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Isolation and properties of the glycolytic enzymes from *Zymomonas mobilis*

The five enzymes from glyceraldehyde-3-phosphate dehydrogenase through to pyruvate kinase

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1. The five glycolytic enzymes glyceraldehyde-3-phosphate dehydrogenase, phosphoglycerate kinase, phosphoglycerate mutase, enolase and pyruvate kinase were each purified from extracts of *Zymomonas mobilis* cells, by using dye-ligand chromatography as the principal step. Two procedures, producing three and two of the enzymes respectively, are described in detail. 2. *Z. mobilis* glyceraldehyde-phosphate dehydrogenase was found to be similar in most respects to the enzyme from other sources, except for having a slightly larger subunit size. 3. Phosphoglycerate kinase has properties typical for this enzyme; however, it did not show the sulphate activation effects characteristic of this enzyme from most other sources. 4. Phosphoglycerate mutase is a dimer, partially independent of 2,3-bisphosphoglycerate, and has a high specific activity. 5. Enolase was found to be octameric; otherwise its properties were very similar to those of the yeast enzyme. 6. Pyruvate kinase is unusual in being dimeric, and not requiring K^+ for activity. It is not allosterically activated by sugar phosphates, having a high activity in the absence of any effectors. 7. Some quantitative differences in the relative amounts of these enzymes, compared with eukaryotic species, are ascribed to the fact that *Z. mobilis* utilizes the Entner–Doudoroff pathway rather than the more common Embden–Meyerhoff glycolytic route.

INTRODUCTION

Zymomonas mobilis is an anaerobic bacterium which ferments glucose and fructose to ethanol and CO_2 almost exclusively, by a modified form of the Entner–Doudoroff pathway (Gibbs & De Moss, 1954; Swings & De Ley, 1977), a catabolic pathway otherwise restricted to aerobes. The organism ferments very rapidly, since only one ATP molecule is synthesized for each hexose molecule fermented; this necessitates very high activities of the enzymes, and makes *Z. mobilis* a particularly good source for purifying these enzymes.

We have previously presented isolation procedures for a number of enzymes in this pathway, including glucose-6-phosphate dehydrogenase (Scopes *et al.*, 1985), 6-phosphogluconolactonase (Scopes, 1985), 6-phosphogluconate dehydratase (Scopes & Griffiths-Smith, 1984) and 3-deoxy-2-oxo-6-phosphogluconate aldolase (EC 4.1.2.14) (Scopes, 1984). All of these purification procedures made use of selective dye-ligand adsorption chromatography, a method further pursued in the present paper. Most sugar-fermentation pathways in bacteria involve the five enzymes from glyceraldehyde-phosphate dehydrogenase to pyruvate kinase, whether the overall route is by the Embden–Meyerhoff pathway, with which we usually associate them, by the Entner–Doudoroff pathway, or by other, more complex, routes. There are few reports of the properties of these enzymes from species that utilize any pathway other than the Embden–Meyerhoff, which is that employed by higher organisms. Indeed, there are relatively few reports on these otherwise well-studied enzymes from any prokaryotic sources. Thus it is relevant to compare the properties of the *Z. mobilis* enzymes with those of the better-studied enzymes from yeast and mammalian sources.

A key feature of the isolation procedures used in all of our purifications is dye-ligand chromatography used in a differential mode. In most examples two columns are set up, with dye adsorbents chosen so that the first excludes the desired enzyme but binds significant amounts of other proteins; the second column exhibits some selectivity towards the desired enzyme, and binds it, but little more protein. Combined with affinity-elution techniques (Scopes, 1977), this has enabled in most cases a 20–60-fold purification on a protein basis with high recovery, simultaneously disposing of all the polysaccharide, lipid and nucleic acid components present in crude bacterial extracts, which do not adsorb on dye columns. If another purification step is required, ion-exchange and/or gel filtration are usually adequate to complete the purification. By screening a range of dye columns for their ability to bind or exclude the enzymes, optimum dyes for each enzyme can be chosen. It would have been possible to develop a scheme to purify all five enzymes at one time, using a series of dye adsorbent columns. However, this would be neither practical nor necessary, as the source material is readily obtained, and one would rarely want to purify all the enzymes simultaneously. As a compromise, two procedures are described here, which result in the isolation of two and three of the enzymes respectively. The properties of each enzyme are compared with those of the better-studied eukaryotic enzymes; although there are several structural features which differ, the five enzymes are found to be similar to their eukaryotic counterparts in most respects.

MATERIALS AND METHODS

Materials

ATP, ADP, NAD^+, NADH, 3-phosphoglycerate, 2-phosphoglycerate, phosphoenolpyruvate and other

biochemicals were from Sigma Chemical Co., St. Louis, MO, U.S.A.

Zymomonas mobilis strains ZM4 (A.T.C.C. 31821) and ZM6 (A.T.C.C. 29191) were obtained from Dr. P. L. Rogers, University of N.S.W., Sydney, Australia, and were used interchangeably during the development of the isolation procedures; the final preparations and studies of the purified enzymes were carried out with ZM6 cells. Cells were grown and extracted as described previously (Scopes *et al.*, 1985), with the exception that the extraction buffer was different. The general buffer used in these procedures consisted of 15 mM-K_2HPO_4 and 15 mM-NaCl adjusted to the stated pH with a mixture of 2 M-acetic acid and 0.5 M-succinic acid. This is referred to as Buffer A1. $MgCl_2$ was added to the desired concentration after pH adjustment. This buffer can be used over the pH range 4.5–8.0, and its ionic strength is close to 0.05 over the whole range. The procedures also made use of the same buffer but at twice the concentration. This is referred to as Buffer A2.

Screening of dye-ligand columns was carried out as described previously (Scopes, 1984) on separate occasions, with Buffer A1 at pH 6.5, and Buffer A2 ($I = 0.1$) at pH 6.0. Adsorbed enzymes were eluted with 1 M-NaCl in buffer.

Dye adsorbents were made at ambient temperature as described by Atkinson *et al.* (1981), coupled to Sepharose CL-4B (Pharmacia, Uppsala, Sweden). Procion dyes were gifts from I.C.I. Australia; Cibacron dyes were provided by Ciba–Geigy, Australia, and Remazol dyes were from Hoechst, Australia. Cibacron and other dyes were coupled by the procedure described for Procion H-type dyes.

Enzyme assays

Glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12) was assayed in the direction of glyceraldehyde phosphate formation. A K/Mes buffer at pH 6.5 ($I = 0.05$, including 30 mM-KCl and 3 mM-$MgCl_2$) contained 5 mM-3-phosphoglycerate, 1 mM-ATP, 0.15 mM-NADH, 10 mM-β-mercaptoethanol, and 10 units of phosphoglycerate kinase/ml. Phosphoglycerate kinase (EC 2.7.2.3) was assayed in exactly the same conditions, except that the coupling enzyme was 10 units of glyceraldehyde-phosphate dehydrogenase/ml. Phosphoglycerate mutase (EC 5.4.2.1) was assayed in the same buffer containing 2 mM-3-phosphoglycerate, 0.5 mM-ADP, 0.15 mM-NADH, 10 mM-β-mercaptoethanol and 10 units each of enolase, pyruvate kinase and lactate dehydrogenase/ml. Enolase (EC 4.2.1.11) was assayed in 30 mM-triethanolamine buffer, pH 7.5, containing ADP, KCl and $MgCl_2$ as above, and 10 units each of pyruvate kinase and lactate dehydrogenase/ml; 2-phosphoglycerate was added to a concentration of 0.4 mM. Pyruvate kinase (EC 2.7.1.40) was assayed in the pH 6.5 Mes buffer with addition of an extra 0.1 M-KCl (the extra KCl was subsequently found to be unnecessary; see below), containing 0.5 mM-ADP, 0.15 mM-NADH, 0.5 mM-phosphoenolpyruvate and 10 units of lactate dehydrogenase/ml. Enzyme activities are expressed in units (μmol/min) at 25 °C.

Electrophoresis

Electrophoresis was carried out on 0.5 mm-thick polyacrylamide gels in an LKB Multiphor apparatus, in a Tris chloride/Tris glycinate discontinuous-buffer system.

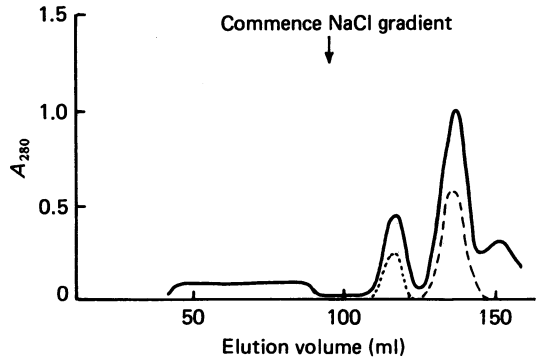

Fig. 1. Elution of phosphoglycerate kinase and phosphoglycerate mutase from DEAE-Trisacryl column

The fraction obtained by affinity elution from the Cibacron Red 3-BA column was applied, after dialysis into 20 mM-Tris/HCl buffer. A gentle gradient in NaCl eluted phosphoglycerate kinase (·····) and phosphoglycerate mutase (----); ——, A_{280}.

Determination of M_r

Gel filtration for molecular size was carried out on a Sephacryl S-200 column (2 cm² × 100 cm) in Buffer A2. The column was calibrated with rabbit muscle pyruvate kinase (M_r 228000), lactate dehydrogenase (M_r 140000), yeast enolase (M_r 88000), bovine serum albumin (M_r 67000) and yeast phosphoglycerate kinase (M_r 45000).

Other methods

Protein was determined by the dye-binding method of Sedmak & Grossberg (1977). Absorption coefficients for the purified enzymes were determined by the A_{205}/A_{280} method (Scopes, 1974). Ultrafiltration of protein samples was carried out in stirred cells (Amicon Corp.) under N_2 pressure, by using YM 10 membranes.

RESULTS

General behaviour of the enzymes on dye-ligand columns

The two screenings were carried out at pH 6.5, $I = 0.05$, and at pH 6.0, $I = 0.1$. Despite the higher ionic strength, binding of proteins was somewhat greater at pH 6.0, and each enzyme (with the exception of enolase) bound to more dyes at the lower pH. The order of binding to dyes for these five enzymes was glyceraldehyde-phosphate dehydrogenase > pyruvate kinase > phosphoglycerate mutase ⩾ phosphoglycerate kinase > enolase. Enolase did not bind to any of 60 dyes that we have tried in these buffers. Glyceraldehyde-phosphate dehydrogenase was analysed in greatest detail in the pH 6.5 screening; it totally bound to 50% of the dyes, partially to 15% and not at all to 35%. Of the latter, we selected Procion Green H-4G as an adsorbent which bound a substantial amount of protein to be the first, 'negative', column for this enzyme. Of the totally adsorbing dyes, Procion Violet H-3R appeared to be the most suitable, in terms of its relative protein-binding ability and recovery of active enzyme on elution with 1 M-NaCl.

Pyruvate kinase has not been screened on all the columns, owing to a degree of instability of this enzyme in crude extracts. However, at pH 6.0 it bound to some 40% of the dyes investigated, and very specifically to Procion Yellow MX-4R. Extracts made at pH above 7.5

Table 1. Isolation of pyruvate kinase, phosphoglycerate kinase and phosphoglycerate mutase from 60 g wet wt. of *Z. mobilis* cells

For details of procedure, see the text.

		Pyruvate kinase		Phosphoglycerate kinase		Phosphoglycerate mutase	
	Protein (mg)	(k-units)	Specific activity (units/mg)	(k-units)	Specific activity (units/mg)	(k-units)	Specific activity (units/mg)
Extract	2980	70	24	60	20	110	37
Not adsorbed on Yellow MX-4R	1750	0	—	60	34	105	60
Not adsorbed on Red 3-BA	1200	—	—	0		0	—
Eluted from Yellow MX-4R by phosphoenolpyruvate	160	45	280	—	—	—	—
Eluted from Red 3-BA by ATP+3-phosphoglycerate	265	—	—	55	210	92	350
Eluted from DEAE-Trisacryl							
(kinase)	65	—	—	50	800	0	—
(mutase)	77	—	—	0	—	84	1090
After gel filtration	—	—	—	—	—	70	2000

lose pyruvate kinase activity, but this is restored slowly on adjustment to pH 6–6.5. Because of the high specificity of both binding and the elution (with phosphenolpyruvate) from the Procion Yellow column, it was not necessary to use a 'negative' column for this enzyme. Nevertheless, Scarlet MX-G, which removes glucokinase and glucose-6-phosphate dehydrogenase as well as 30% of proteins altogether (Scopes *et al.*, 1985), has frequently been used for this purpose.

Phosphoglycerate mutase and phosphoglycerate kinase behave very similarly on the dye adsorbents. Binding mainly to the 30% of dyes that have the highest protein-binding ability, their binding is strongly affected by pH, showing relatively weak interactions at pH 6.5 compared with pH 6.0. There is some selectivity; for example, at pH 6.0 phosphoglycerate kinase is totally adsorbed on Procion Blue H-GR, but the mutase passes through, whereas on Drimarene Brilliant Blue K-BL (Santoz) the converse pattern occurs. Biospecific elution of these enzymes has only been partially successful, reflecting a considerable extent of non-specific interactions between enzyme and dyes. In the purification scheme described below, a dye that binds both enzymes, Cibacron Red 3-BA, was chosen. The enzymes could be eluted separately by substrates, the mutase with 2 mM-3-phosphoglycerate and the kinase with 2 mM-3-phosphoglycerate plus 1 mM-ATP. But neither was particularly pure at this stage, and each required further steps, including ion exchange and gel filtration. Alternatively both enzymes could be eluted together (with 3-phosphoglycerate and ATP), and were separated from each other in the subsequent ion-exchange-chromatographic step.

As enolase did not bind to any columns, it could be purified after passage through dye adsorbents from any of the procedures, though preferably after passage through a dye adsorbent that removed at least 75% of the protein. At pH 7.5–8.0 it would adsorb to DEAE ion exchangers at $I = 0.05$–0.1. The procedure adopted in this paper combines the glyceraldehyde-phosphate dehydrogenase purification with the enolase for convenience. The only other major fermentative enzymes of *Z. mobilis* that do not bind to any of our 60 dye columns at pH 6.0 are phosphoglucose isomerase (EC 5.3.1.9) and pyruvate decarboxylase (EC 4.1.1.1).

An isolation procedure for pyruvate kinase, phosphoglycerate kinase and phosphoglycerate mutase

Two 160 ml columns (16 $cm^2 \times 10$ cm), containing Procion Yellow MX-4R–Sepharose CL-4B (Reactive Orange 14), dye content 4 mg/g wet wt. in the first, and Cibacron Red 3-BA–Sepharose CL-4B (C.I. 18105, Reactive Red 4), dye content 5 mg/g wet wt. in the second, were equilibrated with Buffer A2, pH 6.0, containing 5 mM-$MgCl_2$. Then 300 ml of extract made with Buffer A2 and adjusted to pH 6.0 was run into the two columns connected in series, and washed in with 500 ml of Buffer A1, pH 6.0. (By lowering the ionic strength during the wash phase, leakage of phosphoglycerate kinase was minimized.) The Yellow column was removed and washed with Buffer A1 at pH 6.5, also containing 5 mM-$MgCl_2$. Then 150 ml of the same buffer containing 0.2 mM-phosphoenolpyruvate was applied, followed by a further 200 ml of buffer without substrate. Pyruvate kinase was affinity-eluted by its substrate, and, provided that the pre-elution wash satisfactorily removed contaminating proteins, the enzyme fraction was > 90% pure pyruvate kinase, of specific activity 280–300 units/mg. Active fractions were concentrated by ultrafiltration before being salted out with $(NH_4)_2SO_4$. Turbid $(NH_4)_2SO_4$ suspensions developed fine needle-like crystals after a few weeks.

The Red column was eluted after further washing, with 200 ml of 2 mM-3-phosphoglycerate plus 1 mM-ATP in Buffer A1, followed by more buffer. This resulted in both phosphoglycerate kinase and mutase being eluted, together with substantial amounts of other proteins. The active fractions were combined, ultrafiltered to about 30 ml, and dialysed overnight against 2 litres of 20 mM-Tris/HCl, pH 8.0. The sample was then applied to a DEAE-Trisacryl column (LKB Produkter, Bromma, Sweden) of dimensions 4 $cm^2 \times 10$ cm, pre-equilibrated

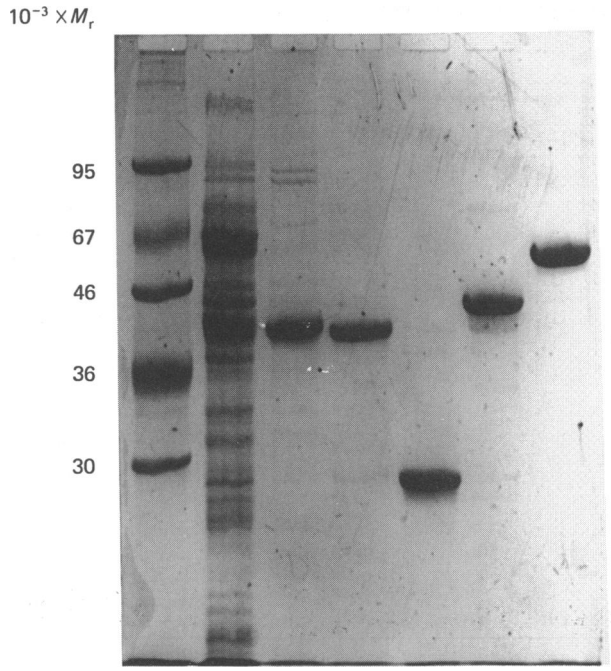

Fig. 2. SDS/polyacrylamide-gel electrophoresis of purified enzymes

Tracks, from left to right; standard mixture; crude *Z. mobilis* extract; glyceraldehyde-phosphate dehydrogenase; phosphoglycerate kinase; phosphoglycerate mutase; enolase; pyruvate kinase.

in the Tris buffer. Both enzymes were adsorbed. A linear gradient (total vol. 400 ml) of 0–0.10 M-NaCl in the Tris buffer was started, and phosphoglycerate kinase was eluted quickly as a sharp peak, followed shortly afterwards by a peak containing the mutase (Fig. 1). The kinase was essentially pure at this stage, but the mutase still contained an impurity, which could be removed on gel filtration. After ultrafiltration of the phosphoglycerate mutase fraction to 4–5 ml, it was applied to a 500 ml column of Cellufine GCL-2000 sf (Amicon Corp.), equilibrated in Buffer A2. Specific activities of the enzymes were comparable with or somewhat greater than those of enzymes purified from other sources. The purity and subunit sizes of the enzymes is illustrated in the SDS/polyacrylamide gel in Fig. 2. A summary of the isolation procedure is given in Table 1.

An isolation procedure for glyceraldehyde phosphate dehydrogenase and enolase

A two-column (negative+positive) procedure for glyceraldehyde-phosphate dehydrogenase has been evolved, with Buffer A1 at pH 6.5. The negative column used was Procion Green H-4G, which binds a large proportion of the applied protein at this pH, yet allows the dehydrogenase through. Procion Violet H-3R proved to be very successful at adsorbing the enzyme, and was used as the positive column. The enzyme could be eluted specifically with NADH or NAD^+ after increasing the ionic strength with 0.2 M-NaCl.

Extract (400 ml) was run on two columns connected in series, the first being 16 $cm^2 \times 10$ cm of Procion Green H-4G–Sepharose CL-4B (Reactive Green 5), dye content 0.5 mg/g wet wt. of gel, and the second the same dimensions of Procion Violet H-3R (Reactive Violet 1), dye content 7 mg/g wet wt. of gel. The flow rate was 300 ml/h. The extract was made with Buffer A1 and adjusted to pH 6.5, and the column buffer consisted of Buffer A1 at pH 6.5, containing 5 mM-$MgCl_2$ and 10 mM-β-mercaptoethanol. The columns were washed with buffer, then the Green column was removed, after checking that no more glyceraldehyde-phosphate dehydrogenase was coming from the Green column. The Violet column was then washed with the same buffer to which 0.2 M-NaCl had been added, and then the eluate monitored until little more protein was being washed out; this required about 500 ml of buffer. Glyceraldehyde-phosphate dehydrogenase was eluted by adding 0.2 mM-NADH (or 1 mM-NAD^+) to the NaCl-containing buffer. The active enzyme was collected, ultrafilter-concentrated, and finally salted out with $(NH_4)_2SO_4$. Turbid $(NH_4)_2SO_4$ suspensions developed crystals over a period of months, which were bipyramidal and grew up to 1 mm in size. The crystals were yellow, as was the concentrated eluate from the column, indicative of a normal Racker band (Racker & Krimsky, 1952). The specific activity of the purified enzyme was 180–200 units/mg, comparable with the highest values for this enzyme from other sources.

Enolase did not bind to either column. After collection of the non-adsorbed fraction from the Violet column, it was adjusted to pH 8.0 with 1 M-Tris base, and run on a column of DEAE-Trisacryl (dimensions 8 $cm^2 \times 12$ cm) in 50 mM-Tris/HCl buffer, pH 8.0, containing 5 mM-$MgCl_2$ and 10 mM-β-mercaptoethanol, and washed in with buffer. A linear NaCl gradient was commenced (total vol. 1000 ml) of 0–0.2 M-NaCl in the Tris buffer. Enolase was eluted at about 0.1 M-NaCl; the active fractions were collected and ultrafilter-concentrated to about 15 ml. This fraction was applied to the column of Cellufine GCL-2000 sf (see above), pre-equilibrated in Buffer A2 plus 5 mM-$MgCl_2$ and β-mercaptoethanol. Enolase was eluted unexpectedly early, although well after the void volume, the position corresponding to an M_r of 300000–400000 (Fig. 3). Enolase was essentially pure after the gel filtration, as illustrated in Fig. 2.

Enolase is thus purified at the first step by differential

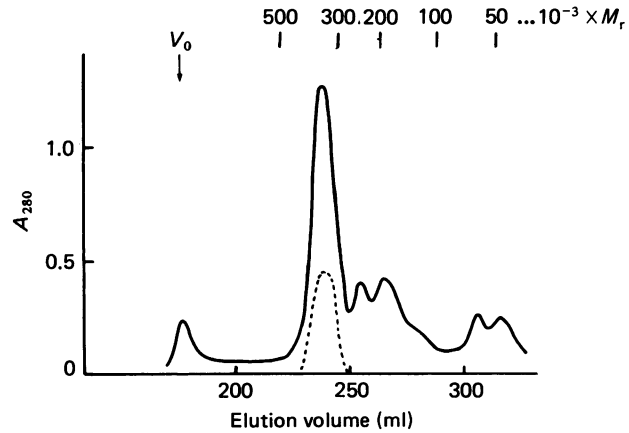

Fig. 3. Purification of enolase on Cellufine GCL-2000 sf column

The enzyme (----) was eluted at a position corresponding to M_r between 300000 and 400000. An approximate M_r calibration is indicated. ——, A_{280}.

Table 2. Isolation of glyceraldehyde-phosphate dehydrogenase and enolase from 70 g wet wt. of *Z. mobilis* cells

For details of procedure, see the text.

	Protein (mg)	Glyceraldehyde-phosphate dehydrogenase (k-units)	Glyceraldehyde-phosphate dehydrogenase Specific activity (units/mg)	Enolase (k-units)	Enolase Specific activity (units/mg)
Extract	5100	60	11.8	11.7	2.3
Not adsorbed on Green H-4G	1650	51	31	11.0	6.7
Not adsorbed on Violet H-3R	390	0	—	9.8	25
Eluted from Violet H-3R by NADH	200	41	205	—	—
Eluted from DEAE-Triacryl	135	—	—	8.5	63
After gel filtration (enolase)	46	—	—	7.3	160

column chromatography, in which the first (negative) column is a dye-ligand adsorbent, and the second (positive) column is an anion exchanger. The nature of the first dye-ligand adsorbent is not important, provided that it binds at least 65%, and preferably 75%, of the protein in the stated conditions. We have also used Procion Green HE-4BD and Remazol Brilliant Blue R as well as the two mentioned in the procedure above. A summary of the purification procedure is given in Table 2.

Properties of the five enzymes

Glyceraldehyde-phosphate dehydrogenase from *Z. mobilis* appears to be somewhat larger than usual, at 40000–42000 Da for its subunit (cf. 36000 Da normally), although on gel filtration its size of 150000 ± 10000 suggests that the subunit size may be slightly over-estimated. The enzyme lost activity rapidly without thiol protection. If no β-mercaptoethanol was present in the assay buffer, purified samples lost activity so rapidly that no further activity occurred after about 2 min. Nevertheless, completely inactive preparations that had been stored for several months as $(NH_4)_2SO_4$ suspensions were re-activated within minutes on addition of a thiol compound. Detailed kinetic studies on this enzyme have not yet been carried out.

The *Z. mobilis* phosphoglycerate kinase appears very similar in most respects to other phosphoglycerate kinases, especially those of other bacteria and of plants (D'Alessio & Josse, 1971; Nojima *et al.*, 1979; Fifis & Scopes, 1978). Its specific activity is of the same order (700–900 units/mg), although high concentrations of substrates were not necessary to obtain such values. Its kinetic properties do differ, in that there is a much less pronounced substrate-activation effect (Scopes, 1978) than occurs with the yeast enzyme, and no activation effects with sulphate. In the latter respect it resembles the plant (*Beta vulgaris*) enzyme; at 0.2 mM of each substrate, sulphate only caused inhibition (Fifis & Scopes, 1978). It is interesting that we then reported that the *Escherichia coli* enzyme was activated by sulphate: further investigation of this effect with bacterial phosphoglycerate kinases is warranted. The molecular size of 44000 Da (monomeric) is normal for this enzyme.

The *Z. mobilis* phosphoglycerate mutase is 2,3-bisphosphoglycerate-dependent. But with a 3-phosphoglycerate preparation that allowed no activity with yeast enzyme in the absence of 2,3-bisphosphoglycerate, the *Z. mobilis* preparation was about 20% active. This suggests either that it exhibits some bisphosphoglycerate-independent activity, or that it has an extremely high affinity for a trace amount of bisphosphate that may be present in the substrate, in amounts too low to activate the yeast enzyme. The *E. coli* enzyme, the only other prokaryotic source studied, is reported to be bisphosphoglycerate-dependent (Price & Stevens, 1983). Its

Table 3. Summary of the properties of the five enzymes from *Z. mobilis*

Abbreviations: PGA, phosphoglycerate; PEP, phosphoenolpyruvate.

Enzyme	Specific activity (units/mg)	K_m (mM)	$A_{280}^{1\,mg/ml}$	Subunits No.	Subunits M_r
Glyceraldehyde-phosphate dehydrogenase	205	n.d.	2.1	4	41000
Phosphoglycerate kinase	800	1.5* (3-PGA) 1.1* (ATP)	0.38	1	44000
Phosphoglycerate mutase	2000	1.1 (3-PGA)	1.79	2	26000
Enolase	160	0.08 (2-PGA)	1.03	8	45000
Pyruvate kinase	300	0.08 (PEP) 0.17 (ADP)	0.43	2	57000

* Non-Michaelis–Menten kinetics, with apparent negative co-operativity (Scopes, 1978): Hill coefficient in each case approx. 0.85.

specific activity of close to 2000 units/mg is very high, so that, although it is the glycolytic enzyme of greatest activity in *Z. mobilis* extracts, as a protein it is by no means the most abundant. The subunit size is 26000 Da, and gel filtration gives a value of 52000 ± 2000, showing that it is dimeric. The K_m for 3-phosphoglycerate was determined to be 1.1 mM in the assay sytem described.

Enolases have somewhat lower specific activities compared with other glycolytic enzymes. The highest values for muscle enolases are of the order of 90 units/mg (Wold, 1971), though the yeast enzyme is about twice as active (Westhead, 1966). The *Z. mobilis* enolase resembles that of yeast in this respect. Thus, although enolase makes up about 2% of the protein in the crude extract, it has only just enough activity to account for the glycolytic throughput *in vivo* (Algar & Scopes, 1985). Although dye-ligand adsorbents tend to have particular specificity towards purine nucleotide-binding proteins and to enzymes with small, multiply-negative-charged, substrates, *Z. mobilis* enolase is clearly an exception to the latter class, as (at pH 6.0) it did not bind to any of some 60 dye adsorbents, even those that bound over 75% of the total protein from the extract. This was convenient in that it enabled a large proportion of the protein to be removed before application to the DEAE-Trisacryl column. The enzyme's subunit size of 45000 Da is normal for enolase. However, gel filtration on calibrated columns of Sephacryl S-200 and of Cellufine GCL-2000 has indicated a large native M_r, greater than 300000, suggesting probably eight subunits to the native enzyme. Whereas all eukaryotic enolases described are dimeric (Wold, 1971), as is the *E. coli* enzyme (Spring & Wold, 1971), two *Thermus* sp. enolases are reported to be octamers (Stellwagen *et al.*, 1973). The K_m for 2-phosphoglycerate in the assay buffer described is 0.08 mM, which is comparable with the value for the yeast enzyme.

Z. mobilis pyruvate kinase, unlike many other pyruvate kinases, is not subject to allosteric control. Possible effectors such as glucose 6-phosphate, 6-phosphogluconate and fructose 1,6-bisphosphate had no effect on the K_m values; normal Michaelis–Menten kinetics were observed in their absence, with the K_m for ADP (at 1 mM-phosphoenolpyruvate) being 0.17 mM, and that for phosphoenolpyruvate (at 1 mM-ADP) 0.08 mM. The enzyme was found not to require K^+ ions; this has also been noted for the enzyme from *Mycobacterium* (Kapoor & Venkitasubramanian, 1983). The specific activity of the purified enzyme at 300 units/mg is comparable with that of the mammalian muscle enzyme, and higher than reported for the *E. coli* enzymes (Valentini *et al.*, 1979).

The subunit size of pyruvate kinase corresponded exactly to that of a rabbit muscle enzyme standard at 57000 Da. However, on gel filtration the enzyme emerged clearly as a dimer, at 115000 ± 5000 Da. This feature distinguishes the *Z. mobilis* pyruvate kinase from all others that have been described, which are tetrameric.

A summary of the properties of the five enymes is given in Table 3.

DISCUSSION

The five enzymes from glyceraldehyde-phosphate dehydrogenase to pyruvate kinase are common to most, if not all, sugar-degrading pathways. The amounts of these enzymes have been measured in a number of eukaryotic species and tissues, and have been found to be in relatively constant proportions to each other (Pette *et al.*, 1962). This is despite the fact that the amounts of enzymes vary considerably between different tissues, and that they differ in amount from each other. The latter point is a reflection of the thermodynamics and kinetics of the reactions catalysed; for instance relatively large amounts of phosphoglycerate kinase are needed to overcome the natural inhibition by high ATP concentrations in the cell, and to be able to interact with the very low concentrations of 1,3-bisphosphoglycerate that exist in glycolysing tissues. On the other hand, enolase, which catalyses a simple uninhibited one-substrate reaction, can act at close to its $V_{max.}$ *in vivo*. In *Z. mobilis* we find that the ratios between the enzymes do differ significantly from those reported for eukaryotic sources. Glyceraldehyde-phosphate dehydrogenase is not present at such a high activity as in yeast (Welch & Scopes, 1985; Algar & Scopes, 1985), although the glycolytic rate in *Z. mobilis* is greater than in yeasts (Lee *et al.*, 1979). The reason may be found by considering the preceding reactions in the two species; the virtual absence of triosephosphate isomerase in *Z. mobilis* means that the 3-deoxy-2-oxo-6-phosphogluconate aldolase is able to provide a relatively high concentration of glyceraldehyde phosphate. On the other hand, in yeast, the equilibrium of fructose-1,6-bisphosphate aldolase together with the triosephosphate isomerase reaction results in relatively low concentrations of glyceraldehyde phosphate, so yeasts need very high activities of glyceraldehyde-phosphate dehydrogenase to deal with the low concentration of substrate. Phosphoglycerate kinase is also present at higher activities in yeast; again the reason may be related to the substrate concentrations *in vivo*, but also it seems likely that the ATP/ADP ratio in yeast may be higher, and so more inhibitory. Unfortunately direct measurements of the concentrations of these compounds *in vivo* are not readily obtained, so these suggested reasons for *Z. mobilis* being able to manage with less of the enzymes than yeasts cannot be confirmed at present. The activities of the other three enzymes, phosphoglycerate mutase, enolase and pyruvate kinase, are all comparable with those in yeast. Thus the relative ratios of glyceraldehyde-phosphate dehydrogenase and phosphoglycerate kinase, compared with the other three enzymes, are lower in *Z. mobilis* than in eukaryotic species. It seems likely that this is a result of *Z. mobilis* utilizing the Entner–Doudoroff pathway.

Those structural and kinetic properties of the *Z. mobilis* enzymes that have been determined indicate that, although the subunit molecular sizes are highly conserved, the number of subunits in the active enzyme in two cases, enolase (eight) and pyruvate kinase (two), is unusual. The *Z. mobilis* glyceraldehyde-phosphate dehydrogenase subunit appears to be about 10% larger than usual, but the number (four) in the active enzyme is normal (Harris & Waters, 1976). Moreover it has characteristic properties, including tightly bound NAD^+ (evidenced by the yellow colour; Racker & Krimsky, 1952) and highly sensitive thiol groups, which can be reversibly oxidized. Phosphoglycerate kinase is normal structurally and kinetically, except for the lack of sulphate activation which occurs with most other phosphoglycerate kinases (Fifis & Scopes, 1978). Phosphoglycerate mutase is dimeric; this is a common

occurrence in an enzyme known to exist in monomeric, dimeric and tetrameric forms (Grisolia & Carreras, 1975). However, it is unusual in being partly 2,3-bisphosphoglycerate-independent; this has only been reported previously in plants and fungi (Price & Stevens, 1983). The *Z. mobilis* enzyme's specific activity is very high, a factor reflected in the high activity of this enzyme in the cell itself (Algar & Scopes, 1985).

Z. mobilis enolase's octameric structure is shared only by *Thermus* sp. enolase (Stellwagen *et al.*, 1973), though no doubt other prokaryotic enolases will be found with this feature. In all other respects it behaves very similarly to the much-studied yeast enzyme.

The pyruvate kinase described here has some unusual features, including the absence of obvious allosteric effectors. Several bacterial pyruvate kinases have been studied, as they have a controlling role in metabolism. Thus a streptococcal (Abbe & Yamada, 1982), a mycobacterial (Kapoor & Venkitasubramanian, 1981) and a propionibacterial (Smart & Pritchard, 1979) pyruvate kinase are each activated by glucose 6-phosphate. A pseudomonad pyruvate kinase is activated by 6-phosphogluconate (Chuang, 1976). Although both these are key metabolites in *Z. mobilis*, we have not been able to demonstrate any significant effect of either on the activity of its pyruvate kinase. Rather, the enzyme behaves much like the rabbit muscle enzyme, with no compulsory effectors; also, their affinities for substrates and their specific activities are very similar. However, unlike the muscle enzyme it does not require univalent metal cations for activity; in this respect it resembles the mycobacterial enzyme (Kapoor & Venkitasubramanian, 1983).

We have now succeeded in purifying all of the glycolytic enzymes of *Z. mobilis* responsible for converting glucose and fructose into ethanol and CO_2, using dye-ligand chromatography as the initial step, and in some examples the only step. A major advantage of using dye adsorbents at the first step is that they bind very little other than protein; bacterial extracts contain large amounts of nucleic acids and carbohydrate material which often interfere with normal protein isolation procedures. By combining this advantage with the selectivity observed between the dyes, and with affinity-elution procedures, we have found the method rapid and convenient for a whole range of proteins from bacteria. It has also been used very successfully for isolating yeast, plant and animal proteins in our laboratory and many others.

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