Purification of 3-Phosphoglycerate Kinase from Diverse Sources by Affinity Elution Chromatography

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1. Affinity elution chromatography was used to purify phosphoglycerate kinase from a variety of sources. The choice of buffer pH for the chromatography was made according to the relative electrophoretic mobility of the enzyme from the species concerned. 2. Outlines of the methods used to isolate the enzyme from over 20 sources are presented. The enzyme was purified from the muscle tissue of a variety of mammals, fish and birds, from liver of several animals, from yeast, *Escherichia coli*, and plant leaves. The more acidic varieties of the enzymes were purified by conventional gradient elution from ion-exchangers as affinity elution procedures were not applicable. 3. The structural and kinetic parameters investigated show that phosphoglycerate kinase is evolutionarily a highly conservative enzyme; there were few differences in properties regardless of source or function (glycolytic, gluconeogenic or photosynthetic). 4. A detailed comparison of the enzyme preparations purified from bovine muscle and bovine liver failed to detect any significant differences between them; the evidence indicates that they are genetically identical.

Phosphoglycerate kinase (ATP-3-phospho-D-glycerate 1-phosphotransferase, EC 2.7.2.3) has been isolated from a variety of sources, including the muscle tissue of rabbit, pig, horse and chicken (Scopes, 1969; Krietch & Bücher, 1970; Blake *et al.*, 1972; Gosselin-Rey, 1963), yeast (Scopes, 1971), bovine liver (Bojanovski *et al.*, 1974), erythrocytes (Hashimoto & Yoshikawa, 1962; Yoshida & Watanabe, 1972), *Escherichia coli* (D'Alessio & Josse, 1971) and silverbeet leaves (Cavell & Scopes, 1976). Most of the more recent procedures included ion-exchange chromatography as a major step; in many cases adsorption on CM-cellulose or phosphocellulose was followed by elution with an ionic strength gradient.

Affinity elution chromatography is defined as elution from an adsorbent by use of a ligand that binds specifically to the protein in question, and displacing it from the column. Methods for purifying each of the glycolytic enzymes by affinity elution chromatography have been described (Scopes, 1977a). The present paper describes the use of affinity elution chromatography for purifying phosphoglycerate kinase from many different sources, indicating the advantages and limitations of the technique. Some comparative properties of the purified enzymes are also described.

Many phosphoglycerate kinases can be purified in three steps, namely $(\text{NH}_4)_2\text{SO}_4$ fractionation, ion-exchange chromatography and gel filtration. A heat treatment after the $(\text{NH}_4)_2\text{SO}_4$ step can also be beneficial (Scopes, 1969). The main property that

varies between the enzyme from one species and another is the net charge, which affects the ion-exchange chromatography. Not only may the relative behaviour of the enzyme be altered, but also for the same reasons the behaviour of other contaminating proteins changes, so that a procedure that may be ideal for one species may be quite unsuitable for another. The affinity elution procedure described previously (Scopes, 1977a) has been refined and developed for this enzyme, using the substrate 3-phosphoglycerate as eluting ligand. A preliminary report of this work has been presented (Fifis & Scopes, 1977).

Materials and Methods

Tissues from various animals were obtained as soon as possible after death; rabbits, rats and chickens were killed in the laboratory. Bovine, sheep and pig materials were fresh from the local abattoir; fish were purchased at the local market. Muscle tissue from the other animals and birds was obtained when available, Australian species with the help of the Victorian Department of Fisheries and Wildlife.

E. coli K12-3000 was grown at 37°C in a medium containing both glucose and glycerol as carbon sources, the cells were harvested by centrifugation (200 000 g-min), disrupted with a French Press (100 MPa) and centrifuged (350 000 g-min) to remove cell debris.

Muscle tissues were homogenized in 3 vol. of 30 mM-potassium phosphate, pH 7.2, containing 2 mM-

EDTA. Liver was homogenized in the same buffer 50% saturated with $(\text{NH}_4)_2\text{SO}_4$. Homogenates were centrifuged at 4800g for 30min or, in the case of the liver homogenates, at 12000g for 30min, and the residues discarded. The extracts obtained were fractionated with $(\text{NH}_4)_2\text{SO}_4$ as described below for each individual source.

Yeast was cytolysed with aq. NH_3 as described previously (Scopes, 1971). Silver beet and spinach leaves were extracted by the method of Cavell & Scopes (1976).

Phosphoglycerate kinase was assayed in the presence of 10mM-3-phosphoglycerate and 4mM-ATP as described previously (Scopes, 1969). E_{act} values were calculated from effects of temperature on the activity. A thermostatically-controlled cell (temperatures 10 to 50°C) was used, and the activity measured in the presence of 3mM-3-phosphoglycerate and 3mM-ATP (series 1), and with the addition of 40mM- Na_2SO_4 in series 2. U.v.-absorption measurements at neutral and alkaline pH were carried out on the purified preparations to determine the amount of protein and absorption coefficients (Scopes, 1974) and for measuring tryptophan and tyrosine content (Goodwin & Morton, 1946). Vertical starch-gel electrophoresis was carried out in a slab gel at pH 6.5. The buffer in the electrode trays consisted of 16.7mM-magnesium citrate and 50mM-histidine (free base) adjusted to pH 6.5 with citric acid. The gel buffer was the same diluted 10-fold with water. Electrophoresis was carried out for 3h at 5mA/cm². In this buffer system most phosphoglycerate kinases ran as discrete bands of material, many of them moving upwards towards the cathode. Enzyme activity was detected by u.v. fluorescence (Beutler, 1969); protein was stained with 2% Nigrosine for 30s before washing with methanol/acetic acid/water (30:5:65, by vol.). Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis was carried out as described by Scopes & Penny (1971). Principal marker proteins for molecular-weight estimation were glyceraldehyde phosphate dehydrogenase (EC 1.2.1.12; 36000 mol.wt.), creatine kinase (EC 2.7.3.2, 41000 mol.wt.), pyruvate kinase (EC 2.7.1.40, 57000 mol.wt.) and bovine serum albumin (68000 mol.wt.).

Tryptic peptide 'maps' were produced after digestion of samples by the method of Uyeda & Kurooka (1970). Electrophoresis was in 75mM-imidazole acetate buffer, pH 6.5, at 2500V for 1h, and chromatography in butan-1-ol/acetic acid/water (60:15:25, by vol.) (Smith *et al.*, 1957).

Results

General procedures

As Scopes (1977a) reported, phosphoglycerate kinase can be eluted from CM-cellulose by any of

its four substrates. Adenine nucleotides are less suitable than 3-phosphoglycerate both because they absorb at 280nm, making the detection of eluted protein more difficult, and because they are less specific; several other kinases are also liable to be eluted. 1,3-Bisphosphoglycerate is not easy to prepare in a suitable relatively salt-free form, and it also causes elution of glyceraldehyde phosphate dehydrogenase, which usually adsorbs on CM-cellulose columns at neutral pH values. 3-Phosphoglycerate binds tightly to phosphoglycerate kinase (K_d at low ionic strength approx. 10 μM ; Roustan *et al.*, 1973; R. K. Scopes, unpublished work), and the only other enzyme it is likely to elute, phosphoglycerate mutase, is in many cases too acidic to adsorb on CM-cellulose. The procedures described below made exclusive use of 3-phosphoglycerate.

It is necessary for the enzyme to be adsorbed on the ion-exchanger at a pH at which it remains stable; in practice a value of 5.8 is the lowest usable pH at 4°C. If the enzyme of a particular species does not adsorb at this pH, other techniques must be used; these will be described separately. The optimum pH for affinity elution depends on the pI, as it is necessary to reach conditions where the enzyme is only weakly adsorbed. In practice this value is at a pH at or slightly above the pI (see the Discussion section). To determine the pI value, isoelectric focussing can be used; however, there are dangers of obtaining anomalous results with this enzyme (Yoshida *et al.*, 1972). Similarly for electrophoretic methods, especially in citrate buffers as we have used, anomalously low pI values can appear as a result of anion binding. Nevertheless, electrophoresis in the magnesium citrate buffer described above has given relative pI values that correspond to ion-exchange behaviour. Small samples of the crude extracts of tissue were clarified by adjusting to pH 5.5 with 1M-acetic acid, centrifuging off any precipitate, and re-adjusting to pH 6.5 before dialysis against the electrophoresis buffer for a few hours. The samples were then applied to the starch-gel slab. After electrophoresis, the location of phosphoglycerate kinase was established by specific staining. A diagram of the relative mobilities of a representative selection of enzymes from various species is shown in Fig. 1, and the mobilities of all species investigated are listed in Table 1. Alongside Fig. 1 is a scale to indicate pH values suitable for affinity elution chromatography of the enzymes, corresponding to the mobilities. For example, rabbit muscle can be adsorbed on CM-cellulose at pH 7.2 or less, but not completely at a higher pH. Affinity elution can then be carried out from about that pH value up to 8.0, the optimum value. Above pH 8.0, the enzyme is slowly washed from the column without applying the substrate (Scopes, 1977a).

Preliminary fractionation of the tissue extract was

Fig. 1. Mobilities of phosphoglycerate kinases from various sources on starch-gel electrophoresis at pH6.5, in magnesium citrate/histidine buffer
1, Chicken muscle; 2, rabbit muscle; 3, rabbit liver; 4, pig muscle; 5, bovine muscle; 6, bovine liver; 7, snapper muscle; 8, yeast; 9, Eastern grey kangaroo; 10, toad; 11, *E. coli*; 12, spinach.

Table 1. List of species investigated, and the relative mobilities of their phosphoglycerate kinases on starch-gel electrophoresis in magnesium citrate/histidine buffer, pH 6.5
Positive mobility is towards the anode.

Species	Mobility (mm)	
	Major band	Other bands
Pig (<i>Sus scrofa</i>)	+3	
Ox (<i>Bos taurus</i>)	-11	
Sheep (<i>Ovis aries</i>)	-11	
Horse (<i>Equus caballus</i>)	-15	
Rabbit (<i>Oryctolagus cuniculus</i>)	-14	
Rat (<i>Rattus norvegicus</i>)	+3	
Eastern grey kangaroo (<i>Macropus giganteus</i>)	+12, +7	+3
Western grey kangaroo (<i>Macropus fuliginosus</i>)	+12, +7	+3
Pademelon (<i>Thylogale billardieri</i>)	+5	
Wombat (<i>Vombatus ursinus</i>)	+3	
Brushtail possum (<i>Trichosurus vulpeculus</i>)	+7	
Ringtail possum (<i>Pseudocheirus peregrinus</i>)	+7	
Toad (<i>Bufo vulgaris</i>)	+14	+16, +12
Emu (<i>Dromaios novaehollandiae</i>)	-13	
Chicken (<i>Gallus gallus</i>)	-20	-14
Pigeon (<i>Columba livia</i>)	-9	
Carp (<i>Cyprinus carpio</i>)	+15	+19
Sea trout (<i>Salmo trutta</i>)	-17	
Rainbow trout (<i>Salmo gairdnerii</i>)	-17	
Bream (<i>Abramis brama</i>)	+8	
Snapper (<i>Chrysophrys suttlatus</i>)	-8	-3
Silverbeet (<i>Beta vulgaris</i>)	+37	
Spinach (<i>Spinacia oleracea</i>)	+37	
Baker's yeast (<i>Saccharomyces cerevisiae</i>)	+5	
<i>Escherichia coli</i>	+50	

carried out with (NH₄)₂SO₄. Most of the muscle enzyme from the majority of species is obtained in the 60–75% saturation (pH 6.0) fraction, but, in cases where there was less enzyme present, 60–80% saturation was required; in some cases the 55–80% satd. fraction was used to improve recovery. As a result, the degree of purification was small at this step, but it served to concentrate the enzyme into a volume that could be rapidly desalted. In some cases, a heat treatment at slightly acid pH (5.5) was carried out after the (NH₄)₂SO₄ fractionation, which denatured a substantial portion of the contaminating proteins (see Table 2). The fraction was then adjusted to pH 7.0 with 1M-Tris, and desalted by passage through a large column (16cm² × 90cm) of Sephadex G-25 pre-equilibrated with 0.2mM-EDTA (acid form adjusted to pH 8.0 with Tris). The emergence of (NH₄)₂SO₄ from the column was checked by spot testing with barium acetate. The de-salted fraction was then made to the appropriate pH by addition of 10mM-Tris base, and lowering the pH with a 1M solution of the zwitterionic buffers [Mes (4-morpholine-ethane sulphonic acid) or Mops (4-morpholine-propane sulphonic acid)] to be used for adsorption in ion-exchange chromatography. The sample was diluted with buffer if necessary to a protein concentration of less than 15mg/ml.

The column sizes were normally 16cm² in cross section, and from 5 to 10cm tall for each litre of extract being used. After application of the sample, the column was washed with 100ml of adsorption buffer, then with 2 to 3 column volumes of pre-elution buffer. Flow rates were from 300 to 400ml/h. Generally the pre-elution buffer was of a higher pH to loosen the adsorbed phosphoglycerate kinase before

elution by substrate. It also contained an extra 0.3–0.5 mM-EDTA, to give comparable ionic properties with 0.5 mM-3-phosphoglycerate. However, it was latterly discovered that phosphoglycerate kinase has a strong affinity for multivalent anions, EDTA included; more recently, we have adopted the alternative procedure of adding ionic strength by including 3 mM-sodium acetate. After this pre-elution wash, the buffer was changed to one containing 0.5 mM-3-phosphoglycerate instead of EDTA or acetate, and phosphoglycerate kinase was then eluted, usually with the substrate front.

The peak containing phosphoglycerate kinase activity was collected, and concentrated either by salting out with $(\text{NH}_4)_2\text{SO}_4$ (60 g/100 ml), or by ultrafiltration. To remove any remaining contaminants the sample was then subjected to gel-filtration chromatography: Sephadex G-100, G-150 and Sephacryl S-200 are all satisfactory for this purpose; the buffer used was 10 mM-triethanolamine, pH 7.5, containing 0.1 M-NaCl. Most of the probable contaminating proteins have molecular weights larger than phosphoglycerate kinase. In some cases this gel-filtration step was unnecessary as the enzyme was already virtually pure, and could be crystallized directly.

The final preparation was salted out with $(\text{NH}_4)_2\text{SO}_4$, taken up in a small volume of 30 mM-

imidazole buffer, pH 7.0, made turbid with saturated $(\text{NH}_4)_2\text{SO}_4$, and left in the cold-room. In most cases the enzyme was crystallized; with some species this occurred overnight, with others it took longer, up to several months. Crystal forms were mostly needles, varying from a sub-microscopic sheen to large chunky shapes of volume up to 0.5 mm^3 (horse and bovine muscle enzymes). The avian phosphoglycerate kinases (chicken, pigeon, emu) did not crystallize in these conditions.

In a few cases there has been more than one electrophoretic form of the enzyme in one tissue. For instance, many marsupials contain traces of the genetically distinct phosphoglycerate kinase-B (Cooper *et al.*, 1971) in muscle tissue, and many species contain minor components, probably epigenetic modifications of the main enzyme type. In the case of chicken muscle, two components are present in similar amounts, which can be separated by a gradient of 3-phosphoglycerate (Scopes, 1977b). This substrate gradient scheme has also been used successfully to separate the phosphoglycerate kinase-A and phosphoglycerate kinase-B from ram testes (Stewart & Scopes, 1975, 1978).

When using tissues such as liver or muscles from some large animals, which contain large amounts of pigmented protein (myoglobin and haemoglobin), it was beneficial to pass the fraction through a column

Table 2. Outline of purification schemes involving affinity elution chromatography

Buffer compositions were: 10 mM-Tris adjusted to pH with Mes (pH 6.0–6.8); 10 mM-Tris adjusted to pH with Mops (pH 7.0–7.6); 10 mM-NaOH adjusted to pH with Tricine {*N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine} (pH 8.0).

Enzyme source	$(\text{NH}_4)_2\text{SO}_4$ fraction (% saturation)	Adsorption buffer (pH)	Elution buffer (pH)	Specific activity after gel filtration (units/mg)	Recovery (%)	Yield (mg/kg)
Pig muscle	60–75*	6.0	6.5	700	45	500
Bovine muscle	60–75*	6.5	7.2	710	62	250
Bovine liver	55–80	6.5	7.2	650	25	11
Sheep muscle	60–75*	6.5	7.2	680	50	300
Horse muscle	60–75*	7.0	8.0	700	56	400
Rabbit muscle	60–75*	6.5	8.0	700	50	350
Rabbit liver	55–80	6.5	8.0	540	36	28
Rat muscle	60–75*	6.0	6.5	460	42	350
Rat liver	55–80	6.0	6.5	300	20	60
Wombat muscle	60–75*	6.5	6.5	700	55	300
Brushtail possum muscle	60–75*	6.0	6.5	700	30	190
Ringtail possum muscle	60–75*	6.0	6.5	690	44	240
Emu muscle	60–75	6.5	8.0	720	35	160
Chicken muscle	60–75	6.5	8.0	680	55	480
Pigeon muscle	60–75	6.5	8.0	620	35	110
Sea trout muscle	55–80†	6.5	8.0	690	50	570
Rainbow trout muscle	55–80†	6.5	8.0	720	47	510
Bream muscle	55–80†	6.5	7.2	560	45	320
Red snapper muscle	55–80†	6.5	7.2	690	25	160
Yeast	60–75	6.0	7.0	700	52	650‡

† The $(\text{NH}_4)_2\text{SO}_4$ fraction was dissolved in a pH 5.5 acetate buffer and heat-treated for 10 min, pH 5.5, at 40°C (†) or 45°C (), cooled, and the denatured protein removed by centrifugation.

‡ Calculated on the basis of equivalent wet weight of yeast.

Table 3. Outline of purification schemes not involving affinity elution chromatography

Enzyme source	(NH ₄) ₂ SO ₄ fraction (% saturation)	DEAE-cellulose chromatography buffer	Specific activity after gel filtration (units/mg)	Recovery (%)	Yield (mg/kg)
Eastern grey kangaroo muscle	55-75*	20mM-Tris/HCl, pH 8.2 KCl gradient to 50mM	600	18	90
Western grey kangaroo muscle	55-75*	20mM-Tris/HCl, pH 8.2 KCl gradient to 50mM	650	20	85
Toad muscle	55-80	20mM-Tris/HCl, pH 8.2 KCl gradient to 100mM	470	40	170
Spinach and silverbeet†	55-75	20mM-Potassium phosphate, pH 7.0 Potassium phosphate gradient to 80mM	780	30	30
<i>E. coli</i>	55-85	20mM-Tris/HCl, pH 8.2 KCl gradient to 200mM	480	50	440

* See footnote to Table 1.

† Method of Cavell & Scopes (1976).

of DEAE-cellulose at pH 8.0 before further lowering the pH for CM-cellulose chromatography (Blake *et al.*, 1972). This removed all haemoglobin, often myoglobin also, as well as many other acidic proteins, but in very few cases does phosphoglycerate kinase adsorb on DEAE-cellulose, if it also adsorbs on CM-cellulose at a pH as high as 6.0. This step was included in all preparations from liver, and from bovine and horse muscle.

Outlines of the purification procedures used for each of the phosphoglycerate kinases are presented in Tables 2 and 3. The purified samples used in the comparative studies were made by these procedures, with a crystallization and/or gel-filtration step at the end. However, somewhat different (NH₄)₂SO₄ fractions, or omission of heat treatments, would still in most cases lead to equally pure preparations, such is the specificity of the subsequent affinity elution chromatography. The steps before chromatography can be regarded merely as a 'cleaning-up' process to obtain a fraction suitable for application to the column.

Table 3 describes the preparation of those types of the enzyme that cannot be adsorbed on CM-cellulose at pH 5.8; these are sufficiently acidic to adsorb on DEAE-cellulose at pH 8.2, and conventional gradient-elution procedures from this adsorbent have been used.

Comparative properties of phosphoglycerate kinases

Despite the wide diversity of sources of these phosphoglycerate kinase preparations and the physiologically different functions, e.g. glycolytic, gluconeogenic and photosynthetic, the enzymic and physical properties vary very little. The specific activities of all pure preparations have proved to be identical, within experimental error, and where the enzyme has not been completely purified the specific activity has been lower by an amount

expected from the degree of contamination as judged by gel electrophoresis. Molecular weights, as determined by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis, have always been between 45000 and 48000 [*E. coli* and other bacterial enzymes being slightly smaller (see, e.g., Suzuki & Imahori, 1974)], and Sephadex-gel filtration of the purified preparations has always indicated that the native molecular weights correspond to monomers of this size. Complete amino acid composition of each preparation has not been determined, but contents of tryptophan and tyrosine (u.v. methods) were measured, and cysteine by using Ellman's (1959) reagent. The u.v. determinations of tryptophan and tyrosine were carried out in combination with protein determination based on measurement of *A*₂₈₀ and *A*₂₀₅ (Scopes, 1974). All readings were corrected for light scattering by subtraction of any absorption detected above 320nm. The results of these determinations are given in Table 4.

Peptide 'mapping'

Two-dimensional tryptic peptide 'mapping' has given an indication of the differences in primary structure between the preparations for each species. Mammalian phosphoglycerate kinases are very similar, and no consistent difference could be seen between muscle and liver preparations from the same species. The 'maps' are complex; theoretically between 50 and 60 peptides should be obtained on tryptic digestion. In the system we used, between 40 and 50 peptides could be clearly distinguished. The complexity of these 'maps' makes direct comparison difficult; tracings of four examples, which have been repeated several times, are shown in Fig. 2. These show that whereas there are a few significant differences between the rabbit and bovine muscle enzymes, the differences between the bovine muscle

Table 4. *Some structural and kinetic parameters for phosphoglycerate kinases*

For the energy of activation determinations, both conditions (1) and (2) contained 3 mM of each substrate, but in state (2) 40 mM- Na_2SO_4 was included in the reaction buffer.

Enzyme source	Amino acid content (mol/mol)			K_m for MgATP (mM)	$E_{act.}$ values (kJ/mol)	
	Tryptophan	Tyrosine	Cysteine		(1)	(2)
Pig muscle	4	4	6	0.33	29	—
Bovine muscle	4	5	6	0.30	29	31
Bovine liver	4	5	6	0.31	29	31
Sheep muscle	4	5	6	0.28	32	32
Horse muscle	4	4	7	0.34	28	30
Rabbit muscle	4	4	8	0.36	27	30
Rabbit liver	4	4	8	—	—	—
Rat muscle	4	6	5	0.25	29	—
Eastern grey kangaroo muscle	4	5	6	0.29	31	33
Western grey kangaroo muscle	4	6	7	0.34	30	34
Pademelon muscle	4	6	5	0.23	26	31
Wombat muscle	4	6	6	0.29	27	—
Brush-tail possum muscle	3	3	4	0.30	32	33
Ringtail possum muscle	3	3	4	0.33	31	33
Emu muscle	3	3	7	0.28	23	27
Chicken muscle	3	3	6	0.33	25	29
Pigeon muscle	3	3	4	0.18	25	30
Sea trout muscle	4	5	4	0.30	27	—
Rainbow trout muscle	4	5	4	0.30	26	28
Bream muscle	3	5	4	0.31	21	—
Red snapper muscle	3	5	4	0.33	23	28
Silverbeet	2	6	1	0.42	13	25
Spinach	2	6	1	0.35	13	26
Yeast	2	7	1	0.20	18	29
<i>E. coli</i>	2	8	1	0.24	7	—

and liver enzymes cannot be regarded as significant. It may be noted that spots corresponding to the positions of free lysine and free arginine are present in all four examples, except for the arginine spot in the yeast 'map.' There is also an *N*-terminal proline peptide (as deduced from the colour) in exactly the same position in every 'map' run (16 species).

Kinetic measurements

It has been observed that phosphoglycerate kinases do not always give linear plots as expected from Michaelis-Menten kinetics (Larsson-Raznikiewicz, 1967; Yoshida & Watanabe, 1972; Orr & Knowles, 1974). A detailed investigation of these effects for the yeast enzyme is presented elsewhere (Scopes, 1978). Linear plots can be obtained in the presence of high concentrations of anions, especially multi-valent ones, so in comparing parameters of the enzymes from different species, we have included 40 mM- Na_2SO_4 in the assay mixture, and report the apparent K_m values for MgATP under these conditions. The values were almost all in the range of 0.2 to 0.4 mM (Table 4) and there is no obvious evolutionary trend. The energy of activation for each enzyme was also determined, both in the presence

and in the absence of 40 mM- Na_2SO_4 (Table 4). It can be seen that all mammals had $E_{act.}$ values of 30 ± 3 kJ/mol in both conditions, whereas the birds and fish had lower values in the absence of SO_4^{2-} . The plant and microorganism phosphoglycerate kinases had very low apparent $E_{act.}$ values in the absence of SO_4^{2-} , which probably reflects a temperature effect on the substrate affinity as well as on the catalytic process. The presence of SO_4^{2-} (one effect of which is to lower the substrate concentrations required for near maximum velocity) resulted in the $E_{act.}$ for phosphoglycerate kinase from these species being closer to that for the enzymes from animal species. Further kinetic investigations included the effects of SO_4^{2-} on the enzyme activity, similar to those described for the yeast enzyme (Scopes, 1978). With the sole exceptions of the plant 'photosynthetic' enzymes, all phosphoglycerate kinases were activated by SO_4^{2-} ions at sub-saturating substrate concentrations (Fig. 3).

Discussion

Phosphoglycerate kinase exists in virtually every biological tissue, functioning either in glycolysis,

Fig. 2. Tryptic peptide 'maps' for four phosphoglycerate kinase preparations; for details see the text (a) Bovine muscle enzyme; (b) bovine liver enzyme; (c) rabbit muscle enzyme; (d) yeast enzyme. Black spots contained tryptophan (detected by fluorescence); R, position of free arginine; K, position of free lysine; hatched spots represent a proline peptide common to all species; X marks the origin.

Fig. 3. Activation and inhibition of phosphoglycerate kinases by Na_2SO_4

The substrate conditions were 0.2 mM of both MgATP and 3-phosphoglycerate. ●, Yeast enzyme; ○, silverbeet enzyme; △, rabbit muscle enzyme. Out of 23 species, only the spinach and silverbeet enzymes were not activated in the 0 to 10 mM- Na_2SO_4 range; the enzymes from other sources behaved similarly to the muscle and yeast enzymes illustrated.

gluconeogenesis or photosynthetic carbon fixation. It has now been isolated from a wide variety of species; although in mammals, for reasons of convenience, muscle has been the tissue of choice, genetic evidence suggests that apart from the phosphoglycerate kinase-B isoenzyme present in male reproductive tissues, all tissues have the same genetic form, phosphoglycerate kinase-A. Thus it can be assumed that, although human muscle enzyme has not been included in the present paper, its properties are as described elsewhere for the human erythrocyte enzyme (Hashimoto & Yoshikawa, 1962; Yoshida & Watanabe, 1972). But it may still be that some species do have another distinct isoenzyme in some tissues; it has been suggested that bovine liver enzyme is distinct from the muscle enzyme, on the evidence of its different properties from those of the rabbit muscle enzyme (Bojanovski *et al.*, 1974). A later paper from this group describing the properties of the bovine liver enzyme (Kulbe *et al.*, 1975) did not present any further evidence that

it differed from the bovine muscle enzyme; we believe them to be identical. The peptide maps of the muscle enzyme of animals are all very similar. And so, although we cannot see any conclusive difference between bovine liver and muscle enzyme by this technique, neither are there many differences between the maps of bovine and rabbit muscle. Nevertheless, the genetically distinct isoenzymes phosphoglycerate kinase-A and phosphoglycerate kinase-B of sheep do have a few significant differences in peptide mapping (Stewart & Scopes, 1978). We feel that a genetically distinct isoenzyme present in liver for gluconeogenesis would have evolved at least by early vertebrate development, and would have diverged from phosphoglycerate kinase-A sufficiently to be detected by peptide mapping.

The technique of affinity elution chromatography has greatly simplified the purification of the enzyme from many different sources. For purifying another enzyme from many sources, the procedure to be adopted is as follows. First establish a procedure for one convenient species as described by Scopes (1977a). Then determine the relative isoelectric point by an electrophoretic procedure on the crude extract of each new species' tissue, using a specific staining method. The pH for adsorption and elution can then be chosen according to these results without necessarily having to go through the full trial-and-error procedure described before.

The reported isoelectric points for both rabbit and yeast enzymes are close to 7.0 (Krietsch & Bücher, 1970). Although the starch-gel electrophoretic system used here suggests that the yeast enzyme is rather more acidic than the rabbit enzyme, it is unlikely that either are positively charged at pH 8.0. Yet the rabbit enzyme remains adsorbed on the CM-cellulose at pH 8.0, but is instantly displaced on the addition of substrate. It is proposed that for this enzyme at least, a localized positively charged area at the active site (all its substrates are strongly negatively charged), is attracted to the adsorbent even if the overall charge of the protein is negative. Ion-exchange adsorption at the active sites of enzymes having charged substrates then becomes more like true affinity adsorption chromatography, and the addition of substrate displaces the enzyme by competing with the adsorbent for the active site. Phosphoglycerate kinase is known to have lysine, arginine and histidine residues (Markland *et al.*, 1975; Hjelmgren *et al.*, 1976; Tanswell *et al.*, 1976) at or close to its active site, and in view of the instant displacement of the enzyme from the column on addition of substrate, this active-site binding by electrostatic interaction seems most likely. It may be possible to develop even better adsorbents, based on normal affinity chromatography materials with charged groups on spacer arms.

The consistency of the properties of phospho-

glycerate kinase regardless of source is remarkable, especially considering its various physiological roles. X-ray crystallographic studies have shown that the enzyme has a bi-lobal structure, with an adenine nucleotide binding site in the cleft between them, but associated with one lobe only. The function of the second lobe has been speculated on; it would seem that its size and structure must be important since otherwise many changes in this lobe would be expected to have occurred during evolution, which would be manifested in the structural properties of the enzymes from the widely divergent sources studied to date.

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