Purification of the fructose 1,6-bisphosphate-dependent lactate dehydrogenase from *Streptococcus uberis* and an investigation of its existence in different forms

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The fructose 1,6-bisphosphate [Fru(1,6) P_2]-dependent lactate dehydrogenase in cells of *Streptococcus uberis* N.C.D.O. 2039 was purified by a procedure that included chromatography on DEAE-cellulose and Blue Sepharose CL-6B in phosphate buffers. The enzyme appeared to interact with Blue Sepharose through NADH-binding sites. The homogeneous enzyme had catalytic properties that were generally similar to those of other Fru(1,6) P_2 -dependent lactate dehydrogenases, and it had no catalytic activity in the absence of Fru(1,6) P_2 . Its existence in different forms, depending on conditions, was investigated by ultracentrifugation, analytical gel filtration and activity measurements. It consisted of subunits with M_r 35900 $\pm$ 500 and, in the presence of adequate concentrations of Fru(1,6) P_2 , phosphate or NADH, it existed as a tetramer, whereas when these ligands were in lower concentrations or absent, the subunits were in a concentration-dependent association–dissociation equilibrium. Dissociation occurred slowly and inactivated the enzyme, and although added ligands reversed the dissociation, the lost activity was at best only partly restored. An exception occurred when dissociation was caused by a decrease in temperature, in which case the lost activity was fully restored at the original temperature. The tetramer also lost activity at certain ligand concentrations without dissociating. The results together indicated (i) the presence on the enzyme of two classes of binding site for both Fru(1,6) P_2 and NADH, and (ii) the likelihood that phosphate bound at the same sites as Fru(1,6) P_2 . Two different ligands together were much more effective at preventing inactivation and dissociation than was expected from their effectiveness when present separately. It was concluded that tetrameric forms of the enzyme rather than the enzyme in association–dissociation equilibrium were involved in the regulation of its activity *in vivo*.

INTRODUCTION

Lactic acid is produced by many bacterial species, but those in the genera *Streptococcus*, *Lactobacillus*, *Leuconostoc* and *Pediococcus* are known as lactic acid bacteria because of their special ability in this respect. The presence of little or no synthetic activity for lactic acid in extracts of many lactic acid bacteria was a puzzling feature of their biochemistry until Wolin (1964) found in streptococci that the lactate dehydrogenases (EC 1.1.1.27) were activated by Fru(1,6) P_2 . However, very few of the Fru(1,6) P_2 -dependent lactate dehydrogenases of streptococci have been purified and investigated in detail (Garvie, 1980), the only exceptions being the enzymes from *S. lactis* (Crow & Pritchard, 1977; Hardman *et al.*, 1985) and *S. cremoris* (Jago *et al.*, 1971; Dynon *et al.*, 1972; Crossley *et al.*, 1979).

No doubt the lactate dehydrogenases mediate and control lactate production (Yamada & Carlsson, 1975; Garvie, 1980), but activation by Fru(1,6) P_2 may be only part of the control mechanism, because phosphate and other metabolites besides Fru(1,6) P_2 affect their activity. Jago *et al.* (1971) concluded from changes in the gel-filtration behaviour of the *S. cremoris* enzyme that activation by Fru(1,6) P_2 involved conformational changes

in its structure, and they also observed that dissociation of the ligand-free enzyme was partly prevented by Fru(1,6) P_2 and NADH. If the streptococcal enzymes undergo reversible changes in structure and activity, depending on metabolite concentration, this is likely to be the means whereby lactate production is controlled *in vivo*.

The lactate dehydrogenase in extracts of *S. uberis*, an organism associated with bovine mastitis, was activated by Fru(1,6) P_2 and inhibited by phosphate, and appeared to be typical of the streptococcal lactate dehydrogenases (Garvie & Bramley, 1979). We have isolated and characterized this enzyme, and have investigated it for properties that depend on metabolite concentration and which therefore might be used to control its activity *in vivo*.

MATERIALS AND METHODS

DE-52 ion-exchange DEAE-cellulose was obtained from Whatman Biochemicals, Maidstone, Kent, U.K., Sephadex G-150 (Superfine grade), Sephacryl S-200 (Superfine grade) and Blue Sepharose CL-6B from Pharmacia Fine Chemicals, Milton Keynes, Bucks.,

Abbreviations used: Fru(1,6) P_2 , fructose 1,6-bisphosphate; N.C.D.O., National Collection of Dairy Organisms.

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U.K., 3 M-mercaptoethanesulphonic acid from Pierce and Warriner (U.K.), Chester, Cheshire, U.K., enzyme substrates and sugar phosphates from Boehringer Corporation (London), Lewes, East Sussex, U.K., guanidinium chloride (Aristar grade) from BDH Chemicals, Poole, Dorset, U.K., and other organic chemicals from Sigma (London) Chemical Co., Poole, Dorset, U.K.

'Phosphate buffer' refers to 50 mM-potassium phosphate buffer, pH 7.0, unless otherwise stated.

Enzyme assay

In the 'standard' assay, lactate dehydrogenase activity was measured at 25 °C in a mixture that contained 5 μ mol of sodium pyruvate, 0.134 μ mol of NADH, 0.5 μ mol of Fru(1,6) P_2 and 50 μ mol of Tris/HCl buffer, pH 7.0, in a total volume of 1 ml. The reaction was initiated by adding enzyme, and the rate of absorbance decrease at 340 nm was measured. Rates were linear with time for fully active enzyme, but with enzyme partly inactivated by dilution or other adjustments of the conditions, the rates often increased with time after an initial linear period. In these cases, the initial rate was used. One unit of enzyme was defined as the amount which oxidized 1 μ mol of NADH/min.

Polyacrylamide-gel electrophoresis

Disc electrophoresis was done with 7% (w/v)-polyacrylamide gels prepared in 25 mM-Tris/phosphate buffer, pH 7.0, and 50 mM-buffer in the electrode compartments. A constant current of 2.5 mA/tube was applied for 90 min. When electrophoresis was complete, the gels were either fixed in 40% (w/v) trichloroacetic acid and stained for protein with Coomassie Blue G-250, or stained for lactate dehydrogenase activity as described by Garvie (1978) to detect residual NADH. Electrophoresis in the presence of SDS was done with 5% (w/v)-acrylamide gels prepared in 50 mM-sodium phosphate buffer, pH 8.0, containing 5 mM-2-mercaptoethanol, 5 mM-EDTA and 0.1% (w/v) SDS. The same buffer mixture was used as the electrode-compartment buffer, but with an increased concentration of SDS (3%, w/v) to dissolve the protein.

Amino acid analysis

The enzyme was reduced and carboxymethylated at pH 8.5 (Crestfield *et al.*, 1963), and the product isolated by gel filtration. The modified protein was hydrolysed under N_2 in 3 M-mercaptoethanesulphonic acid for 24 h (Penke *et al.*, 1974). Amino acid analyses were obtained with an LKB automatic analyser type 4102 (LKB Instruments, Croydon, Surrey, U.K.).

Ultracentrifugation

Runs were performed at 25 °C in a Beckman Model E analytical ultracentrifuge equipped with Rayleigh interference optics. The high-speed equilibrium method of Yphantis (1964) was used for M_r determinations, with a type AN-H rotor and cells with 12 mm six-channel filled-Epon centrepieces and sapphire windows. The method described by Edelstein & Schachman (1967) was employed for the simultaneous determination of M_r and partial specific volume of the undissociated enzyme. Baseline fringe patterns were obtained as recommended by Teller (1973) for the Yphantis 6-channel cell assembly. Baseline measurements were smoothed graphically with a curve drawn through the points.

When plots of $\ln(\text{fringe displacement})$ against r^2

(Yphantis, 1964) were linear, a line was fitted to the points by linear regression. When such plots were not linear, a curve was fitted to the points by solving the orthogonal polynomial

$$r^2 = A + B/(1 + D \ln C_r) + C \ln C_r,$$

in which C_r is the protein concentration at radius r . Differentiation of this equation allowed the gradient to be calculated at each experimental point, and the corresponding weight-average relative molecular mass (M_w) obtained. A graphical method was used to estimate protein concentration at the meniscus at sedimentation equilibrium from the measurements of fringe displacement below 140 μ m, and the result was used to calculate the number-average relative molecular mass (M_n) at the meniscus (Teller, 1973).

Low-speed synthetic-boundary runs were performed to obtain the relationship between absorbance and fringe displacement for determining the specific absorption coefficient of the enzyme.

Solutions for ultracentrifugation were prepared from fully active enzyme precipitated with $(NH_4)_2SO_4$ (see the Results section) and equilibrated with buffer by dialysis. Enzyme for determination of subunit M_r was dialysed against water until salt-free, freeze-dried, and dissolved in guanidinium chloride solution.

Analytical gel filtration

Experiments were performed with a column (50 cm $\times$ 2.4 cm) of Sephadex G-150 (Superfine grade), equilibrated with 50 mM-Tris/HCl buffer, pH 7.0, containing 0.1 M-KCl and ligands as required. The column was calibrated for M_r estimation with well-characterized proteins (Andrews, 1964, 1965). Enzyme (0.1 mg), precipitated from phosphate buffer with $(NH_4)_2SO_4$, was applied to the column in 3 ml of the equilibration buffer/ligand solution, together with 4 mg each of bovine serum albumin and bovine γ -globulins to act as markers. Enzyme activity in the effluent fractions (3 ml each) was measured with the standard assay.

Organism, culture conditions and preparation of cell-free extract

S. uberis was obtained from the N.C.D.O. at this Institute as strain N.C.D.O. 2039. Cultures were first grown in a yeast/glucose/peptone broth (Garvie, 1978) at 30 °C, then, for enzyme preparation, 100 ml lots from 12 h cultures were used to inoculate stirred 8-litre batches of the same medium, which were incubated at 37 °C for 16 h. The pH was kept at 6.0 or above by the addition of 2 M-NaOH. The cells were harvested with a continuous-action rotor (MSE, Crawley, Sussex, U.K.) run at 18000 rev./min and 4 °C, with a flow-rate of 150 ml/min, then washed twice in ice-cold 0.85% NaCl solution and collected by centrifugation (40000 g, 1 h, 4 °C). The pellets were stored at -20 °C. An 8-litre culture usually yielded 60–80 g (wet wt.) of bacteria.

Cell pellets were thawed and the bacteria, suspended in phosphate buffer containing 0.1 M-KCl at a concentration of 0.5 g (wet weight)/ml, were disrupted with a Braun homogenizer type 2870. Glass beads (50 g; 0.115–0.118 mm diam.), phosphate buffer (10 ml) and cell suspension (20 ml) were placed in each homogenizer bottle, shaken for 2.5 min at 2700 rev./min with intermittent CO_2 cooling and the homogenate decanted. The pooled homogenates were stirred for 1 h at 4 °C

Table 1. Purification of Fru(1,6) P_2 -dependent lactate dehydrogenase from *Streptococcus uberis* N.C.D.O. 2039

Experimental details are given in the text. Protein was determined (a) from A_{280} and A_{280} measurements (Warburg & Christian, 1941) or (b) with $A_{280,1\text{ cm}}^{1\%}$ 6.45 (see the Results section).

Step	Volume (ml)	Protein (mg)	Lactate dehydrogenase activity		
			Total (units)	Specific (units/mg of protein)	Recovered (% overall)
Cell-free extract	400	470(a)	56 300	120	100
Pooled fractions from ion-exchange chromatography	250	250(a)	43 400	174	77
Pooled fractions from chromatography on Blue Sepharose	33	35(a)	31 400	952	56
Pooled fractions from preparative gel filtration	18	17.6(b)	24 600	1422	44

before the cell debris was removed by centrifugation (40 000 g , 1 h, at 4 °C). The supernatant was used for enzyme purification. More efficient cell breakage was obtained with pellets frozen and thawed before homogenization, and stirring the suspension of broken cells for 1 h at 4 °C gave up to 20% more enzyme, possibly by improving the diffusion of contents from the broken cells.

Enzyme purification

All procedures were performed at 4 °C.

Ion-exchange chromatography. Cell-free extract (400 ml) was dialysed against phosphate buffer containing 0.1 M-NaCl and applied to a column (60 cm $\times$ 4.5 cm) of DE-52 ion-exchange cellulose equilibrated with the same buffer/salt mixture. The column was washed with the equilibration solution (3 litres), then a linear gradient of NaCl (0.1–0.6 M) in phosphate buffer was applied. Lactate dehydrogenase activity emerged as a sharp peak, with the maximum at 0.2 M-NaCl. Fractions (25 ml) were collected.

Chromatography on Blue Sepharose CL-6B. Fractions from ion-exchange chromatography which contained > 1000 units of lactate dehydrogenase activity were combined, dialysed against 50 mM-sodium acetate buffer, pH 5.5, containing 50 mM-KH₂PO₄, and applied to a column (18 cm $\times$ 2.3 cm) of Blue Sepharose CL-6B equilibrated with same buffer/phosphate mixture. The column was then eluted successively with 250 ml of each of the following solutions: equilibration mixture; acetate buffer, pH 5.5, containing 0.5 M-KH₂PO₄; acetate buffer, pH 6.5, containing 0.1 M-KH₂PO₄; 5 mM-potassium phosphate buffer, pH 7.0, containing 2 μ M-NADH. The effluent was collected in 10 ml fractions and the enzyme, eluted with the last solution, was collected in tubes containing 1 ml of 0.5 M-potassium phosphate buffer, pH 7.0. Fractions containing enzyme were combined and the protein precipitated with (NH₄)₂SO₄ (0.7 g/ml).

Preparative gel filtration. Enzyme precipitated with (NH₄)₂SO₄ was collected by centrifugation (150 000 g , 1 h), dissolved in 3 ml of phosphate buffer containing 0.1 M-NaCl, and applied to a column (100 cm $\times$ 2.5 cm) of Sephacryl S-200 (Superfine grade) gel which was equilibrated with the same buffer/salt mixture. Small amounts of protein emerged before and after the main

protein peak, which was symmetrical and coincided with the peak of lactate dehydrogenase activity. Fractions containing enzyme with a specific activity of at least 1200 units/ A_{280} unit were combined and the protein precipitated with (NH₄)₂SO₄ as described above.

Storage. The enzyme retained full activity for several weeks when precipitated from phosphate buffer with (NH₄)₂SO₄ and stored in the supernatant solution at –5 °C.

RESULTS AND DISCUSSION

Enzyme purification

Although the catalytic mechanism of the *S. uberis* enzyme involved compulsory ordered formation of the ternary complex with NADH and pyruvate (Williams, 1981), attempts to use affinity chromatography on oxamate-Sepharose in its purification, as applied to other lactate dehydrogenases by O'Carra & Barry (1972) and Kelly *et al.* (1978), were unsuccessful. The *S. uberis* enzyme, in phosphate buffer or Fru(1,6) P_2 solution, was irreversibly adsorbed to an affinity gel in which oxamate was linked to the matrix by a hexamethylene chain and not adsorbed at all to one in which oxamate was linked by a hydrophilic chain (O'Carra *et al.*, 1974), whether NADH was present in the buffer or not. Both affinity gels gave good results with lactate dehydrogenase from rabbit muscle.

Blue Sepharose CL-6B adsorbed the enzyme from phosphate buffer but not from solutions containing 0.5 mM-Fru(1,6) P_2 , and about ten times as much enzyme was adsorbed at pH 5.5 as at pH 7.0. Enzyme at pH 5.5 remained bound when adsorbed proteins were eluted with a high phosphate concentration. At pH 7.0 the enzyme leaked off slowly in 50 mM-phosphate and more rapidly in 5 mM-phosphate, but the yield was low. NADH added to the buffer effected sharp elution of the enzyme in high yield, indicating that it interacted with the bound dye through NADH-binding sites, as do mammalian lactate dehydrogenases (Thompson *et al.*, 1975). However, the interaction occurred only in the presence of phosphate, when the enzyme was not catalytically active, and not in the presence of Fru(1,6) P_2 , when the enzyme was catalytically active and must then have possessed binding sites for NADH as substrate.

Table 2. Amino acid composition of lactate dehydrogenases

(a) Present work, mean of three determinations; (b) calculated from values based on subunit M_r 70000 (Jago *et al.*, 1971); (c) calculated from values based on M_r 140000 for the native enzyme (Pesce *et al.*, 1967). Values are for (a) subunit M_r 36000 or (b,c) subunit M_r 35000 and are rounded off to the nearest integer.

Amino acid	Enzyme source . . .	Composition (residues/subunit)		
		<i>Streptococcus uberis</i> (a)	<i>Streptococcus curvatus</i> (b)	Rabbit muscle (c)
Asp		36	36	30
Thr		14	16	12
Ser		18	18	21
Glu		38	36	30
Pro		12	9	10
Gly		26	24	27
Ala		40	37	22
Cys		2	1	4
Val		31	33	34
Met		1	6	9
Ile		20	19	21
Leu		24	25	35
Tyr		10	10	7
Phe		14	13	7
His		6	7	10
Lys		22	22	26
Arg		10	10	9
Trp		6	2	6
Total residues		330	324	320

The results of a typical enzyme purification are shown in Table 1. The specific activity of the product is similar to that of Fru(1,6) P_2 -dependent lactate dehydrogenases isolated from *S. cremoris* (Dyner *et al.*, 1972) and *S. lactis* (Crow & Pritchard, 1977) by different procedures.

Homogeneity of the purified enzyme

After being run on polyacrylamide-electrophoresis gels, in both the absence and presence of SDS, the enzyme gave a single band when stained for protein. A single band, coincident with the protein band, was also obtained when gels run in the absence of SDS were stained for lactate dehydrogenase activity. The sedimentation behaviour of the enzyme (see below) also indicated that it was homogeneous.

Amino acid composition

The amino acid composition of the enzyme is given in Table 2, together with those of the Fru(1,6) P_2 -dependent enzyme from *S. curvatus* and the rabbit muscle enzyme for comparison. The two streptococcal enzymes differ mainly in their methionine and tryptophan contents. They differ from the rabbit muscle enzyme more than they do from each other.

Specific absorption coefficient

Assuming a displacement of 4.1 fringes for a solution containing 1 mg of protein/ml (Babul & Stellwagen, 1969), absorbance measurements on solutions of the enzyme, correlated with fringe displacements in the ultracentrifuge, gave $A_{280,1\text{ cm}}^{1\%}$ 6.45.

Catalytic activity of the purified enzyme

No catalytic activity was detected when Fru(1,6) P_2 was omitted from the standard assay mixture, or when it was replaced by fructose, fructose 1-phosphate, fructose 6-phosphate, glucose 1,6-bisphosphate (all 5 mM) or phosphate (50 mM). The enzyme was active in the presence of Fru(1,6) P_2 both in pyruvate oxidation and lactate reduction, but the K_m values for pyruvate and NADH (1.75 mM and 24 μ M respectively) were much lower than those for lactate and NAD⁺ (109 mM and 230 μ M respectively) (Williams, 1981). Pyruvate concentrations above 5 mM were inhibitory, and no activity was detected when NADPH was substituted for NADH. The effect of pH on activity was determined with the standard assay mixture containing as buffer either 50 mM-Tris/HCl or 50 mM-sodium acetate, adjusted to different pH values. The enzyme had activity maxima at pH 5.5 and pH 7.0, with 1.4 times the activity at pH 5.5 as at pH 7.0, and was inactive at pH values below 4.3 and above 8.0. The enzyme in solution was less stable stored at pH 5.5 than at pH 7.0. The addition of EDTA or Mn²⁺ (0.5 mM or 50 mM of either) had no effect on its activity at either pH 5.5 or pH 7.0, indicating that it did not have a cation requirement similar to that of Fru(1,6) P_2 -dependent lactate dehydrogenases from *S. faecalis* (Crow & Pritchard, 1977) or *Lactobacillus* species (Hensel *et al.*, 1977).

Effect of phosphate concentration and temperature on enzyme specific activity

Enzyme in buffers containing at least 30 mM-phosphate showed little or no change in specific activity when diluted

Fig. 1. Effects of changing the conditions on the specific activity of Fru(1,6) P_2 -dependent lactate dehydrogenase from *Streptococcus uberis* in buffers containing less than 30 mM-phosphate

(a) Enzyme was incubated in 7.5 mM-phosphate buffer, pH 7.0 (●). At zero time, portions were diluted either 10-fold (▲) or 100-fold (○) with the same buffer, and the activity of the diluted enzyme measured at intervals with the standard assay. At A, 10-fold diluted enzyme was diluted a further 10-fold with the same buffer, and the activity measured at intervals (△). At B and C respectively, 10 mM-Fru(1,6) P_2 or 50 mM-phosphate (final concn.) was added to 10-fold diluted enzyme, and the activity measured at intervals (■, □). The experiments and the activity measurements were all performed at 25 °C. (b) Enzyme was incubated in 5 mM-phosphate buffer, pH 7.0, at 25 °C (●). At D, a portion of the solution was cooled at 0 °C, and its activity measured at intervals (▲) at 25 °C. At E, a portion of the cold solution was warmed to 25 °C and its activity measured at intervals (△), again at 25 °C. In both (a) and (b) the initial enzyme concentration was about 50 nM, and the enzyme solutions contained 1 mg of bovine serum albumin/ml and 1 mM-2-mercaptoethanol. The points are means of two determinations.

with similar buffers, and gave linear reaction rates when added to the standard assay mixture. By contrast, enzyme equilibrated in buffers containing less than 30 mM-phosphate had lower specific activities than did enzyme in the more concentrated buffers, and when diluted with similar buffers it slowly lost more activity until a constant specific activity was reached (Fig. 1a). Activity lost on dilution was slowly, but not completely, restored when the enzyme was incubated with a higher phosphate concentration or with Fru(1,6) P_2 . When the concentration of diluted enzyme was held constant and the phosphate concentration varied, graphs of residual activity against phosphate concentration were sigmoidal, the more so the more dilute the enzyme. Analyses with the Hill equation

Fig. 2. Sedimentation behaviour of Fru(1,6) P_2 -dependent lactate dehydrogenase from *Streptococcus uberis* in buffers containing $^1\text{H}_2\text{O}$ and $^2\text{H}_2\text{O}$

The graphs show the distribution of protein at sedimentation equilibrium in 50 mM-phosphate buffer, pH 7.0, containing 0.5 mM-Fru(1,6) P_2 and 0.1 M-KCl, dissolved in either (○) $^1\text{H}_2\text{O}$ or (●) $^2\text{H}_2\text{O}$. The initial enzyme concentration was 0.8 mg/ml. Further details are given in the text.

Fig. 3. Dissociation and reassociation of Fru(1,6) P_2 -dependent lactate dehydrogenase from *Streptococcus uberis*

The open symbols show the relationship between M_w and protein concentration at sedimentation equilibrium in the ultracentrifuge for the ligand-free enzyme. The initial protein concentrations were (mg/ml): ○, 1.28; △, 0.64; □, 0.32. The closed symbols show the same relationship for ligand-free enzyme (initial concentration 0.4 mg/ml) with added ligands: ●, 5 μM -Fru(1,6) P_2 ; ▲, 50 μM -NADH; ■, 5 mM-phosphate. Duplicate experiments gave similar results. Further details are given in the text.

showed that curves obtained with 10 mM- and 1 mM-enzyme (final concentrations) were fitted by K_d values for phosphate of 80 ± 20 mM and 21 ± 9 mM and Hill coefficients (h) of 2.9 ± 0.1 and 4.9 ± 0.2 respectively. The labile nature of the active site on unliganded enzyme may account, at least in part, for the nature of these curves and the difficulty in their interpretation.

The specific activity of enzyme in buffers containing less than 30 mM-phosphate decreased when the solution was cooled, even though all the enzyme assays were performed at 25 °C, but the original activity was restored when the enzyme solution was returned to its initial temperature (Fig. 1b).

Sedimentation behaviour

M_r of the undissociated enzyme. When the enzyme was dissolved in $^1\text{H}_2\text{O}$ or $^2\text{H}_2\text{O}$, in the presence of phosphate and Fru(1,6) P_2 , plots of $\ln$ (fringe displacement) against r^2 at sedimentation equilibrium were linear over the whole range of measurement (Fig. 2). The values obtained for M_r and partial specific volume from the slopes of the graphs were 144 000 and $0.749 \text{ cm}^3/\text{g}$ respectively. Partial specific volume calculated from the amino acid composition (Table 2), as described by Schachman (1957), was $0.747 \text{ cm}^3/\text{g}$. Runs in phosphate buffer containing 0.1 M-KCl, at initial protein concentrations ranging from 0.25 to 1.0 mg/ml, also indicated homogeneity and a similar M_r . The combined results gave M_r $144\,000 \pm 2\,000$, and M_n at the meniscus was not significantly different.

Subunit M_r . Values of M_r for the denatured enzyme were obtained with enzyme (0.5–1.0 mg/ml) dissolved in 6 M-guanidinium chloride containing 10 mM-2-mercaptoethanol. Plots of $\ln$ (fringe displacement) against r^2 were linear at sedimentation equilibrium and gave M_r $35\,900 \pm 500$ when a partial specific volume of $0.737 \text{ cm}^3/\text{g}$, obtained as described by Lee & Timasheff

(1974) from the amino acid composition, was used in the calculations.

Since the undissociated enzyme had M_r 144 000, it evidently consisted of four similar or identical subunits, as do most lactate dehydrogenases.

Sedimentation behaviour of ligand-free enzyme and the effect of adding ligands. Since the effect of phosphate concentration on activity indicated that the enzyme dissociated at low phosphate concentrations, the dissociation of ligand-free enzyme was investigated. Ligand-free enzyme was prepared by dialysis against 50 mM-Tris/HCl buffer, pH 7.0, containing 0.1 M-KCl, and solutions containing three different concentrations of enzyme were centrifuged simultaneously (Hoagland & Teller, 1969). At sedimentation equilibrium, plots of $\ln$ (fringe displacement) against r^2 were curved upwards. Plots of M_w at points on the curves against protein concentration for the three different initial protein concentrations were nearly superimposable (Fig. 3), indicating that dissociation of the enzyme was in reversible equilibrium. The M_w values showed that, even in dilute solution, the enzyme was only partly dissociated, but the value for M_n at the meniscus ($60\,000 \pm 3\,000$; mean of six determinations) indicated that some monomer was present. By contrast, dissociation of the ligand-free enzyme from *S. cremoris* was irreversible, and only resulted in dimer formation (Jago *et al.*, 1971).

The results of adding ligands to the dialysed enzyme are shown in Table 3 and Fig. 3. Only tetramer was detected after Fru(1,6) P_2 was added, except when the Fru(1,6) P_2 concentration ($5 \mu\text{M}$) was less than the enzyme subunit concentration ($11 \mu\text{M}$), and even in this case dissociation was observed only in the meniscus region where the protein concentration was about $1 \mu\text{g}/\text{ml}$. The result suggests that there were strong interactions between liganded and unliganded monomers, or cooperativity between them in tetramer formation. Addition of phosphate or NADH also resulted in tetramer

Table 3. Effect of ligand concentration on the M_r and stability of Fru(1,6) P_2 -dependent lactate dehydrogenase from *Streptococcus uberis*

The experiments were performed at 20 °C. The results are means of two determinations, and deviations from the means were < 5% for M_r , M_w and M_n , and < 10% for recovery of activity. (a) Ligands were added to ligand-free enzyme in 50 mM-Tris/HCl buffer, pH 7.0, and the solutions equilibrated by dialysis. (b) Enzyme precipitated from phosphate buffer with $(\text{NH}_4)_2\text{SO}_4$ was dissolved in the buffer/ligand solution, pH 7.0, used to equilibrate the column. (c) The result is shown in Fig. 3. Further details are given in the text.

Ligand	Ligand concn. (mM)	Sedimentation analysis (a)		Analytical gel filtration (b)	
		$10^{-3} \times M_w$ at protein concns. > 90 $\mu\text{g}/\text{ml}$	$10^{-3} \times M_n$ at meniscus	$10^{-3} \times$ apparent M_r	Recovery of activity (%)
Fru(1,6) P_2	0.005	144(c)	105	—	—
	0.05	144	145	—	0
	0.1	—	—	135	18
	0.2	—	—	135	50
	0.5	146	144	135	105
Phosphate	5.0	136–141(c)	73	115	6
	20	—	—	126	80
	50	141	86	135	100
NADH	0.05	147(c)	83	135	17
Phosphate + Fru(1,6) P_2	5.0 } 0.02 }	—	—	135	62
Phosphate + NADH	5.0 } 0.002 }	—	—	135	78

formation, but even in 50 mM-phosphate some dissociation was detected in the meniscus region.

Gel-filtration behaviour and ligand binding

Enzyme dissociation and loss of activity at various ligand concentrations was investigated by analytical gel filtration. Since the dissociation was reversible, the dissociating enzyme moved through the column more slowly than the tetramer, as a single band containing the equilibrium mixture of tetramer and dissociated forms (Andrews, 1964). The mean concentration of enzyme eluted from the column was about 3 µg/ml, a value comparable with that in the meniscus region in the ultracentrifuge. Activity measurements on the effluent fractions were used to estimate the amount of active enzyme present. The results are shown in Table 3.

The tetramer had an apparent M_r of 135000 and, as in the ultracentrifuge, the enzyme existed in this form in the presence of Fru(1,6) P_2 , phosphate or NADH if the ligand concentration was high enough. When phosphate and either Fru(1,6) P_2 or NADH were present together, at concentrations too low to prevent dissociation and loss of most or all of the activity when they were present separately, tetramer formation and the recovery of activity were greatly enhanced.

When the ligands were present singly, the results obtained in the presence of Fru(1,6) P_2 or NADH were different from those obtained with the enzyme in phosphate, in that in some concentrations of Fru(1,6) P_2 or NADH the enzyme lost much of its activity but remained in tetrameric form, whereas in phosphate the loss of activity and dissociation occurred together. The results with Fru(1,6) P_2 and NADH may be explained if the enzyme possesses two classes of binding sites for each ligand, and ligand bound with higher affinity maintains the enzyme in tetrameric form whereas that bound with lower affinity protects the catalytic sites. Two classes of binding site for NADH also explain the behaviour of the enzyme in chromatography on Blue Sepharose, with the higher-affinity sites rather than the lower-affinity substrate-binding sites being involved in the retention of the enzyme on the gel. The higher-affinity sites appeared to be accessible to the matrix-bound dye when the enzyme was in tetrameric form in phosphate, but not when it was in tetrameric form in Fru(1,6) P_2 or dissociated. Although the gel-filtration results indicated a single class of binding site for phosphate, phosphate might bind at the same two classes of site as does Fru(1,6) P_2 , because it both inhibited the catalytic activity (Garvie & Bramley, 1979) and stabilized the tetramer.

Conclusions

Since unliganded forms of the enzyme readily undergo irreversible inactivation, whether existing as tetramer or as subunits in association-dissociation equilibrium, such forms seem unsuitable to act in a mechanism to control activity. In any case, little if any of the enzyme may occur in unliganded forms *in vivo*. In *S. lactis*, the intracellular concentrations of Fru(1,6) P_2 were 10 mM and 25 mM respectively in cells converting 1 and 95% of glucose to lactose (Thomas *et al.*, 1979), and in *S. faecalis* the intracellular phosphate concentrations were of

similar magnitude (Harold & Spitz, 1975). With the *S. uberis* enzyme, synergism between different ligands in stabilizing the tetramer and preventing irreversible inactivation indicated that comparatively low metabolite concentrations can maintain it in forms which would have full catalytic activity when the Fru(1,6) P_2 concentration was high enough. However, if a variety of phosphate-containing metabolites inhibit the *S. uberis* enzyme as they do the *S. mutans* enzyme (Yamada & Carlsson, 1975), and metabolites bind at more than one class of site with synergistic action, control of the catalytic activity is a complex process.

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