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# Purification and Properties of a Fructose-1,6-Diphosphate-Activated Lactate Dehydrogenase from *Streptococcus faecalis*

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An L-(+)-lactate dehydrogenase was purified approximately 35-fold from crude extracts of *Streptococcus faecalis*. The purified enzyme had an absolute and specific requirement for fructose-1,6-diphosphate (FDP) for catalytic activity. The concentration of FDP required for 50% maximal activity was about 0.045 mM. The activator was bound to the enzyme more effectively at *p*H 5.8 than it was at a neutral or alkaline *p*H. Activation appeared to involve a conformational change in the enzyme which made the substrate and coenzyme sites more accessible to the respective reactants. Among the evidence supporting this hypothesis was the fact that FDP lowered significantly the apparent $K_m$ for both pyruvate and reduced nicotinamide adenine dinucleotide. Moreover, the enzyme, which was quite heat stable in the absence of any of the reactants, was rendered heat labile by FDP.

Wolin (26) demonstrated that lactate dehydrogenases from several members of the genus *Streptococcus* were activated specifically by fructose-1,6-diphosphate (FDP) and suggested that this glycolytic intermediate might function as a positive allosteric effector for the enzymes studied. A number of strains of *Bifidobacterium bifidum* have also been shown to possess lactate dehydrogenases which are activated by FDP (22). During a course of studies on lactate metabolism in various group D streptococci, we found that reduced nicotinamide adenine dinucleotide (NADH)-linked, L-(+)-specific lactate dehydrogenases from several representative organisms were similarly dependent on FDP for catalytic activity. In this communication we wish to report a procedure for partially purifying the enzyme from *Streptococcus faecalis* and results of some preliminary studies which were oriented toward resolving the mechanism by which FDP activates this enzyme.

## MATERIALS AND METHODS

**Organism and culture conditions.** The organism employed in this study was obtained from Jack London under the designation of *Streptococcus faecium* NCTC 7171. It has been learned recently that the culture was originally mislabeled and that the organism actually is a typical strain of *S. faecalis* (London, *personal communication*). In a preliminary report of this work (Wittenberger and Angelo, Bacteriol. Proc., p. 118, 1969) we incorrectly referred to this organism as *S. faecium*.

The organism was grown anaerobically ($K_2CO_3$-pyrogallol seal) in a complex medium of the following composition: $K_2HPO_4 \cdot 3H_2O$, 0.5 g; tryptone (Difco), 0.25 g; yeast extract, 0.25 g; and distilled water to 100 ml. The *p*H of the medium was 7.5. Glucose was sterilized separately and added aseptically to the above medium at a final concentration of 0.4%. A 2-liter, 8-hr culture was used to inoculate a 20-liter carboy which was completely filled with medium, and cells were harvested from the stationary phase of growth after 18 to 20 hr of incubation at 37 C. The cells were then washed twice with 10 mM potassium phosphate buffer (*p*H 6.2) and were stored at −20 C either as a wet cell paste or, in most cases, as a lyophilized powder. The specific activity of the lactate dehydrogenase was the same in extracts prepared from cells stored in either manner.

**Chemicals and enzymes.** The cofactors, hexose mono- and diphosphates and calcium phosphate gel employed in this study were all products of the Sigma Chemical Co., (St. Louis, Mo.). The calcium phosphate gel was suspended in distilled water to give about 35 mg (dry wt)/ml and stored at 5 C. It was important that the gel suspension not be prepared too far in advance of use. Suspensions that were much more than 10 days old were found to absorb irreversibly the lactate dehydrogenase from *S. faecalis*. Deoxyribonuclease and ribonuclease were purchased from Worthington Biochemical Corp., Freehold, N.J.

**Protein determinations.** Protein was determined either by the biuret method (9) or by the method of Warburg and Christian as described by Kalckar (12).

**Enzyme assay.** Lactate dehydrogenase activity was routinely measured by following the decrease in absorption at 340 nm resulting from the pyruvate-dependent oxidation of NADH. The standard assay system contained potassium phosphate buffer (*p*H 6.2), 100 mM; potassium pyruvate, 5.0 mM; NADH, 0.1 mM; FDP, 1 mM; and water to a final volume of 1.0 ml. Reactions were initiated by the addition of enzyme, and all measurements were made with a model DB spectrophotometer (Beckman Instruments, Inc., Fullerton, Calif.) equipped with a Sargent model SRL recorder. All assays were carried out at room temperature. Initial reaction rates were linear and were proportional to enzyme concentration within the ranges employed. One unit is the amount of enzyme required to catalyze the oxidation of 1 μmole of NADH per min under the above assay conditions. Specific activity is expressed as units per milligram of protein.

**Preparation of cell extracts.** Two grams (dry weight) of stored cells was suspended in 30 to 40 ml of cold 10 mM potassium phosphate buffer (*p*H 6.2), and the suspension was disrupted by treatment with a Branson Sonifier for 20 min at maximum voltage output. Three passages of the cell suspension through a French pressure cell was also a satisfactory means of cellular disruption. The specific activity of the enzyme was the same in extracts prepared by either procedure. Somewhat better cell breakage was achieved with the Sonifier, however, and this method of cellular disruption was employed routinely.

The disrupted cell suspension was centrifuged in the cold for 30 min at 30,000 × *g*, and the supernatant fluid was collected by decantation. The crude, cell-free extract usually contained 20 to 27 mg of protein per ml and was diluted with 10 mM potassium phosphate buffer, *p*H 6.2, to contain about 10 mg of protein per ml.

**Purification of lactate dehydrogenase.** All steps were carried out at 4 C unless otherwise specified.

**Step I, heat treatment.** Five-millimeter volumes of the diluted cell-free extract were added to tubes (10 by 150 mm), which were equilibrated previously in a 67 C water bath. Tubes containing the extract were allowed to incubate at 67 C for 5 min, after which time they were removed and quickly cooled in an ethyl alcohol-ice bath. This process was repeated until all of the extract had been so treated. The heated extract was then pooled and centrifuged in the cold at 30,000 × *g* for 30 min, and the pellet was discarded.

**Step II, deoxyribonuclease-ribonuclease treatment.** To the somewhat opaque supernatant fluid from step I was added deoxyribonuclease and ribonuclease (50 μg each per ml) and magnesium chloride to a final concentration of 1 mM. The mixture was dialyzed overnight in the cold against distilled water. The turbid mixture was removed from the dialysis tubing and centrifuged at 30,000 × *g* for 30 min, and the clear supernatant fluid was collected by decantation. It should be noted that on a few occasions the supernatant fluid from this step remained turbid and, in these cases, the subsequent ammonium sulfate steps failed to give a satisfactory fractionation.

**Step III, first ammonium sulfate precipitation.** Solid ammonium sulfate was added slowly to the clear supernatant fluid from step II to 70% saturation, while the *p*H was maintained at 6.2 with 1 M ammonium hydroxide. The turbid mixture was then centrifuged at 30,000 × *g* for 15 min and the supernatant fluid was discarded. The pellet was redissolved in a volume of 10 mM potassium phosphate buffer, *p*H 6.2, equal to that at the beginning of step II. The 280 to 260 nm optical density ratio of this fraction was 0.700 or greater. A ratio of at least 0.700 was found to be critical to the success of the following ammonium sulfate steps.

**Step IIIa, ammonium sulfate fractionation.** To the step III fraction was added solid ammonium sulfate to 35% saturation as described previously and the protein precipitate was removed by centrifugation and discarded.

**Step IV, second ammonium sulfate precipitation.** Solid ammonium sulfate was added to the supernatant fluid from step IIIa to 60% saturation as described previously. The protein precipitate was collected by centrifugation and was redissolved in a minimal volume of 10 mM potassium phosphate buffer, *p*H 6.2. This fraction was stored overnight at −20 C.

**Step V, Sephadex chromatography.** The step IV fraction was thawed and placed on a Sephadex G-200 column (2.5 by 33 cm) that was previously equilibrated with 10 mM potassium phosphate buffer, *p*H 6.2. The column was eluted with the same buffer, and 5-ml effluent fractions were collected. The column procedure was carried out at room temperature, but the effluent fractions were placed in ice as soon as they were collected. The activity and protein elution profiles are shown in Fig. 1. Those effluent fractions containing the highest specific activity were pooled (fractions 12 through 17 in this case).

**Step VI, concentration with calcium phosphate gel.** To the pooled Sephadex G-200 fractions was added 0.25-ml of a calcium phosphate gel suspension in water (40 mg dry weight/ml) and the mixture was allowed to stand in the cold for about 15 min. The mixture was then centrifuged and the pellet was discarded. To the supernatant fluid was added 0.7-ml of the gel suspension as described previously, and the mixture was again centrifuged. The supernatant fluid was discarded, and the pellet was washed once with a small volume of distilled water. The enzyme was then eluted from the gel with 0.5 M potassium phosphate buffer, *p*H 6.2. This was accomplished by resuspending the gel in 5-ml of the buffer and allowing the suspension to stand for 20 min with occasional stirring. The suspension was then centrifuged, and the supernatant fluid was collected by decantation. This elution procedure was repeated twice more and the supernatant fluids showing the highest specific activity were pooled and stored at −20 C. It should be noted that the gel adsorption and elution procedure described was that used for this particular preparation. We have found some variability among different preparations with respect to the amount of gel required to adsorb the enzyme and have accordingly found it expedient to determine this empirically for each prepa-

ration. A summary of the results of the complete purification procedure is given in Table 1.

Although the purified enzyme was stable to storage for at least several weeks under the above conditions, it tended to lose activity over the course of the day once it was thawed. Purified preparations, therefore, were routinely stored in small portions and thawed only as required. Storage of the enzyme in a low concentration of potassium phosphate buffer, *p*H 6.2, or repeated freezing and thawing resulted in a substantial loss of activity. Neither β-mercaptoethanol (1 mM) nor dithiothreitol (2 mM) protected against the activity losses, and inclusion of these compounds in the standard assay system had no effect on catalytic activity. It was also found that purified enzyme diluted in distilled water, although exhibiting a small initial loss in activity, was thereafter more stable over the course of several hours than was enzyme diluted in 0.5 M potassium phosphate buffer (*p*H 6.2). Dilutions where required, were made in distilled water in all of the studies herein reported.

## RESULTS

**Activation of the enzyme by FDP.** The dependence of the *S. faecalis* lactate dehydrogenase on FDP for catalytic activity is shown in Fig. 2. The concentration of FDP required for 50% maximal velocity, $(M)_{0.5\ V}$ (3), is about 0.045 mM under these assay conditions. This is comparable to the value we calculated for the *S. bovis* lactate dehydrogenase from the data of Wolin (26).

It should be noted that when crude extracts of *S. faecalis* were employed as a source of the enzyme, the $(M)_{0.5\ V}$ value for FDP was often higher than that shown in Fig. 2 and was also quite variable from one extract to the next. Furthermore, specific enzyme activity in different crude extracts was often found to vary considerably even at fully saturating concentrations of FDP. No single explanation is presently available for the erratic behavior of this enzyme in crude extracts.

**Coenzyme specificity and substrate stereo specificity.** The purified enzyme was specific for

TABLE 1. *Summary of purification procedure*

| Fraction[a] | Protein | Enzyme activity | Specific activity |
|---|---|---|---|
| | *mg* | *units* | *units/mg* |
| Cell-free extract | 666.8 | 2,964 | 4.45 |
| I. Heat treatment | —[b] | 2,473 | —[b] |
| II. Deoxyribonuclease-ribonuclease treatment | 172.4 | 1,783 | 10.34 |
| III. First $(NH_4)_2SO_4$ precipitate | 112.7 | 1,793 | 15.91 |
| IV. Second $(NH_4)_2SO_4$ precipitate | 40.5 | 1,359 | 33.56 |
| V. Sephadex eluate pooled | 22.5 | 1,283 | 57.02 |
| VI. Ca-phosphate gel eluate | 4.9 | 779 | 158.98 |

[a] See text for complete description of Fractions.
[b] Not determined.

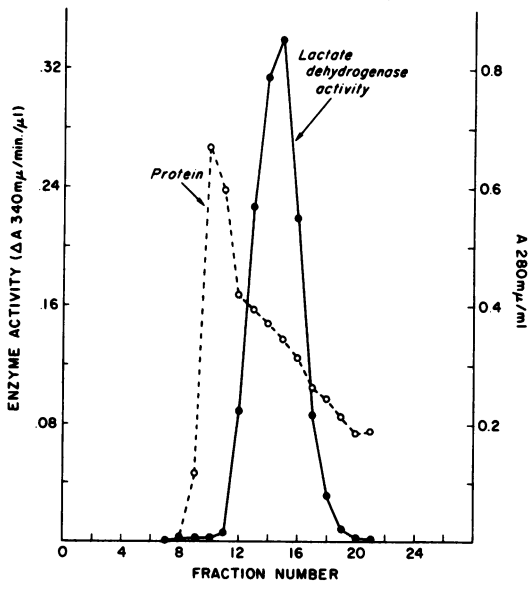


FIG. 1. *Elution profile of Streptococcus faecalis lactate dehydrogenase from Sephadex G-200 column.*

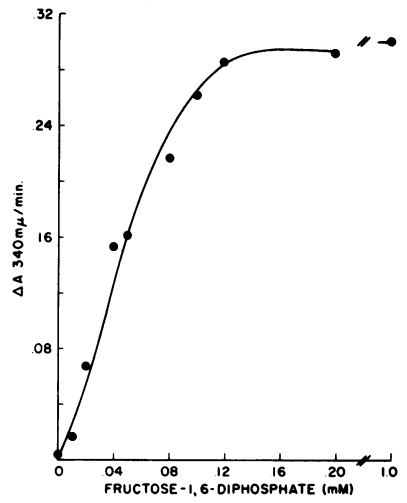


FIG. 2. *Dependence of Streptococcus faecalis lactate dehydrogenase upon fructose-1,6-diphosphate for catalytic activity. The standard NADH-pyruvate assay was employed except that the fructose-1,6-diphosphate concentration was varied as shown. Each reaction was initiated by the addition of fraction VI (Table 1) enzyme (0.18 mg of protein).*

NADH as the coenzyme; reduced nicotinamide adenine dinucleotide phosphate was completely inactive as a reductant for pyruvate when tested in the standard NADH-pyruvate assay. The enzyme exhibited specificity for the L-(+) stereoisomer of lactate. It catalyzed the oxidation of L-(+)-lactate, but not D-(−)-lactate, when either nicotinamide adenine dinucleotide (NAD) or the 3-acetylpyridine analogue of NAD served as the coenzyme. The specific activity, however, was always markedly higher with the 3-acetylpyridine analogue. The assay conditions employed were as described previously (25) except that 1 mM FDP was included in the reaction mixture and the pH of the buffer used was 7.0. The reduction of either coenzyme or analogue with L-(+)-lactate was completely dependent upon FDP.

**Specificity for the activating ligand.** The lactate dehydrogenase from *S. bovis* has been shown to be highly specific for FDP as the activating ligand (26). The enzyme from *S. faecalis* also exhibited specificity for FDP. None of the following compounds could substitute for FDP when tested in the standard NADH-pyruvate assay at final concentrations of 1 mM: fructose, fructose-1-phosphate, fructose-6-phosphate, glucose, glucose-6-phosphate, ribose, ribose-5-phosphate, ribulose-1,5-diphosphate, or glyceraldehyde-3-phosphate. Significantly, a mixture of fructose-1-phosphate and fructose-6-phosphate, each at a final concentration of 1 mM, also failed to activate the enzyme. Moreover, at this concentration the fructose monophosphate esters neither individually nor in combination inhibited activation of the enzyme by FDP. It is concluded, therefore, that these compounds either do not bind to the enzyme at the activator site or if they do bind at this site, they do so far less effectively than does FDP.

**Effect of pH on the FDP saturation curve.** The kinetic response of the enzyme to increasing concentrations of FDP was influenced markedly by the *p*H at which the assays were carried out. $V_{max}$ decreased with changes in *p*H between 5.8 and 7.5, and the $(M)_{0.5\ v}$ value for FDP increased with *p*H changes in this range (Fig. 3). As the *p*H moved from the more acidic range toward neutrality, therefore, the enzyme appeared to have a markedly decreased affinity for the activating ligand. It is interesting to speculate that these in vitro effects of *p*H on the catalytic properties of the enzyme might serve to explain why certain streptococci produce more lactate from glucose at an acid *p*H than they do at an alkaline *p*H (11). At an alkaline *p*H, the lactate dehydrogenase would be expected to bind the activator poorly, thus forcing the cell to dispose of the reducing equivalents generated at the glyceraldehyde phosphate dehydrogenase step in an alternate manner.

**Multiple binding sites for the activator.** The kinetic response of the enzyme to increasing concentrations of FDP was sigmoidal rather than hyperbolic (Fig. 3). This type of response is typical of a number of allosteric enzymes (3, 19) and has been interpreted to be the consequence of cooperative interactions between multiple binding sites for the ligand in question. A graphic analysis of the data in Fig. 3 according to the equation (3):

$$\log [v(V - v)] = n \log (S) - \log K$$

yielded linear relationships between the parameters $\log [v/(V - v)]$ and log (FDP) for each of the *p*H values at which the FDP concentration series was determined (Fig. 4). The slope of the lines ($n$), which is a measure of the minimal number of FDP-binding sites and the interaction strength between them (2, 3), ranged between 1.60 and 2.10. The data suggest, therefore, that the *S. faecalis* lactate dehydrogenase may possess at least two cooperative binding sites for the activator.

**Effect of FDP on the apparent $K_m$ for pyruvate and NADH.** In an effort to gain some general insight into the nature of the FDP activation, a series of kinetic studies was carried out to determine what effect the activator might have on the apparent affinity of the enzyme for its substrate and coenzyme. The results of these studies are summarized in Fig. 5 and 6, where it may be seen that the activator lowered significantly the apparent $K_m$ for both pyruvate and NADH without affecting $V_{max}$. Activation, therefore, appeared to involve a conversion of the enzyme from a state of low or nonaffinity for its substrate and coenzyme to one of relatively high affinity for the respective reactants.

**Effect of FDP on thermal stability of the enzyme.** It was considered that the effect of FDP on the apparent affinity of the enzyme for its substrate and coenzyme may have been the consequence of an FDP-mediated change in the conformational state of the protein. Such a change could have rendered the substrate and coenzyme sites more accessible to the respective reactants. On the basis of the observation that the lactate dehydrogenase was relatively heat stable in the absence of any of the reactants, it was reasoned that, if the activator did mediate an allosteric transition to a less constrained configuration, this might be manifested by an increased sensitivity of the enzyme to thermal inactivation. The results of an experiment designed to test this possibility showed (Table 2) that the enzyme was significantly more heat labile in the presence of FDP than it was in its absence. This decreased thermostability was not observed when any one of the following com-

pounds was substituted for FDP under experimental conditions otherwise identical to those described in Table 2: glucose-6-phosphate, fructose-6-phosphate, fructose-1-phosphate, or fructose. The enzyme was rendered heat labile in the absence of FDP, however, when incubated with relatively high concentrations of potassium phosphate buffer (200 to 600 mM, pH 6.2). Qualitatively similar results were obtained with sodium sulfate, sodium arsenate, and potassium chloride. None of these compounds was able to function as an activator, however, and it seems likely that their heat-labilizing effect was due to some ion-mediated change in the structure of the enzyme that was not related to activation. It is well documented that the structure of various macromolecules is strongly affected by the ionic environment in which they are placed (7, 21, 23). Moreover, ionic effects specifically on the structure of lactate dehydrogenases from mammalian sources have also been reported (4, 6, 15, 16).

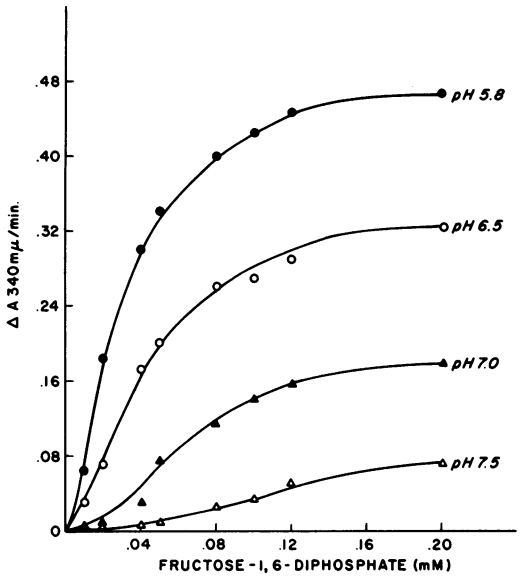


FIG. 3. *Effect of pH on the fructose-1,6-diphosphate saturation curve for Streptococcus faecalis lactate dehydrogenase. The standard NADH-pyruvate assay was employed except that the concentration of fructose-1,6-diphosphate was varied as shown and the pH of the phosphate buffer used was as indicated. Each reaction was initiated with fraction VI (Table 1) enzyme (0.18 mg of protein).*

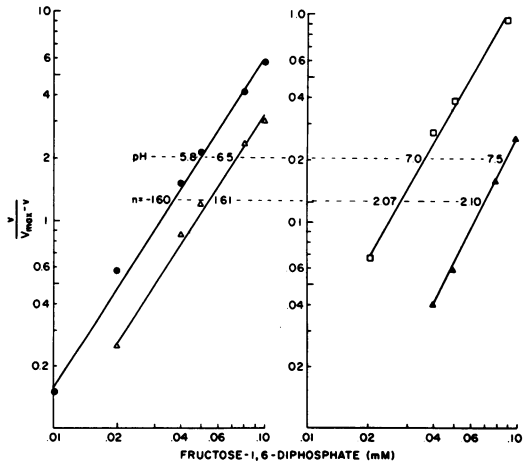


FIG. 4. *Effect of pH on the apparent kinetic order of the reaction catalyzed by the Streptococcus faecalis lactate dehydrogenase with respect to the activator, fructose-1,6-diphosphate. Data from Fig. 3.*

**Effect of coenzyme or substrate on FDP-medi-**

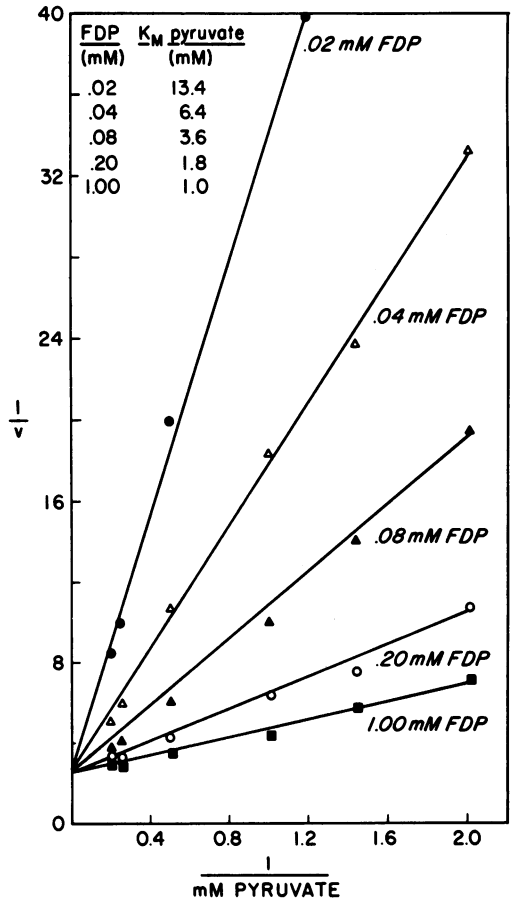


FIG. 5. *Effect of fructose-1,6-diphosphate on the apparent $K_m$ of the Streptococcus faecalis lactate dehydrogenase for pyruvate. The standard NADH-pyruvate assay was employed except that the concentrations of fructose-1,6-diphosphate and pyruvate were varied as shown. Each reaction was initiated with fraction VI (Table 1) enzyme (0.18 mg of protein).*

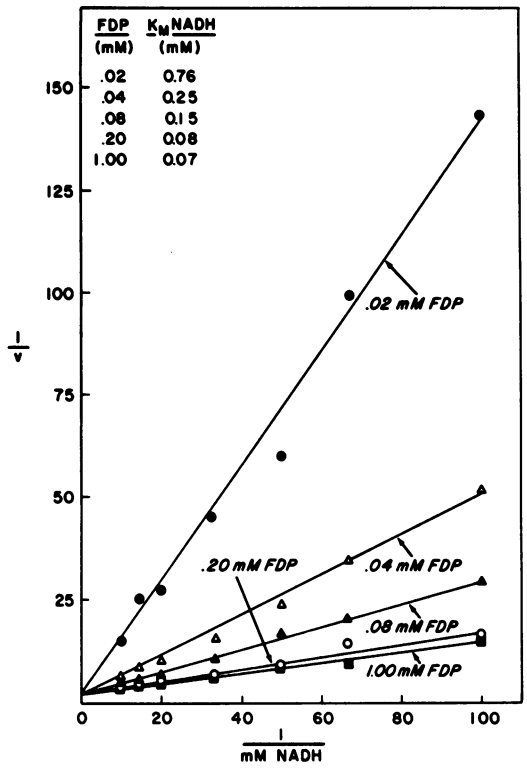


FIG. 6. *Effect of fructose-1,6-diphosphate on the apparent $K_m$ of the Streptococcus faecalis lactate dehydrogenase for NADH. The standard NADH-pyruvate assay was employed except that the fructose-1,6-diphosphate and NADH concentrations were varied as shown. Each reaction was initiated with fraction VI (Table 1) enzyme (0.18 mg of protein).*

**ated thermal inactivation of the enzyme.** If the observed increased heat sensitivity of the enzyme in the presence of FDP was indeed due to the activator mediating a conformational change which resulted in an "exposure" of the substrate and coenzyme sites, it seemed possible that the reactants might offer some protection against the deleterious effect of heat. NADH did afford the enzyme a marked degree of protection against the thermolabilizing effect of FDP (Table 3), although pyruvate did not. Inasmuch as the kinetic studies indicated that FDP increased the apparent affinity of the enzyme for pyruvate as well as NADH (Fig. 5 and 6), this observation appeared inconsistent with the concept that activation involved an "exposure" of both the coenzyme and the substrate sites. Within this concept, however, two alternative explanations for the failure of pyruvate to protect were considered. First, it was possible that the NADH site was thermolabile, whereas the pyruvate site was relatively thermostable. The inability of NADH to protect completely could have been due to yet another thermolabile site on the enzyme. Alternatively, it was possible that both the pyruvate and NADH sites were thermolabile but that pyruvate failed to protect because there was an obligatory ordered sequence of binding the reactants to the enzyme, i.e., pyruvate binding might occur only after NADH was bound. Ordered sequence mechanisms (with NADH binding first) have been established for lactate dehydrogenases from other sources (1, 17, 20, 27). If the latter explanation were correct, it seemed likely that a combination of NADH and pyruvate would confer a greater degree of heat stability to the enzyme than would NADH alone. Unfortunately this combination could not be tested directly, since the reactants would be more or less instantaneously converted to NAD and lactate in the presence of FDP. In an attempt to circumvent this difficulty, oxamate was substituted for pyruvate. Oxamate is a well-known competitive inhibitor of other L-(+)-lactate dehydrogenases with respect to pyruvate (5, 17, 18; Hakala, Glaid, and Schwert, Fed. Proc., p. 213, 1953), and we have found this to be the case for the *S. faecalis* enzyme as well (Wittenberger and Angelo, *unpublished observation*). Oxamate alone, like pyruvate, imparted no protection against heat inactivation. Moreover, oxamate plus NADH afforded the enzyme no greater protection than did the coenzyme alone (Table 3). There can be little doubt that oxamate did bind to the enzyme, since it inhibited the reac-

TABLE 2. *Effect of FDP on the thermal stability of S. faecalis lactate dehydrogenase*

| Incubation time at 67 C | Enzyme activity minus FDP[a] | Loss of activity[b] | Enzyme activity plus FDP[a] | Loss of activity[b] |
|---|---|---|---|---|
| *min* | | % | | % |
| 0 | 0.405 | 0 | 0.424 | 0 |
| 4 | 0.381 | 5.9 | 0.146 | 65.6 |
| 7 | 0.356 | 12.1 | 0.058 | 86.3 |
| 10 | 0.297 | 26.7 | 0.040 | 90.6 |

[a] As indicated above, FDP (final concentration of 1 mM) was added to one test tube (1 by 7 cm) and an equivalent volume of distilled water was added to another. The purified enzyme (1.27 mg of protein/ml) was next diluted 1:50 in distilled water, and 0.2 ml of this dilution was added to each of the two test tubes. The total volume of each tube was 0.4 ml. Both tubes were then placed in a 67 C water bath, and 0.05-ml samples were removed from each at the times indicated above. Samples were assayed immediately for lactate dehydrogenase activity in the standard NADH-pyruvate assay system. Enzyme activity: $\Delta A/340$ nm per min.

[b] Enzyme activity in zero time samples was taken as 100% of the maximum rate.

tion almost completely at the concentration employed in the incubation mixture. Furthermore, this analogue has been shown by direct binding studies to form a ternary complex with NADH and beef heart lactate dehydrogenase (24) and NADH plus oxamate has been reported to protect a D-(−)-lactate dehydrogenase from *Polysporidylium pallidum* against an alteration of structure induced by ammonium sulfate (8).

The results shown in Table 3 do not permit a definitive conclusion regarding the possibility that the reaction catalyzed by the *S. faecalis* enzyme may follow a compulsory ordered sequence mechanism. They do suggest, however, that the coenzyme binding site is thermolabile, whereas the pyruvate site is relatively more thermostable, and that the failure of NADH and oxamate to protect the enzyme completely may reflect one or more additional thermolabile sites on the enzyme.

TABLE 3. *Protection of S. faecalis lactate dehydrogenase against FDP-mediated thermal inactivation by NADH*

| Tube | Additions to incubation mixture[a] | Per cent loss of initial activity[b] | | |
|---|---|---|---|---|
| | | 4[c] | 7 | 10 |
| 1 | None | 0 | 8.2 | 22.9 |
| 2 | FDP | 59.8 | 82.3 | 90.5 |
| 3 | FDP + NADH | 7.5 | 29.0 | 46.3 |
| 4 | FDP + pyruvate | 59.4 | 84.3 | 93.9 |
| 5 | FDP + oxamate | 62.2 | 76.5 | 94.6 |
| 6 | FDP + NADH + oxamate | 17.7 | 27.7 | 43.6 |

[a] The compounds shown were added to test tubes (1 by 7 cm) and were each present in the following final concentrations (final volume, 0.4 ml): FDP, 1 mM; NADH, 0.1 mM; potassium-pyruvate, 5 mM; potassium-oxamate, 20 mM. The concentration of oxamate employed gave greater than 95 percent inhibition of enzyme activity when tested in the standard NADH-pyruvate assay system. The purified enzyme (0.892 mg of protein/ml) was next diluted 1:5 in distilled water and 0.2 ml of this dilution was added to each of the six test tubes. All tubes were then placed in a 67 C water bath, and samples (0.05 ml) were removed from each at zero time, 4 min, 7 min, and 10 min, as shown above. Each sample was quickly diluted 1:10 in ice cold distilled water and 0.03 ml of each of the diluted samples was then assayed immediately for lactate dehydrogenase activity in the standard NADH-pyruvate system. The dilutions employed reduced the level of oxamate in the final assay system to a noninhibitory concentration.

[b] Enzyme activity ($\Delta$A340 nm/min) in zero time samples was taken as 100% of the maximum rate.

[c] Minutes at 67 C.

## DISCUSSION

The activation of certain microbial lactate dehydrogenases by FDP was first reported by Wolin (26), who demonstrated that the enzymes from *S. bovis*, *S. faecalis*, *S. agalactiae*, and *S. thermophilus* all required this compound for maximum catalytic activity. In studies which will be reported in detail elsewhere, we have extended Wolin's original observation to include strains of *S. salivarius*, *S. lactis*, *S. faecium*, and *S. mutans* (10). It appears likely, therefore, that this is a property common to lactate dehydrogenases from all or most of the streptococci. It should be emphasized, however, that this is not a property unique to this group of organisms. A number of strains of *B. bifidum* have also been shown to possess lactate dehydrogenases which depend upon FDP for catalytic activity (22).

The precise mechanism by which FDP effects the activation of the *S. faecalis* lactate dehydrogenase remains to be resolved. Certain features of the process, however, have been characterized. Fructose diphosphate clearly lowers the apparent $K_m$ for both pyruvate and NADH (Fig. 5 and 6), and it increases markedly the sensitivity of the enzyme to heat inactivation (Table 2). If the results from the thermal inactivation studies are interpreted correctly as reflecting a conformational change that is related to catalytic function, the simplest explanation of these data which is also consistent with the kinetic studies would appear to be that the enzyme undergoes a transition to a less constrained configuration when it interacts with FDP. Whether this proposed transition in state involves an alteration in the tertiary structure of the enzyme or an alteration in its quaternary structure, or both, requires further investigation. In the presumed altered conformational state, both the coenzyme and the substrate sites may be more "exposed," thereby accounting for the observed decrease in apparent $K_m$ values for the reactants and the increased thermal sensitivity of the enzyme. The fact that the FDP-mediated thermolabilization of the enzyme could be largely reversed by NADH (Table 3) indicates that the "exposed" coenzyme site is thermolabile. Inasmuch as oxamate plus NADH failed to provide the enzyme with a greater degree of protection against the FDP effect than did NADH alone, it seems likely that the pyruvate site is relatively thermostable and that the lactate dehydrogenase possesses one or more thermolabile sites in addition to the coenzyme site.

Wolin (26) has speculated on the physiological significance of the FDP activation of the *S. bovis* enzyme, and our studies on the *S. faecalis* lactate dehydrogenase add little to his original discussion

in this regard. It should be pointed out, however, that FDP appears to be a key compound in the regulation of several catabolic activities in *S. faecalis*. In addition to its demonstrated role here as a specific activator for the lactate dehydrogenase, it is one of several glycolytic intermediates that have been implicated in the repression of an inducible lactate oxidase system in this organism (13). Moreover, it has been shown that FDP is one of several compounds which act as negative effectors for an inducible malate dehydrogenase (decarboxylating) from *S. faecalis* (14). It seems reasonable to assume, therefore, that fluctuations of cellular pool levels of FDP would influence qualitatively and quantitatively the course of carbon dissimilation in *S. faecalis* and perhaps in other streptococci as well. A study of factors which might affect cellular pool levels of FDP in streptococci would appear to be a fruitful area for future investigation.

## LITERATURE CITED


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