BBA 66430

PHYSICOCHEMICAL STUDIES ON THE LACTATE DEHYDROGENASE OF *STREPTOCOCCUS CREMORIS* US3 THE EFFECTS OF MODIFIERS

G R JAGO*, L W NICHOL**, K O'DEA AND W H SAWYER

*Russell Grimwade School of Biochemistry, University of Melbourne, Parkville, Victoria 3052 (Australia), and *CSIRO Division of Food Research, Dairy Research Laboratory, Highett, Victoria 3190 (Australia)*

(Received May 17th, 1971)

SUMMARY

A purified preparation of the lactate dehydrogenase (L-lactate NAD^+ oxidoreductase, EC 1 1 1 27) from *Streptococcus cremoris*, Strain US3, is shown to have an amino acid composition and physical properties similar to those previously reported for a lactate dehydrogenase from *Bacillus subtilis* In the pH range 5 0–7 0 and at pH 8 0 in phosphate buffer, the *S cremoris* enzyme is characterized by a mol wt of 140 000 and frictional ratio 1 26 In contrast, at pH $\geqslant$ 7 8 in triethanolamine–HCl buffer, the enzyme undergoes a relatively slow unfolding and dissociation resulting in complete conversion at pH 9 0 to an enzymically inactive form of mol wt 70 000 and frictional ratio 1 80 Sedimentation velocity experiments at pH 8 0 in triethanolamine–HCl buffer show that the substrate NADH and the activator Fru-1,6-P_2 partially stabilize the 140 000 form of the enzyme

Further examination of the size and shape properties of the enzyme at low total concentrations was made by the method of frontal analysis in Sephadex chromatography It was found that in phosphate buffer an increase of pH in the range 6 0–8 0 (or the addition of NADH or Fru-1,6-P_2 at the fixed pH 7 0) decreased the observed elution volume, while the addition of the inhibitor ATP increased it These results are correlated with initial velocity studies to conclude that the modifiers, ATP and Fru-1,6-P_2, induce conformational changes in the structure of the enzyme, the unfolded form being more active

INTRODUCTION

The lactate dehydrogenases (L-lactate NAD^+ oxidoreductase, EC 1 1 1 27) of several species of the genus Streptococcus have been shown to be markedly activated by the glycolytic intermediate, fructose 1,6-diphosphate (Fru-1,6-P_2)[1–3] This is in contrast to mammalian lactate dehydrogenases and to that of many other bacterial species The lactate dehydrogenase of the Group N *Streptococcus cremoris* US3 was

** Present address Department of Physical Biochemistry, The John Curtin School of Medical Research, The Australian National University, Canberra, A C T , Australia

found[4] to have, in the absence of Fru-1,6-P_2, a pH optimum of 8.0 which is outside the range of pH (5–7) where growth of this group of organisms is possible. However, in the presence of Fru-1,6-P_2, there was not only a marked activation of the enzyme but also a change in the pH optimum to a broad plateau of activity between pH 5.0 and 7.0. It has also been observed[2,4] with a partially purified extract of the enzyme that ATP operates as an inhibitor.

It has been suggested that Fru-1,6-P_2 may function as a modifier by inducing conformational changes in the enzyme facilitating substrate and co-enzyme binding[3]. On the other hand, polymerization of the H_4 isoenzyme of beef heart lactate dehydrogenase has been implicated in the explanation of allosteric effects observed in kinetic studies performed with this enzyme[5] and it is possible that modifiers may operate by perturbing such polymerization equilibria[6]. In an attempt to elucidate the mode of action of the modifiers, ATP and Fru-1,6-P_2, on *S. Cremoris* lactate dehydrogenase, this work presents results pertaining to the size and shape properties of a purified form of the enzyme found in the presence and absence of the modifiers.

MATERIALS AND METHODS

Purification of the enzyme

S. cremoris Strain US3 was grown in 5-l batches of broth containing tryptone, 30 g, yeast extract, 10 g, lactose, 30 g, KH_2PO_4, 5 g, beef extract, 2 g and water to 1 l. The broth was incubated at 30° for 16 h, the pH being maintained at 6.3 by the addition of 10 M NaOH supplied through a magnetic valve connected to a Radiometer Titrator, Model TTTA3. Cell-free extracts were prepared by extruding cells, previously washed in 0.9% NaCl and resuspended in 0.01 M sodium phosphate buffer (pH 7.0) (1 g wet wt. per 4 ml buffer), through a French pressure cell[7] at 10 tons/inch^2 and were dialysed against the same phosphate buffer at 4°. The following purification steps were also carried out at 4°.

Nucleic acids were precipitated from the dialysed cell-free extract (approx. 250 ml) by the dropwise addition of streptomycin sulphate (15% solution, w/v) until no further precipitation occurred. The precipitate was removed by centrifugation and solid ammonium sulphate was added to the supernatant to give 40% saturation. The precipitate was removed as before and the ammonium sulphate concentration in the supernatant was increased to 60% saturation. The precipitate was collected and dissolved in 0.05 M sodium phosphate buffer (pH 7.0) and dialysed overnight against the same buffer. A 60-ml aliquot of the dialysed fraction was applied to a DEAE-cellulose (Whatman DE-11) column (120 cm × 1.3 cm) equilibrated in 0.05 M sodium phosphate buffer (pH 7.0). The enzyme was eluted with a NaCl gradient (0–0.5 M) in the same buffer at a flow rate of 0.6 ml/min. Fractions of 15 ml were collected and examined for enzymic activity and protein concentration. Fractions (45–55) exhibiting the highest specific activity were pooled and concentrated in a Diaflow apparatus using a 10 000 mol. wt. cut-off membrane to a final volume of 10 ml. The concentrated enzyme solution was dialysed against 0.05 M sodium phosphate in 0.1 M NaCl (pH 7.0) and applied to a DEAE-Sephadex A-50 column (25 cm × 1.3 cm) previously equilibrated with 0.05 M sodium phosphate in 0.1 M NaCl (pH 7.0). The enzyme was eluted with a NaCl gradient (0.1–0.5 M) in the above buffer at a flow rate of 0.6 ml/min and 10-ml fractions were collected. Fractions 34 and 35 were pooled, concentrated in

TABLE I

STEPS INVOLVED IN THE PREPARATION OF LACTATE DEHYDROGENASE FROM CELL-FREE EXTRACTS OF *S cremoris* US3

Treatment	*Total activity (defined units)*	*Total protein (mg)*	*Total nucleic acids (mg)*	*Specific activity (units/mg)*	*Purification factor*
Cell-free extract	62 000	5630	2040	11	–
Streptomycin sulphate	60 500	3040	280	20	2
Ammonium sulphate (40–60%)	43 000	1840	55	27	2 5
DEAE-cellulose	46 000	128	0 4	180	16 4
DEAE-Sephadex	21 100	26 5	0	800	73

the Diaflow apparatus to 5 ml, dialysed against 0 05 M sodium phosphate buffer (pH 7 0) and further fractionated by ammonium sulphate according to the method of JAKOBY[8] The staining of polyacrylamide disc electrophoresis gels for protein and enzymic activity[9,10] indicated that the 35–40% ammonium sulphate fraction alone exhibited a single protein band corresponding to a single activity band This fraction had a specific activity of 1000–1200 units/mg and was used in all subsequent experiments

A summary of the initial purification steps with measures of their relative efficiency is given in Table I, wherein only the parameter, specific activity, requires further comment Lactate dehydrogenase activity was estimated by measuring the initial rate of oxidation of NADH at 340 nm from traces obtained using a Zeiss spectrophotometer, Model PMQ11, coupled to a Rikadenki recorder, Model B14 The reaction mixture (total volume 3 ml) contained 250 μmoles of triethanolamine–HCl buffer (pH 8 0), 0 4 μmole of NADH, 20 μmoles of sodium pyruvate and 0 1 ml of enzyme solution A change in absorbance at 340 nm of 0 1 absorbance unit per min was defined as 1 unit of lactate dehydrogenase activity The specific activity was defined as units of lactate dehydrogenase activity per mg of protein Estimates of protein concentration were calculated from the absorbance measurements at 280 nm and 260 nm, employing the nomogram devised by Adams (California Corp for Biochemical Research, Los Angeles)

Initial velocity studies

Samples of purified lactate dehydrogenase were stored in sodium phosphate buffer (pH 7 0, $I = 0$ 1) containing 35% ammonium sulphate In this environment it was shown that lactate dehydrogenase activity was fully maintained for at least 21 days In preparation for a series of kinetic experiments, the ammonium sulphate was removed by dialysis at 0–4° against sodium phosphate buffer (pH 7 0, $I = 0$ 1) and the enzyme was diluted with the same buffer to give a concentration suitable for addition to the assay mixture Kinetic experiments were completed within 2 h following dilution Relative values of enzyme concentrations were determined spectrophotometrically at 280 nm, assuming a specific extinction coefficient ($E^{1\%}_{280\ nm}$) of 10 The diluted enzyme solution (0 1 ml) was added to 2 9 ml of assay mixture contained in a cuvette, thermostated at 30° In most experiments the components of the assay mixture were dissolved in triethanolamine–HCl buffer (pH 8 0, final concentration

0 08 M) and the pH of all mixtures was confirmed to be 8 0 immediately after the completion of the experiment (duration approx 1 min) other buffered media employed are specified in the text The initial velocity of the reaction, v, was determined as described above In all studies pyruvate and NADH were used as initial substrates

Isoelectric point

Horizontal slab polyacrylamide gel electrophoresis was used to estimate the isoelectric point of the enzyme and was carried out according to the method of CARNEGIE *et al* [11] using 5% gels and buffers of $I = 0\ 1$ The voltage was chosen to give a current through each gel of 40–45 mA and the temperature was maintained at $-5°$ to prevent overheating In each experiment, performed in duplicate, the sample was introduced at the origin in the same buffer used to equilibrate the gel At the conclusion of the experiments (duration 2 h), the gels were stained with Amido black 10B for protein

Amino acid composition

Samples of purified enzyme were treated by two different procedures prior to acid hydrolysis and subsequent amino acid analysis, performed by the method of SPACKMAN[12] using a Beckman 120B amino acid analyser First, 2 mg of protein was treated at 30° for 90 min with a 40-fold molar excess of iodoacetic acid in Tris buffer (pH 8 3) containing 8 M urea A 5-fold molar excess of mercaptoethanol was added and the sample, a carboxymethyl derivative, was dialysed against 1 mM HCl for 16 h Samples of about 200 μg were then individually freeze–dried in preparation for acid hydrolysis Secondly, duplicate samples (approx 200 μg) were dialysed against distilled water, freeze–dried and oxidized with performic acid (0 4 ml) for 4 h at 4° These oxidized samples were freeze–dried Samples of S-carboxymethylated enzyme were hydrolysed in evacuated sealed tubes in duplicate for 24, 48 and 72 h at 110° in 6 M HCl containing 1% phenol Duplicate samples of the oxidized form were hydrolysed in the same environment for 24 h Tryptophan, in the unhydrolysed protein, was determined spectrophotometrically[13]

Ultracentrifuge studies

Sedimentation velocity experiments were performed in a Spinco Model E ultracentrifuge at 59 780 rev /min employing a schlieren optical system with bar angle fixed at 70° Solutions of lactate dehydrogenase were dialysed in the cold for at least two days against buffers specified in the text and then equilibrated for a further 2 h at the temperature selected for each experiment In experiments performed at pH 8 0 in triethanolamine–HCl buffer in the presence of added modifiers or substrates, the same dialysis and temperature equilibration procedures were used Thus, sufficient time was allowed to permit a comparison between the extent of structural transitions observed in this medium in the presence and absence of modifiers or substrates Values of the weight-average sedimentation coefficient, $\bar{s}_{20,w}$, were calculated from the rate of movement of the square root of the second moment of refractive index gradient curves[14], corrected to 20° in water[15] Corresponding concentrations were estimated refractometrically Values of sedimentation coefficients reported as $s_{20,w}$ were computed from the rate of movement of maximum ordinates of schlieren peaks

The Archibald procedure as outlined by KLAINER AND KEGELES[16] was used to evaluate weight-average molecular weights from the observed refractive index gradient near the meniscus. Measurement of all schlieren patterns was made with a two-dimensional comparator (Nikon Shadowgraph, model 6C).

Optical rotatory dispersion

ORD studies were carried out with dialysed enzyme samples using a Jasco ORD/UV-5 instrument and a quartz cell (thermostated at 25 ± 1°) of 2 mm path length. Recordings were made in the range 190–260 nm and all spectra were corrected for base-line shifts of pure buffers. Protein concentrations were determined at the completion of ORD studies by the method of LOWRY *et al.*[17].

Frontal analysis on Sephadex G-100

A column of Sephadex G-100 (1.4 cm × 21 cm) was equilibrated at 4° and solvent (at least two bed volumes) containing the added substrate or modifier, where appropriate, was run through the column prior to the addition of approx. 30-ml samples of enzyme in the same environment. The enzyme solutions were dialysed for at least 2 days prior to their addition to the column. Fractions (approx. 0.5 ml) were collected in previously tared tubes, containing 0.2 ml of 20 mM Fru-1,6-P_2 to activate the enzyme. Each tube was monitored for enzymic activity and volumes were determined accurately by weighing individual tubes. The void volume of a particular column was determined using blue dextran before and after each experiment and was shown to be constant.

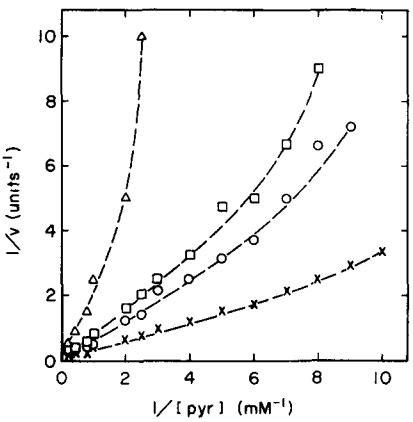
RESULTS

Initial velocity studies

Double reciprocal plots illustrating the effect of ATP on the kinetics of the reaction catalysed by *S. cremoris* lactate dehydrogenase in triethanolamine–HCl buffer (pH 8.0) are shown in Fig. 1. It is apparent that ATP functions as an inhibitor, in agreement with previous findings[2,4]. In addition, the slight upward curvature of the results found in the absence of ATP is markedly accentuated by its addition, the effect systematically becoming more pronounced as the ATP concentration increases. A similar but less pronounced inhibitory effect of ATP was found in sodium phosphate buffer ($I = 0.1$) at the same pH value of 8.0.

The effect of varying enzyme concentration at pH 8.0 in triethanolamine–HCl buffer is shown in Fig. 2, where a logarithmic scale on both axes is used to permit representation of all results on one graph. In the absence of ATP, the specific activity is evidently independent of protein concentration over a 30-fold range. Similarly, no concentration dependence of specific activity was found in additional experiments performed in the absence of ATP at pH values of 7.0, 6.5 and 6.0 employing 0.08 M triethanolamine–acetic acid buffers, although a progressive decrease of specific activity with decreasing pH was observed. In contrast, it is clear from Fig. 2 that at pH 8.0 in the presence of 0.2 mM ATP, a marked concentration-dependence is observed, which is accentuated on increasing the ATP concentration to 0.5 mM.

The effects of Fru-1,6-P_2 as a modifier are illustrated in Fig. 3. The tendency for upward curvature of the plot found in the control experiment in the absence of



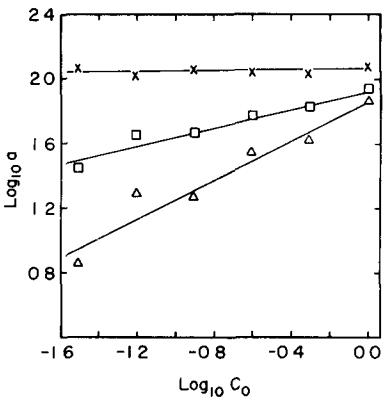
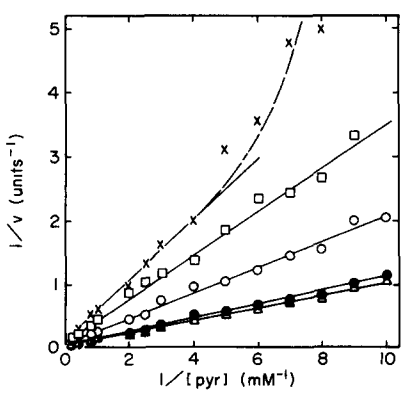


Fig 1 Double reciprocal plots illustrating the inhibitory effect of ATP on the initial velocity of the reaction catalysed by *S cremoris* lactate dehydrogenase The 3 0 ml assay mixture contained 0 08 M triethanolamine–HCl buffer (pH 8 0), 0 03 mg enzyme, 0 133 mM NADH, pyruvate concentration as indicated on the abscissa and the following concentrations of ATP ×, 0 mM ○, 0 1 mM □, 0 2 mM and △, 0 4 mM

Fig 2 The dependence of the initial velocity of the reaction catalysed by *S cremoris* lactate dehydrogenase on the concentration of the enzyme in the presence and absence of ATP, plotted as the logarithm of specific activity (*a* units/mg) *vs* the logarithm of the initial enzyme concentration (c_0 mg/ml) The 3 0 ml assay mixture contained 0 08 M triethanolamine–HCl buffer (pH 8 0), 1 mM pyruvate, 0 133 mM NADH and the following concentrations of ATP ×, 0 mM □ 0 2 mM and △, 0 5 mM



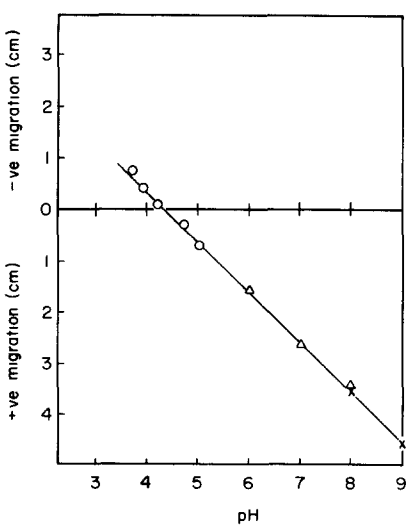


Fig 3 Double reciprocal plots illustrating the activating effect of Fru-1,6-P_2 on the initial velocity of the reaction catalysed by *S cremoris* lactate dehydrogenase The 3 0 ml assay mixture contained 0 08 M triethanolamine–HCl buffer (pH 8 0), 0 015 mg enzyme, 0 133 mM NADH, pyruvate concentration as indicated on the abscissa and the following concentrations of Fru-1,6-P_2 ×, 0 mM □, 0 001 mM ○, 0 002 mM ●, 0 01 mM and △, 0 02 mM

Fig 4 Relative mobilities as a function of pH observed in the polyacrylamide gel electrophoresis of purified samples of *S cremoris* lactate dehydrogenase in buffer of $I = 0\ 1$ at -5° ○ acetate buffers, △, phosphate buffers, ×, triethanolamine–HCl buffers

TABLE II

AMINO ACID COMPOSITION OF *S cremoris* LACTATE DEHYDROGENASE

The values for each hydrolysis, of duration specified in parentheses, are the average of two determinations and are calculated for 70 000 g of protein In the last column values in parentheses are average values with the following exceptions threonine, serine, valine and isoleucine were estimated by standard extrapolation procedures, half-cystine and methionine were estimated as cysteic acid and methionine sulphone after oxidation with performic acid, tryptophan was estimated spectrophotometrically

Residue	*Oxidized protein (24 h)*	*Carboxymethylated protein*			*Residues per monomer unit, mol wt 70 000*
		24 h	*48 h*	*72 h*	
Lysine		46 6	40 4	41 4	43 (43 0)
Histidine		13 7	13 2	13 4	13 (13 4)
Arginine		21 7	19 2	19 8	20 (20 3)
Aspartic acid	72 5	73 6	72 2	70 0	72 (71 9)
Threonine	29 7	29 3	27 5	26 3	31 (31 4)
Serine	35 2	33 7	31 2	29 0	37 (36 7)
Glutamic acid	77 1	73 1	72 4	70 7	72 (72 1)
Proline		20 3	17 4	18 3	19 (18 7)
Glycine	48 1	48 1	47 3	49 8	48 (48 0)
Alanine	80 0	74 5	73 3	72 3	73 (73 4)
Half-cystine	2 8				3 (2 8)
Valine		53 3	58 8	60 5	66 (65 5)
Methionine	11 0				11 (11 0)
Isoleucine	29 2	31 0	33 7	35 9	38 (37 9)
Leucine	49 2	50 0	50 7	50 1	50 (50 2)
Tyrosine		19 1	18 6	19 4	19 (19 0)
Phenylalanine	22 7	25 6	26 1	26 3	26 (26 0)
Tryptophan					5 (4 7)

modifier is eliminated even at low concentrations of Fru-1,6-P_2 which clearly functions as an activator A similar but less pronounced activating effect of Fru-1,6-P_2 was found in a series of experiments conducted in sodium phosphate buffer (pH 8 0, $I = 0\ 1$)

Isoelectric point and amino acid composition of lactate dehydrogenase

The distance of migration of the major protein band in each polyacrylamide gel electrophoresis experiment, performed under standard conditions, is plotted as a function of pH in Fig 4 Only a single band was observed in each experiment, except in those conducted at pH 8 0 and 9 0 in triethanolamine–HCl buffers where a minor component moving with a slightly lower mobility was also observed Although no detailed interpretation of the ordinate values of Fig 4 is possible in terms of net charge, this series of comparative experiments permits estimation of the isoelectric point of the protein as pH 4 3 in acetate buffer, $I = 0\ 1$

The results of the amino acid analyses are summarized in Table II They were used to estimate the mean residue weight (109) of the constituent amino acids and an apparent specific volume of 0 74 for the enzyme

Ultracentrifuge studies

Schlieren patterns obtained on subjecting dialysed solutions of lactate dehydrogenase to sedimentation velocity are shown in Fig 5 The use of a cell containing a

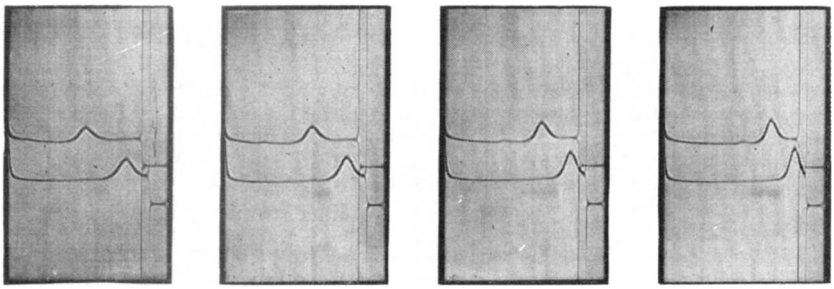


Fig 5 Sedimentation velocity patterns obtained with solutions of *S. cremoris* lactate dehydrogenase at 19°. Sedimentation is from right to left. The upper patterns refer to a 0.26% enzyme solution in phosphate buffer (pH 7.0, $I = 0.1$) and the lower patterns to a 0.32% enzyme solution in triethanolamine-HCl buffer (pH 9.0, $I = 0.01$)

wedge-window permits direct comparison of the behaviour at pH 7.0 (upper pattern) and at pH 9.0 (lower pattern). The result obtained at pH 7.0 was consistently observed with four different preparations of the enzyme and was typical of results obtained at pH 5.0 in acetate buffer and in phosphate buffers of pH 6.0 and 8.0. It reveals a major peak sedimenting with an $\bar{s}_{20,w}$ value of 7.1 S and a small proportion of a 14-S component. In contrast, at pH 9.0 in triethanolamine–HCl buffer, the amount of 14-S component is reduced and the $\bar{s}_{20,w}$ value of the major peak is 3.0 S.

Sedimentation velocity results obtained in other environments are summarized in Table III, together with mol. wt. values obtained at pH 7.0 and 9.0 by the Archi-

TABLE III

WEIGHT-AVERAGE SEDIMENTATION COEFFICIENTS AND MOLECULAR WEIGHTS OF *S. cremoris* LACTATE DEHYDROGENASE

Buffer composition	*Concn (M)*	*pH*	*Enzyme concn (%)*	*Temp. of run*	$\bar{s}_{20,w}$ *(S)*	*Mol. wt.*
NaCl	0.03					
Sodium acetate	0.07	5.0	0.14	19.2°	7.1$_3$	
Acetic acid	0.03					
NaH_2PO_4	0.07	6.0	0.36	20.0°	6.7$_1$	
Na_2HPO_4	0.01		0.32	5.3°	6.7$_3$	
NaH_2PO_4	0.018	7.0	0.39	19.5°	7.0$_8$	138 000 ± 7000
Na_2HPO_4	0.027		0.34	19.3°	6.9$_6$	
			0.27	20.5°	7.1$_5$	
			0.26	19.2°	7.0$_5$	
			0.21	19.5°	7.2$_1$	142 000 ± 8000
NaH_2PO_4	0.002	8.0	0.33	20.0°	6.6$_4$	
Na_2HPO_4	0.033		0.30	5.3°	6.8$_7$	
NaCl	0.09					
HCl	0.01	9.0	0.38	19.1°		71 000 ± 3000
Triethanolamine	0.10					
HCl	0.01	9.0	0.32	18.8	3.0	
Triethanolamine	0.10		0.25	16.9°	3.2	
			0.25	17.1°		69 000 ± 3000

TABLE IV

SEDIMENTATION VELOCITY RESULTS OBTAINED WITH *S cremoris* LACTATE DEHYDROGENASE

Buffer composition	*Concn (M)*	*pH*	*Enzyme concn (%)*	*Temp of run*	*1st peak*		*2nd peak*	
					$s_{20,w}$ (S)	%	$s_{20\,w}$ (S)	%
NaH_2PO_4	0 018	7 0	0 27	20 5°	–	0	$7\,1_5$	100
Na_2HPO_4	0 027							
NaCl	0 08							
HCl	0 02	7 8	0 29	19 2°	$4\,5_9$	55	$7\,3_2$	45
Triethanolamine	0 033							
NaCl	0 05							
HCl	0 05	8 0	0 32	19 5°	$4\,7_1$	57	$7\,2_0$	43
Triethanolamine	0 10		0 31	3 8°	$4\,5_0$	58	$7\,3_6$	42
NaCl	0 09							
HCl	0 01	8 3	0 27	19 2°	$4\,5_3$	67	$7\,2_5$	33
Triethanolamine	0 03							
HCl	0 01							
Triethanolamine	0 10	9 0	0 25	17 0°	$3\,2_0$	100	–	0

bald procedure It is evident that no pronounced or systematic variations of $\bar{s}_{20,w}$ values with concentration or with temperature were observed under the conditions specified Moreover, it is apparent that at pH 9 0 the protein exists in solution as a species of mol wt 70 000 half that of the form (140 000) existing at pH 7 0 It was found that the form existing at pH 9 0 exhibited no enzymic activity Moreover, attempts to dialyse a solution from pH 7 0 or 8 0 (phosphate) to pH 9 0 (triethanolamine–HCl) and back to pH 7 0 (phosphate) indicated that irreversible changes had occurred For example, a spread boundary with $\bar{s}_{20,w}$ of 5 3 S was found at the final pH of 7 0 after the reversal compared to an initial value in the same phosphate buffer of 7 1 S

The question arises whether the large decrease in $\bar{s}_{20,w}$ and mol wt values reported above is solely due to a pH change from 7 0 to 9 0 The results summarized in Table IV indicate the operation of a specific buffer effect in addition to a pH effect At pH values of 7 8, 8 0 and 8 3 in triethanolamine–HCl buffers, two incompletely resolved peaks were seen, the last four columns of Table IV giving estimates of their $s_{20,w}$ values and relative proportions It is clear that the relative proportion of 7-S material decreases systematically with increasing pH The result obtained at pH 8 0 (Table IV), where two peaks were evident, may be contrasted with that obtained at the same pH value in phosphate buffer (Table III) where only 7-S material was evident (except for about 3% of 14-S material) It appears that at pH 8 0 phosphate stabilizes the 7-S form of the enzyme and it was therefore of interest to examine the effects of added substrates and modifiers on the structural integrity of the enzyme at pH 8 0 in triethanolamine–HCl buffer Addition of 10 mM pyruvate in this environment did not alter within experimental error the relative proportion of the peaks reported in Table IV In contrast, there was a marked effect of dialysing the enzyme to pH 8 0 in the presence of 1 mM NADH, the result being shown in Fig 6a The relative

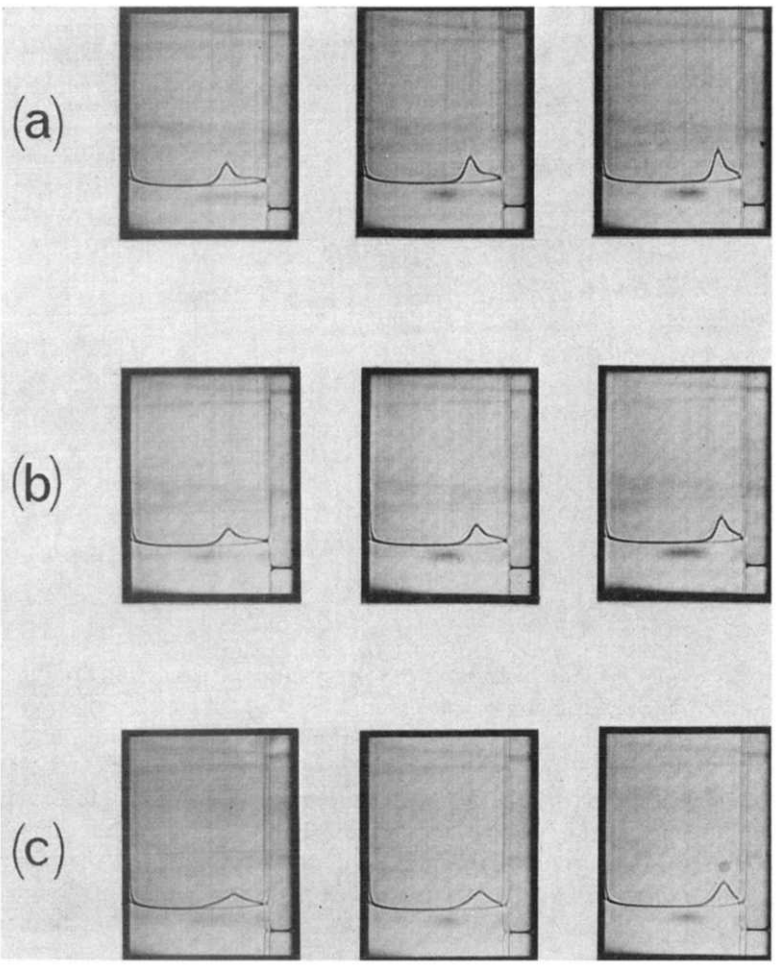


Fig 6 Sedimentation velocity patterns obtained with 0 4% solutions of *S cremoris* lactate dehydrogenase at 20° Sedimentation is from right to left In each case the solution had been dialysed against triethanolamine–HCl buffer (pH 8 0, $I = 0$ 1) containing (a) 1 mM NADH (b) 1 mM Fru-1,6-P_2 and (c) 1 mM ATP

proportion of 7-S material has increased from about 43% found after dialysis in the absence of NADH to about 80% in its presence The remaining 20% of the material evident in Fig 6(a) is characterized by an $\bar{s}_{20,w}$ of approx 4 S The inclusion of 1 mM Fru-1,6-P_2 gave an entirely similar result (Fig 6b) On the other hand, the inclusion of 1 mM ATP in the dialysis procedure gave the result shown in Fig 6(c), where a single spread boundary is apparent with $\bar{s}_{20,w}$ of 5 6 S It was also observed that these substrates and modifiers (studied separately) had no effect on the sedimentation behaviour of the enzyme at pH 7 0 in phosphate, $I = 0$ 1

Optical rotatory dispersion

The observed variation of the mean residue rotation, $[m']_\lambda$, with wavelength, λ, is shown in Figs 7 and 8 for the protein at pH 7 0 and 9 0, respectively Borate

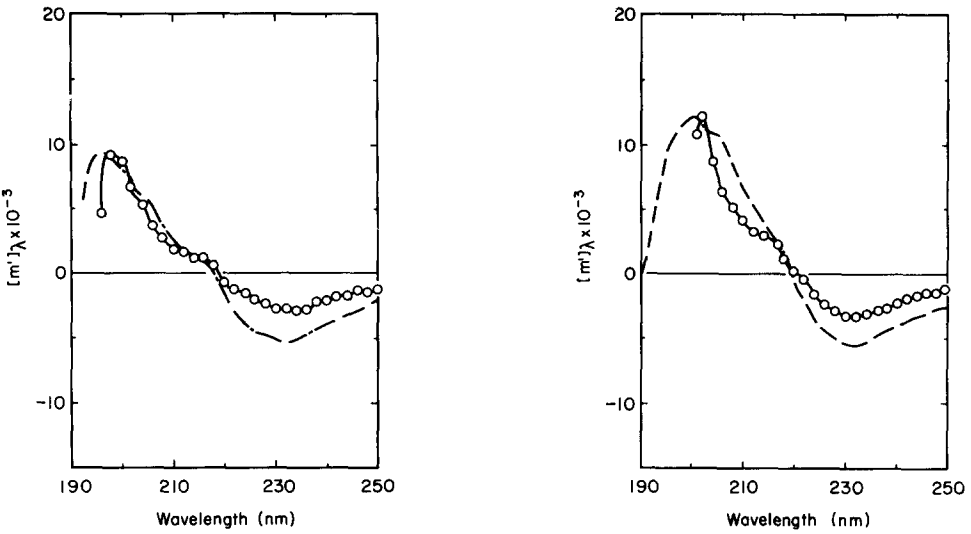


Fig 7 A comparison of ORD spectra obtained with *S cremoris* lactate dehydrogenase with a curve computed for poly-L-lysine ○—○, the spectrum obtained with the enzyme (0 017%) in phosphate buffer (pH 7 0) (0 018 M NaH_2PO_4, 0 027 M Na_2HPO_4) at 25° — —, the simulated spectrum calculated for poly-L-lysine containing 10% α-helix, 50% β structure and 40% random chain

Fig 8 The ORD spectrum of *S cremoris* lactate dehydrogenase (0 0138%) in borate buffer (pH 9 0) (0 005 M $Na_2B_4O_7$, 0 0069 M H_3BO_3) at 25° ○—○, experimental points, and — —, a curve calculated for poly-L-lysine containing 10% α-helix, 40% β structure and 50% random chain

buffer was used for the latter determination since it was found that triethanolamine–HCl buffer absorbed light strongly below 234 nm Estimates of the helical content based on previously derived relationships[18] were 17% at pH 7 0 and 13% at pH 9 0 (calculated from the height of the peak at 202 nm and 198 5 nm, respectively) The shift in the position of these maxima suggests a loss of β structure, a postulate supported by comparison of experimental curves in Figs 7 and 8 (———) with curves simulated[19] for poly-L-lysine (— —) The experimental results obtained at pH 7 0 fit reasonably closely the curve simulated for poly-L-lysine comprising 10% α-helix, 50% β structure and 40% random chain the experimental results obtained at pH 9 0 better fit the set, 10% α-helix, 40% β structure and 50% random chain Although it is not suggested that this type of comparison uniquely defines the conformation of the enzyme, it shows that a conformational change (probably involving a loss of both β structure and α-helical content) occurs on changing the pH from 7 0 to 9 0

Frontal analysis in Sephadex chromatography

A typical elution profile obtained with a solution of lactate dehydrogenase in phopshate buffer (pH 7 0) is shown in Fig 9, where also the median bisector (– – –) of the enzymic activity gradient on the advancing side is indicated The corresponding elution volume (V_e = 14 7 ml) may be used to calculate a distribution coefficient[20], K_{av}, equal to $(V_e - V_0)/(V_t - V_0)$ where V_0 is the void volume and V_e is the total volume of the column Table V summarizes K_{av} values found with a fixed enzyme concentration (0 038 mg/ml) as a function of pH and shows the effect on K_{av} values

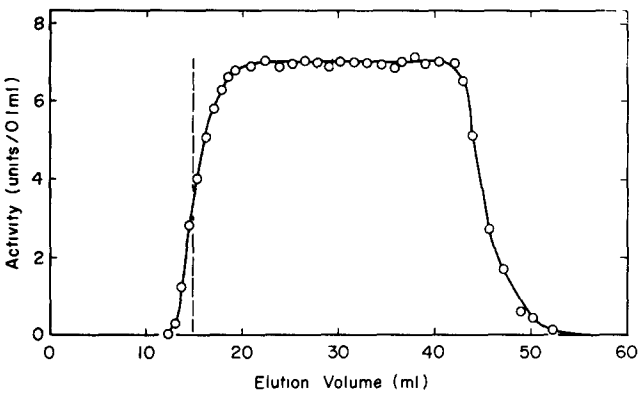


Fig 9 The elution profile obtained from a frontal analysis chromatography experiment on Sephadex G-100 (1 4 cm 21 cm) conducted at 4° A 30-ml sample of *S cremoris* lactate dehydrogenase (0 038 mg/ml in sodium phosphate buffer (pH 7 0, I = 0 1) was applied and collected fractions were weighed to give the abscissa values and assayed for enzymic activity to give the ordinate values

of including modifiers and substrate (NADH) as indicated In four different experiments, all conducted with enzyme alone in sodium phosphate buffer (pH 7 0, I = 0 1), the value of K_{av} was 0 096 ± 0 003 Thus, the decrease of K_{av} with increasing pH (first three rows of Table V) is regarded as significant Comparison of results obtained at pH 7 0 clearly shows that NADH and Fru-1,6-P_2 both decrease K_{av}, while ATP acts oppositely by increasing K_{av} to the largest observed value of 0 11_7

TABLE V

THE DEPENDENCE OF DISTRIBUTION COEFFICIENTS OF *S cremoris* LACTATE DEHYDROGENASE ON pH AND ADDED MODIFIERS

Values of the distribution coefficients, K_{av} (estimated accuracy ± 0 003), were calculated from observed elution volumes obtained in Sephadex G-100 chromatographic experiments performed by the method of frontal analysis (enzyme concentration in the plateau was 0 038 mg/ml)

Environment	*pH*	K_{av}
Sodium phosphate (I = 0 1)	6 0	$0\ 10_2$
Sodium phosphate (I = 0 1)	7 0	$0\ 09_6$
Sodium phosphate (I = 0 1)	8 0	$0\ 08_1$
Sodium phosphate (I = 0 1) containing 0 1 mM Fru-1,6-P_2	7 0	$0\ 07_1$
Sodium phosphate (I = 0 1) containing 0 2 mM NADH	7 0	$0\ 06_3$
Sodium phosphate (I = 0 1) containing 10 mM ATP	7 0	$0\ 11_7$

DISCUSSION

The method presented for purifying *S cremoris* lactate dehydrogenase consistently gave a product which was electrophoretically homogeneous with respect to enzymic and protein content YOSHIDA AND FREESE[21] reported a similar result with a lactate dehydrogenase from *Bacillus subtilis* and thus the behaviour of these bacterial dehydrogenases may be contrasted with that of mammalian lactate dehydrogenases where the occurrence of electrophoretically distinct isoenzymes is well established

The amino acid composition reported in Table II differs only in minor respects from that found with the enzyme from *B subtilis*[21] and moreover, the two dehydrogenases are strikingly similar in physical properties Thus, the *B subtilis* enzyme in phosphate buffer (pH 7 2) is enzymically active, and is characterized by an $s_{20,w}$ of 6 7 S and a mol wt of 146 000 in Tris (pH 8 8) the activity is lost, the molecular weight decreases to 72 000 and ORD measurements indicate conformational unfolding The *S cremoris* enzyme exists at pH 7 0 essentially as a single species (Fig 5) of mol wt 140 000 (the mean of values reported in Table III) and this state of the enzyme prevails in the pH range 5 0–8 0 in the environments specified in Table III The frictional ratio of this form of the enzyme, estimated by standard procedures[15] is 1 26 At pH 9 0 in triethanolamine–HCl buffer the frictional ratio increases to 1 80 and the molecular weight decreases to 70 000, the effects in conjunction explaining the marked decrease of $\bar{s}_{20,w}$ from 7 S to 3 S In what follows the 70 000 species is termed monomer and the 140 000 species dimer although the possibility is not excluded that the monomer may be comprised of subunits not detected in this study It appears, therefore, that increased electrostatic repulsive forces inherent on increasing the pH away from the isoelectric point (pH 4 3) contribute to the dissociation and intramolecular rearrangement within monomer units The postulated conformational change indicated by hydrodynamic measurements is supported by the ORD results (Figs 7 and 8)

The choice of triethanolamine–HCl buffers in the further investigation of sedimentation properties in the alkaline range (Table IV) was dictated by the observation[4] that maximal activity occurred at pH 8 0 in this buffer Therefore, it is of interest that after 2 days of dialysis in this environment, the sedimentation velocity results exhibited behaviour intermediate between that observed at pH 7 0 and 9 0 It is not possible that the two peaks observed in this pH region arose as a result of a rapid equilibration between the monomer and dimer[22] Indeed, the available evidence, including the failure to reverse the 7 S $\rightarrow$ 3 S transition on changing the pH by dialysis, favours the postulate of a relatively slow and irreversible transformation in triethanolamine–HCl buffer toward an unfolded and inactive monomeric form, the extent or rate of the transition being favoured by an increase of pH The results in Figs 6(a) and (b) indicate that at pH 8 0 the substrate NADH and the activator Fru-1,6-P_2 tend to stabilize the folded dimeric form, although the appearance of slower sedimenting material indicates that their protective action in this respect is less effective than phosphate ions The spread boundary evident in Fig 6(c) suggests that ATP is even less effective than NADH and Fru-1,6-P_2 in maintaining the structural integrity of the dimer unit, but the polydispersity indicated prohibits detailed interpretation

Although the above results provide the first indication that a modifier (Fru-1,6-P_2) and a substrate (NADH) may partially determine the size and shape properties of the enzyme at pH 8 0, it is not suggested that the slow 7 S $\rightarrow$ 3 S transition is of importance in relation to the initial velocity studies reported Thus, even kinetic experiments performed in triethanolamine–HCl buffer at pH 8 0 were conducted with enzyme solutions stored at pH 7 0 and were completed in times less than 1 min from the time the enzyme encountered the pH 8 0 medium Thus, it appears from the results discussed that the operative species in kinetic experiments would be the dimer of mol wt 140 000 However, the possibility of a rapid dissociation and/or conformational change occurring on dilution at a fixed pH cannot be excluded This possibility

is related to the question as to the mode of action of the modifiers, ATP and Fru-1,6-P_2, and it becomes necessary to examine two possible explanations

The first involves the proposition that at a fixed pH, dilution of the enzyme (apparently existing as dimer at concentrations equal to or greater than 0·1%) results in a rapidly equilibrating mixture of monomeric and dimeric species In this event, the observed elution volumes (*e g* Fig 9) would be weight-average quantities[22,23] and the variations in K_{av} values (Table V) would reflect shifts in the monomer–dimer equilibrium In these terms, the decrease in K_{av} observed in the presence of NADH and Fru-1,6-P_2 would imply that these materials favoured an active dimeric form Double reciprocal kinetic plots found with a monomer–dimer system in which the active species is favoured are expected to be essentially linear[24,25] as observed (Fig 3) Since K_{av} is increased in the presence of ATP, this inhibitor could be functioning by favouring the less active monomeric form, leading to upward curvature of kinetic plots (Fig 1) also in accord with theoretical prediction[24,25] However, three items of evidence suggest that the proposed monomer–dimer equilibrium is not the sole explanation of all observed effects First, the decrease in K_{av} values (Table V) with increasing pH would imply that dimer was favoured as the net negative charge increased Secondly, no concentration-dependence of specific activity was found in the pH range 6·0–8·0 in the absence of modifiers Thirdly, double reciprocal plots (Fig 3) assumed linearity with an Fru-1,6-P_2 concentration as low as 0·001 mM, which might suggest that conversion to dimer was essentially complete however, further and marked activation was found employing higher concentrations of Fru-1,6-P_2

The second explanation assumes that the dimeric species (140 000) remains undissociated on dilution to concentration levels used in enzyme kinetic and frontal analysis studies Changes in K_{av} values must now be interpreted in terms of shape changes of the enzyme Thus, the decrease in K_{av} values with increasing pH (Table V) suggests that the dimer is susceptible to conformational transitions even in phosphate buffer, an increase in net negative charge resulting in unfolding to forms characterized by smaller elution volumes The lowering of K_{av} values by NADH and Fru-1,6-P_2 would similarly imply unfolding of the enzyme structure, in turn (since Fru-1,6-P_2 is an activator) facilitating the binding of the second substrate pyruvate and the subsequent oxidation–reduction reaction The increase in K_{av} found in the presence of ATP suggests induction of a more compact structure in which the active site is partially masked It would not be expected with a non-associating system for the specific activity at fixed pH values to be concentration-dependent and the dependence observed in the presence of fixed concentrations of ATP is explained on the basis of a changing molar ratio of ATP enzyme

Although the two models discussed are not mutually exclusive in that both size and shape changes may operate at high dilution, it appears definite that conformational changes of the enzyme induced by variation of pH or addition of the modifiers play an important role in regulating the enzymic activity Certainly the concept of unfolding of the enzyme structure to produce a more active form is consistent with several other observations made with this enzyme[2,4] First, it has been found that the activity increases with pH to an optimum value of pH 8·0 in the absence of modifiers suggesting that the degree of unfolding is also optimal at this pH It is entirely consistent that as the pH is further increased, a time-dependent dissociation and further marked unfolding would occur leading to inactivation Thus, it might be expected

that the enzyme would be more stable with respect to time at lower pH values (*e g* pH 6 0) and indeed such behaviour has been observed[4] Secondly, it has been found that phosphate ions inhibit the activity in the presence of Fru-1,6-P_2, an observation explained by the postulate that phosphate ions stabilize the folded dimeric form of the enzyme in opposition to the unfolding effect of Fru-1,6-P_2 Finally, it appears that the unfolding induced by Fru-1,6-P_2 in triethanolamine–HCl buffer not only eliminates any slight cooperativity between the active sites (Fig 3) but also is essentially complete in the pH range 5 0–7 0, since in the presence of 1 mM Fru-1,6-P_2 a broad plateau of enhanced activity was observed in this pH range[4]

ACKNOWLEDGEMENTS

The authors are indebted to Dr B E Davidson of the University of Melbourne for his assistance in performing polyacrylamide gel electrophoresis experiments and in the determination of the amino acid composition of the enzyme K O'D acknowledges the receipt of a Sir John and Lady Higgins research scholarship

REFERENCES

1 M J Wolin, *Science*, 146 (1964) 775
2 R Anders, H A Jonas and G R Jago, *Aust J Dairy Technol*, 25 (1970) 73
3 C L Wittenberger and N Angelo, *J Bacteriol*, 101 (1970) 717
4 H A Jonas, M Sc Thesis, University of Melbourne, 1968
5 G Hathaway and R S Criddle, *Proc Natl Acad Sci U S*, 56 (1966) 680
6 L W Nichol, G D Smith and D J Winzor, *Nature*, 222 (1969) 174
7 C S French and H W Milner, *Methods in Enzymology*, Vol 1, Academic Press New York, 1955, p 64
8 W B Jakoby, *Anal Biochem*, 26 (1968) 295
9 B J Davis, *Ann N Y Acad Sci*, 121 (1964) 404
10 E S Vessell, *Ann N Y Acad Sci*, 94 (1961) 877
11 P R Carnegie, G Lamoureux and B Bencina, *Nature*, 214 (1967) 407
12 D H Spackman, *Fed Proc*, 22 (1963) 244
13 H Edelhoch, *Biochemistry*, 6 (1967) 1948
14 R J Goldberg, *J Phys Chem*, 57 (1953) 194
15 T Svedberg and K O Pedersen, *The Ultracentrifuge*, Oxford Clarendon Press, 1940
16 S M Klainer and G Kegeles, *J Phys Chem*, 59 (1955) 952
17 O H Lowry, N J Rosebrough, A L Farr and R J Randall, *J Biol Chem*, 193 (1951) 265
18 B Jirgensens, in *Optical Rotatory Dispersion of Proteins and Other Macromolecules*, Springer-Verlag, New York, 1969, p 70
19 N Greenfield, B Davidson and G D Fashman, *Biochemistry*, 6 (1967) 1630
20 P Andrews, *Methods Biochem Anal*, 18 (1970) 1
21 A Yoshida and E Freese, *Biochim Biophys Acta*, 99 (1965) 56
22 G A Gilbert, *Proc R Soc London, Ser A*, 250 (1959) 377
23 L W Nichol, A G Ogston and D J Winzor, *J Phys Chem*, 71 (1967) 726
24 L W Nichol, W J H Jackson and D J Winzor, *Biochemistry*, 6 (1967) 2449
25 C Frieden, *J Biol Chem*, 242 (1967) 4045