 to 7.3, whereas activity at pH 6.6 or 7.6 (with both substrates) was only 15% lower than that found at the optimum pH. The enzyme showed a lower $K_m$ and a higher $V_{max}$

TABLE 3. Properties of TDP aldolase and FDP aldolase separated and purified from *S. cremoris* $E_8$

| Aldolase | pH optimum | $K_m$ (mM)[a] | $V_{max}$ (U/mg of protein)[a] | Concn (mM) for 50% inhibition[b] | | | $NaBH_4$ treatment (%)[c] | |
|---|---|---|---|---|---|---|---|---|
| | | | | EDTA | $MgCl_2$ | NaCl | No DHAP | 10 mM DHAP |
| TDP | | | | | | | | |
| TDP as substrate | 7.0–7.3[d] | 0.10 | 44.4 | 20 | 10 | 90 | 24 | 94 |
| FDP as substrate | 7.0–7.3[d] | 0.25 | 22.3 | 18 | 14 | 88 | 23 | 96 |
| FDP | 7.8–8.0[e] | 1.10 | 22.4 | 1.5 | 18.0 | —[f] | 11 | 8 |

[a] $K_m$ and $V_{max}$ values were determined from Lineweaver-Burk plots. For TDP aldolase the FDP or TDP concentrations were varied in 50 mM imidazole-HCl buffer (pH 7.0), and for FDP aldolase the FDP concentration was varied in 50 mM triethanolamine-HCl buffer (pH 7.8).

[b] The standard assay procedures were used except that, for the TDP aldolase assay with FDP as the substrate, the FDP concentration was 0.5 mM. The concentrations of EDTA, $MgCl_2$, and NaCl were varied over the appropriate ranges.

[c] Treatment with $NaBH_4$ is described in the text, and activity after 20 min is expressed as percent activity lost compared with activity at time zero.

[d] The pH optima were determined with 50 mM imidazole-HCl buffer (pH 6.6 to 7.6) and either 0.16 mM TDP or 0.5 mM FDP as the substrate.

[e] The pH optimum was determined with 50 mM Tris-hydrochloride buffer (pH 7.3 to 8.8) in otherwise standard assay conditions.

[f] —, No inhibition at 100 mM.

with TDP as the substrate compared with FDP as the aldolase substrate. The inhibition of activity by EDTA, $MgCl_2$, and NaCl was similar with either FDP or TDP as the substrate. Although EDTA (tetrasodium salt) at a concentration of 18 to 20 mM gave 50% inhibition, it appeared that all, or most, of this inhibition could be due to $Na^+$ since 88 to 90 mM NaCl gave similar inhibition. When EDTA (free acid) was neutralized by imidazole, only slight inhibition (10 to 12%) of the TDP aldolase (with either substrate) occurred with 20 mM EDTA, and this inhibition was accounted for by the imidazole present. When TDP aldolase was assayed with near-saturating FDP (5 mM compared with 0.5 mM FDP used in Table 3), the EDTA concentration required for 50% inhibition increased from 18 to 100 mM. The interrelationship between inhibitor ($Na^+$) and substrate was not investigated further.

Treatment with sodium borohydride gave similar inhibition of TDP aldolase activity with either TDP or FDP as the substrate. Inhibition was markedly increased in the presence of DHAP. This pattern of inhibition was very similar to that found with rabbit muscle aldolase under the same conditions (23 and 94% inhibition of activity in the absence of DHAP and in the presence of 10 mM DHAP, respectively). Neither the 6-phosphate nor the 1-phosphate derivatives of glucose, fructose, and galactose served as substrates for the TDP aldolase when tested at 10 mM concentrations under standard assay conditions.

The activity of TDP aldolase was measured as a function of TDP concentration with or without FDP present (Fig. 2). At near-saturating concentrations of one substrate, the effect on activity of the two substrates was not additive but competitive. This suggests that the cleavage of TDP and FDP is catalyzed by the same enzyme.

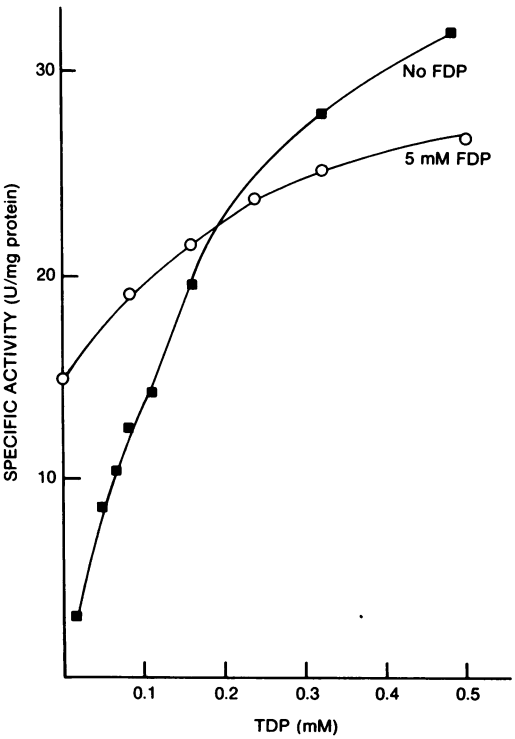


FIG. 2. Competitive effect of FDP and TDP on specific activity of the TDP aldolase. Standard assay conditions were used except that the TDP concentration was varied as indicated in the presence (○) or absence (■) of 5 mM FDP.

TABLE 4. Amino acid composition of TDP aldolase from *S. cremoris* $E_8$

| Amino acid | No. of residues (mol)[a] |
|---|---|
| Aspartic acid | 30.3 (30)[b] |
| Threonine | 16.5 (17) |
| Serine | 25.1 (25) |
| Glutamic acid | 46.0 (46) |
| Proline | 12.5 (13) |
| Glycine | 19.4 (19) |
| Alanine | 34.4 (34) |
| Half-cystine | ND[c] |
| Valine | 25.1 (25) |
| Methionine | (3.6) (4) |
| Isoleucine | (9.2) (9) |
| Leucine | 30.3 (30) |
| Tyrosine | 11.4 (11) |
| Phenylalanine | 10.6 (11) |
| Histidine | 2.7 (3) |
| Lysine | 27.7 (28) |
| Arginine | 10.7 (11) |
| Tryptophan | 3.9 (4)[d] |

[a] The number of amino acid residues per subunit was estimated assuming a molecular weight of 34,500 as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

[b] Numbers in parentheses represent probable numbers of amino acid residues per subunit.

[c] ND, Not detectable, less than 0.1 residue per subunit.

[d] Determined spectrophotometically (11).

The TDP aldolase from *S. lactis* $C_{10}$ grown on lactose was purified, to a specific activity of 12 U per mg of protein, by the same procedure as described for *S. cremoris* $E_8$ except that the Sephacryl S-200 column was not used. The DE-52 gradient separated out two aldolase activity peaks in the same manner as for *S. cremoris* $E_8$ (Fig. 1). The EDTA sensitivities, the pH optima, and the $K_m$ and $V_{max}$ values of the two aldolases from *S. lactis* $C_{10}$ were similar to those studied in more detail from *S. cremoris* $E_8$.

**Amino acid analysis.** Amino acid analysis of the purified TDP aldolase from *S cremoris* $E_8$ revealed the presence of all of the common amino acids except cysteine (Table 4).

**Properties of FDP aldolase.** The properties of FDP aldolase, partially purified from *S. cremoris* $E_8$, were studied in relation to those of the purified TDP aldolase (Table 3). The pH optimum was higher for the FDP aldolase, whereas the $K_m$ value for FDP (1.1 mM) was 4.4 times higher than that obtained with the TDP aldolase. In contrast to the TDP aldolase, the FDP aldolase was sensitive to EDTA and insensitive to NaCl. Furthermore, the presence of DHAP during $NaBH_4$ treatment of FDP aldolase did not increase the degree of inhibition. The FDP aldolase, when assayed under standard conditions, showed no activity with either 0.16 or 1.6 mM TDP.

**Products of the TDP aldolase reaction at equilibrium.** When FDP was incubated with TDP aldolase there was a gradual increase in the concentration of TDP (Fig. 3A). Steady-state concentrations of FDP, TDP, DHAP, and D-GA3P were reached within 240 min such that the FDP/TDP ratio was 7:1 and the $K_{eq}$ was $6.25 \times 10^{-5}$ M. The steady-state concentration of triose phosphates was reached after ~30 min of incubation, whereas at the same time the TDP concentration was only half the steady-state value.

Starting with equimolar concentrations of D-GA3P and DHAP, the initial rate of formation of both FDP and TDP was the same for the first 2 min of incubation (Fig. 3B). Thereafter, the apparent rate of TDP formation decreased much faster than the rate of FDP formation. At equilibrium, the FDP/TDP ratio was 7.1:1 and a $K_{eq}$ value of $5.1 \times 10^{-5}$ M was calculated. The above experiments were both repeated twice, and from a total of six experiments the following average

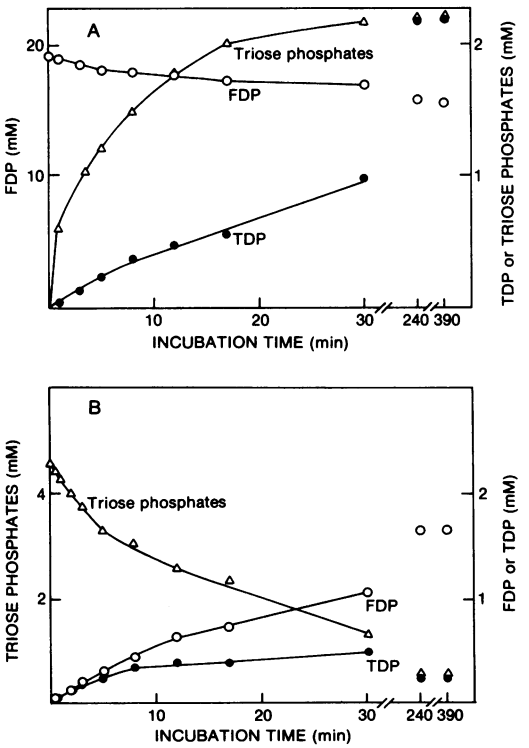


FIG. 3. Products of the TDP aldolase reaction approaching and at equilibrium when (A) the substrate was 19 mM FDP and (B) when the substrates were 2.3 mM D-GA3P and 2.3 mM DHAP. Reaction mixture (4 ml), incubated at 25°C, contained 100 mM imidazole-HCl buffer (pH 7.0) and 50 μg of TDP aldolase. Products were measured as described in the text.

values were obtained: $K_{eq} = 5.8 \times 10^{-5}$ M and FDP/TDP = 6.9:1. No triose phosphate isomerase activity was evident in these experiments as the ratio of GA3P to DHAP was the same (i.e., 1:1) for the duration of the experiment. The L-isomer of GA3P was not a substrate for the TDP aldolase as incubation with 5 mM L-GA3P and 2.5 mM DHAP gave no reaction products. In one experiment, using rabbit muscle aldolase incubated under the same conditions as in Fig. 3A, a $K_{eq}$ value of $5.1 \times 10^{-5}$ M was obtained.

**Intracellular concentrations of FDP and TDP.** The in vivo concentrations of FDP and TDP were measured in strains of *S. lactis* and *S. cremoris* growing on galactose, glucose, or lactose (Table 5). The intracellular concentrations of GA3P and DHAP are not shown but the respective values ranged from 0.3 to 0.7 and 3.6 to 8.0 mM. In the *S. lactis* strains growing on glucose, the TDP concentration was either not detectable or very low (<0.05 mM), whereas the TDP concentration was higher (0.2 to 1.0 mM) in *S. cremoris* strains growing on glucose. Relative to the concentration found in cells growing on glucose, the TDP concentration was higher in cells growing on lactose or galactose where the FDP/TDP ratio was between 4.4:1 and 16.8:1 (average, 8.9:1). *S. lactis* 133 Lac$^-$ is a lactose-negative variant which had no detectable TDP aldolase or tagatose 6-phosphate kinase activity (unpublished data) when grown on galactose. These cells contained no detectable intracellular TDP in contrast to the wild type (Table 5).

## DISCUSSION

Two ketohexose diphosphate aldolases are present in the lactic streptococci. The TDP aldolase is an inducible enzyme, being present at high specific activity when strains are growing on galactose or lactose and low specific activity when cells are growing on glucose. This aldolase has appreciable activity with FDP as the substrate, although the $V_{max}$ and the affinity for FDP are lower than the respective values with TDP as the substrate. The other aldolase, FDP aldolase, shows no activity with TDP.

FDP aldolases have been subdivided into two main classes (27, 29). Aldolases of class I are typified by the rabbit muscle aldolase, which forms a Schiff base between the ε-amino group of a specific lysine residue and the carbonyl group of the substrate. Thus, class I aldolases are irreversibly inhibited by borohydride in the presence of substrate but are not inhibited by EDTA. The TDP aldolase from *S. cremoris* (and *S. lactis*) shows these characteristic properties of a class I aldolase. On the other hand, the FDP aldolase from *S. cremoris* (and *S. lactis*) is not inhibited by sodium borohydride in the presence of substrate but is inhibited by EDTA. These characteristics are possessed by class II (metalloenzyme) aldolases typified by yeast aldolase. Other differences between the two aldolases from *S. cremoris* (and *S. lactis*) include the pH optima, the $K_m$ values for FDP, and the effect of $Na^+$ (Table 3).

The TDP aldolase from *S. cremoris* has the following similarities to the enzyme from *Staphylococcus aureus* (6): (i) both are class I type aldolases; (ii) subunit molecular weights are similar (34,500 and 37,000, respectively); (iii) both have a higher affinity and $V_{max}$ with TDP relative to FDP as the substrate; (iv) the TDP aldolases from both *S. cremoris* and *Staphylococcus aureus* show no activity towards fructose 1-phosphate, in contrast to typical class I aldolases; and (v) both enzymes can operate in the reverse direction, using only DHAP and the D-isomer of GA3P as substrates to form a mixture of D-ketohexose 1,6-diphosphates. This mixture from *Staphylococcus aureus* (6) includes sor-

TABLE 5. Intracellular concentrations of FDP and TDP in strains of *S. lactis* and *S. cremoris* growing on different sugars[a]

| Strain | Growth sugar | Intracellular conc (mM) | |
|---|---|---|---|
| | | FDP | TDP |
| *S. lactis* | | | |
| $ML_3$, $H_1$ | Galactose | 9.3–9.7 | 0.7–1.2 |
| | Lactose | 15.8–16.7 | 3.8–4.0 |
| | Glucose | 15.4–16.0 | ND[b]–0.05 |
| $ML_8$ | Galactose | 27.2 | 1.8 |
| | Lactose | 20.1 | 1.2 |
| | Glucose | 19.2 | ND |
| 7962 | Galactose | 9.1 | 0.6 |
| | Lactose | 3.3 | 0.4 |
| | Glucose | 25.4 | ND–0.05 |
| 133 | Galactose | 20.0 | 1.93 |
| 133 Lac$^-$ | Galactose | 12.3 | ND |
| *S. cremoris*[c] | | | |
| $E_8$, HP, $AM_2$ | Galactose | 7.9, 7.1, 10.7 | 0.7, 1.6, 1.3 |
| | Lactose | 13.3, 17.5, 22.9 | 2.9, 2.8, 2.9 |
| | Glucose | 15.4, 16.7, 22.3 | 0.2, 0.8, 1.0 |

[a] Culture conditions and measurement of intracellular intermediate concentrations are described in the text. Mean values from at least three separate experiments are given.

[b] ND, Not detectable.

[c] The three values are given in order for strains $E_8$, HP, and $AM_2$.

bose 1,6-diphosphate and psicose 1,6-diphosphate in addition to TDP and FDP. In the present study, TDP was measured enzymatically and may include the diphosphates of sorbose and psicose. The only noticeable difference between the TDP aldolase from *S. cremoris* and that from *Staphylococcus aureus* is the apparent molecular weight of the native enzyme as determined by gel filtration. The *Staphylococcus aureus* enzyme gave an apparent molecular weight of 50,000 from Sephadex G100 chromatography (6) compared with a molecular weight of 105,000 for the *S. cremoris* enzyme with Sephacryl S-200 chromatography. Bissett and Anderson (6) reported an anomaly between the molecular weight of the *Staphylococcus aureus* aldolase determined by gel filtration and that determined by a sucrose density gradient (37,000); they concluded that the *Staphylococcus aureus* TDP aldolase is a monomer of molecular weight 37,000. The present data for the *S. cremoris* TDP aldolase are consistent with a trimeric enzyme, but further study is clearly required. No other TDP aldolases have been studied in detail, but they have been identified in *Klebsiella pneumoniae* (25) and *Streptococcus mutans* (14) and have been implicated in *Escherichia coli* (32) and mycobacteria (31).

The present study indicates that the TDP aldolases of lactic streptococci are, like the aldolase from various other bacteria (1, 6, 10, 13, 21, 23, 30), class I aldolases. The relationship of these class I aldolases from bacteria to the more common (class II) bacterial aldolases and to the mammalian class I aldolases (e.g., rabbit muscle) has received limited study (20, 21). Lebherz et al. (20) concluded that the structure and perhaps the mechanism of the class I aldolase from *Micrococcus aerogenes* may be quite different from those of the class I aldolases of eucaryotic cells. The TDP aldolase from *S. cremoris* has some similarities with the *M. aerogenes* aldolase. For example, the cysteine composition of the *M. aerogenes* aldolase was low (no more than 0.3 residue per subunit) and no cysteine was detected in the TDP aldolase from *S. cremoris*. Lebherz et al. (20) suggested that, in contrast to the mammalian enzymes, cysteinyl residues are not involved in the catalytic function of *M. aerogenes* aldolase. This should also apply to the TDP aldolase from *S. cremoris*. In addition to cysteine being different in *M. aerogenes*, histidine, threonine, glycine, methionine, and tyrosine were judged (20) to be significantly different from rabbit A (class I aldolase) and yeast (class II aldolase). Of these amino acids only the histidine and glycine composition in the TDP aldolase from *S. cremoris* was similar to the *M. aerogenes* aldolase. The isoleucine composition of the TDP aldolase was low compared with other aldolases (20). The amino acid composition differences between these two aldolases may indicate some significant differences within the bacterial class I ketohexose diphosphate aldolases, although complete structures would be necessary to substantiate these differences.

The purified TDP aldolase has been used to estimate the intracellular TDP concentration in a number of lactic streptococci (Table 5). In cells growing on galactose or lactose the average intracellular FDP/TDP ratio was 8.9:1, which is similar to the value (6.9:1) found for the purified TDP aldolase at equilibrium. Participation of the Leloir and D-tagatose 6-phosphate pathways in the metabolism of galactose varied in different strains of lactic streptococci (36). The presence of TDP in cells does not necessarily indicate use of the D-tagatose 6-phosphate pathway since this metabolite may arise from a reversal of the TDP aldolase reaction. This is most evident in *S. cremoris* cells growing on glucose, where there are significant concentrations of TDP which are unlikely to arise from phosphorylation of tagatose 6-phosphate. These *S. cremoris* strains (Table 1) have lower TDP aldolase specific activity when grown on glucose relative to galactose or lactose, but activity must be sufficient to form some TDP from the triose phosphates.

Although the intracellular TDP concentration varied with the strain and the growth sugar, the concentration was always considerably less than the intracellular FDP concentration. When *S. lactis* strains $ML_3$ and $H_1$ and *S. cremoris* strains $E_8$, HP, and $AM_2$ are growing on galactose with heterolactic fermentation (36), both the intracellular FDP and TDP concentrations are lower than the respective concentrations when cells are growing on lactose with homolactic fermentation. *S. lactis* $ML_8$ was unique in producing only lactate from galactose (36) and this strain has, in contrast to a decrease, slightly increased intracellular concentrations of both FDP and TDP compared with cells growing on lactose (Table 5). *S. lactis* 7962 is atypical of lactic streptococci since lactose fermentation is heterolactic in batch culture, even less lactate being produced than from galactose (36). This strain had lower intracellular concentrations of both FDP and TDP when growing on lactose compared with galactose. TDP and FDP are the only two known activators of lactate dehydrogenase from lactic streptococci (32, 33), and up to now the contribution of TDP to the total intracellular ketohexose diphosphate concentration was not known. This study has shown that the intracellular FDP concentration is greater than the TDP concentration; thus, FDP will be the more important lactate dehydrogenase activator in vivo.

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