

## PURIFICATION BY AFFINITY CHROMATOGRAPHY, PROPERTIES AND CRYSTALLISATION OF PHOSPHOFRUCTOKINASE FROM THERMOPHILIC MICRO-ORGANISMS

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### 1. Introduction

Phosphofructokinase (PFK) catalyses the inter-conversion of fructose 6-phosphate (F-6P) and fructose 1,6-diphosphate (FDP) in a reaction involving ATP that represents the first unique step in glycolysis (for review see [1]). The activity of PFK is regulated by a number of intracellular metabolites, and a study of its mode of action, and of the mechanisms through which it exercises control over the rate of glycolysis in the cell, is therefore of particular biochemical interest.

PFK has been isolated in pure form from a number of different sources [1]. Enzymes from muscle and from yeast possess complex oligomeric structures (with large subunits with mol. wts of 85 000 [1] and 130 000 [2] respectively) that have hitherto proved difficult to study by methods of protein chemistry and X-ray crystallography. PFKs of microbial origin have been reported [1] to possess lower molecular weights and less complex subunit structures. More specifically earlier work from this laboratory (reference [3] and unpublished results of E. Kolb and J. I. Harris) has shown that PFK from *B. stearothermophilus* with a mol. wt of 130 000 is a thermostable tetrameric enzyme with four identical protein chains each comprising 300 amino acid residues.

The enhanced stability of enzymes from thermophilic micro-organisms can be utilised to advantage in enzyme purification and in studies of enzyme structure and mechanism (cf [4, 5]). For example, in an exploratory study of glycolytic enzymes from the extreme thermophile, *T. aquaticus* (grows optimally at 70–75°C), Hocking and Harris [6] showed that pure glyceraldehyde 3-phosphate dehydrogenase (GPDH)

could be obtained in high yield from partially purified cell extracts by affinity chromatography on NAD-Sepharose. Partial purification of *T. aquaticus* PFK was also achieved. Other examples of the use of Sepharose-linked nucleotides in the purification of dehydrogenases and kinases have since been reported (eg. [7, 8]) and we now describe a method that allows pure GPDH and PFK to be obtained from cell extracts of *T. aquaticus* and of *B. stearothermophilus* by the combined application of NAD-Sepharose and AMP-Sepharose. Some molecular and kinetic properties of the purified PFKs are also given together with a method for crystallising the *B. stearothermophilus* enzyme for X-ray crystallographic analysis.

### 2. Methods and results

'NAD-Sepharose' was prepared and used to purify GPDH as previously described [6]. 'AMP-Sepharose' (*N*<sup>6</sup>-(6 aminoethyl) AMP-Sepharose) was prepared by reacting CNBr-activated Sepharose 4B with 6-chloropurine-5'-riboside phosphate by a method similar to that described by Guilford et al. [9]. *B. stearothermophilus* (NCA 1503) and *T. aquaticus* (ATCC 25104) cells were grown (cf [10]) at 60°C and 70–75°C respectively at M. R. E. Porton. Disruption of cells and extraction of cell paste was carried out as before [6, 11]. Partially purified enzyme fractions were prepared at M. R. E. Porton by a modification (A. Atkinson and C. Bruton, to be published) of the earlier [6, 11] procedures. GPDH [4], [6] and PFK [12] were assayed at 20°C in thermostated cuvettes in a Guilford 222A recording spectrophotometer.

### 2.1. Purification of GPDH and PFK from *T. aquaticus*

The cell extract [6] from *T. aquaticus* was adsorbed batchwise onto DEAE-cellulose (2 ml settled vol. per g cells). The fraction eluting between 0.1 and 0.4 M NaCl in 50 mM Tris-HCl, pH 7.5, containing 5 mM  $\beta$ -mercaptoethanol (ME) 1 mM  $MgSO_4$  and 0.1 mM EDTA (Buffer A) was dialysed against Buffer A and then reabsorbed onto DEAE-Sephadex (1–2 ml settled vol. per g cells). Fractions rich in aldolase (a), GPDH (B) and PFK (c) were eluted with Buffer A containing 180 mM NaCl, 240 mM NaCl, and 350 mM NaCl, respectively.

Fraction (b) (the content of GPDH in different batches of cells varies between 50 and 200 mg per Kg frozen cells) was brought to 1.2 M ammonium sulphate, centrifuged, and the supernatant adsorbed onto NAD-Sephadex (1–2 ml settled vol. /mg GPDH). The column was washed with 100 mM phosphate, pH 6.8, 5 mM  $\beta$ -ME, 1 mM EDTA (Buffer B) containing 500 mM NaCl, and was then eluted with the same buffer containing 10 mM  $NAD^+$  in order to obtain pure GPDH as previously described [6].

A sample of fraction (c) (100 ml containing 380 units PFK, spec. act. 0.43 units/mg protein) was applied to a column of AMP-Sephadex (1–2 ml settled vol. /mg PFK). The column was washed with several column vol. of Buffer A containing 400 mM NaCl until the effluent was free of  $A_{280\text{ nm}}$  absorbing material. It was then eluted with same buffer containing 2 mM ATP/5 mM  $Mg^{++}$  and fractions containing PFK activity (50 ml) were pooled, dialysed against Buffer A and concentrated by pressure dialysis. The recovery of PFK activity was 85–90%. The protein content was estimated by a colorimetric method [13] and from the results of amino acid analyses (table 1). The specific activity was calculated to be 37 units/mg at 20°C.

### 2.2. Properties of *T. aquaticus* PFK

The material eluted from AMP-Sephadex was pure. It gave a single protein band when examined by gel-electrophoresis with [14] and without [15] SDS. The pattern of protein bands given by the fractions applied to and eluted from AMP-Sephadex are shown in fig. 1. The subunit mol. wt. was estimated (from its mobility in a 0.1% SDS/12.5% acrylamide gel) to be 32 000. The mol. wt of the active enzyme (measured by gel-filtration on a calibrated column of

Table 1  
Amino acid composition<sup>a</sup> of the subunit in phosphofructokinase from *T. aquaticus*

	Mean values	Nearest integer
Cysteine <sup>d</sup>	0	0
Aspartic acid	19.29	19
Threonine <sup>b</sup>	15.69	16
Serine <sup>b</sup>	14.56	15
Glutamic acid	35.16	35
Proline	9.81	10
Glycine	43.02	43
Alanine	41.99	42
Valine <sup>c</sup>	28.77	29
Methionine	7.35	7
Isoleucine <sup>c</sup>	21.19	21
Leucine	26.17	26
Tyrosine	4.40	4
Phenylalanine	7.60	8
Lysine	10.41	10
Histidine	4.93	5
Arginine	22.11	22
Tryptophan <sup>c</sup>	0.78	1
Total		313

<sup>a</sup> After 24, 48 and 72 hr hydrolysis calculated for a mol. wt of 32 500.

<sup>b</sup> Extrapolated to 0 hr hydrolysis.

<sup>c</sup> Extrapolated to 120 hr hydrolysis.

<sup>d</sup> Following radioactive carboxymethylation and performic oxidation

<sup>e</sup> Spectroscopic estimation [20].

Sephadex G-200 [16] is in the range of 130 000 to 140 000 showing that *T. aquaticus* PFK, like its counterpart from *B. stearothermophilus* [3] and unpublished results of E. Kolb and J. I. Harris), is a tetramer with four probably identical protein chains each comprising approximately 310 amino acid residues (cf amino acid analysis, table 1) Of particular interest is the total absence of cysteine (in accord with its lack of reactivity towards DTNB and iodoacetate, even in the presence of 6 M guanidine) indicating that SH groups cannot be essential, either for the regulation of the catalytic activity of procaryotic PFK. It would appear that all but essential SH groups (such as for example Cys-149 in *T. aquaticus* GPDH [6]) are deleted in *T. aquaticus* enzymes (cf [17]), presumably because SH groups are possible sites of inactivation in enzymes

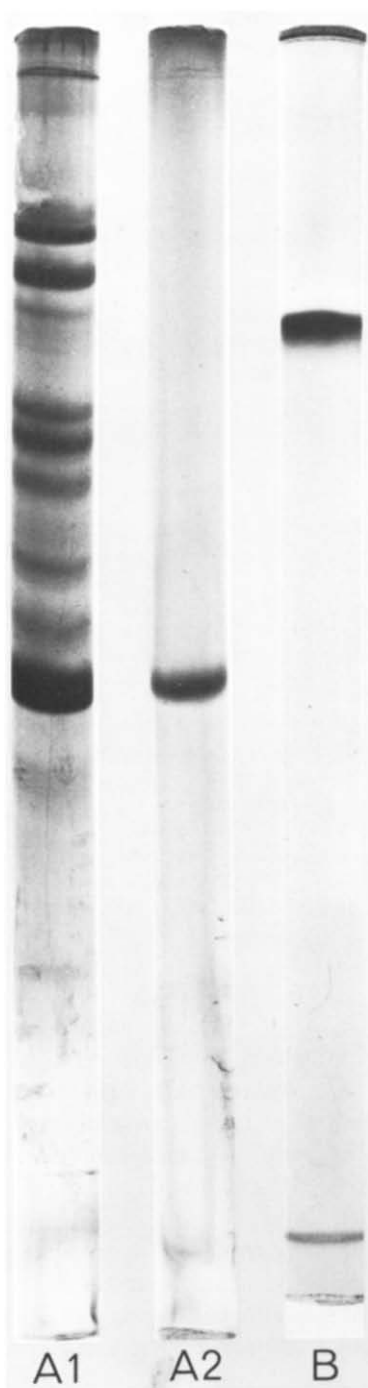


Fig.1. Gel-electrophoresis of *T. aquaticus* PFK. (A) In 0.1% SDS [14]. (1) before and (2) after AMP-Sepharose. (B) Native gel at pH 8.9 [15].

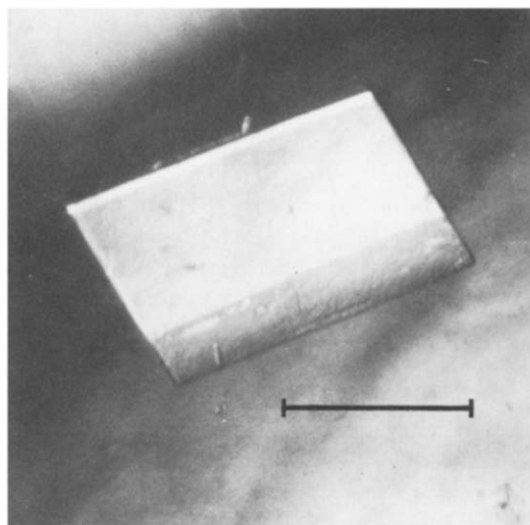


Fig.2. Crystals of *B. stearotherophilus* from 2 M sodium phosphate (pH 6.0). The scale line represents 0.5 mm

that, of necessity, must retain activity under aerobic conditions at temperatures in excess of 70°C.

*T. aquaticus* PFK is remarkably thermostable (a 0.2 mg/ml solution in Buffer A retains full activity after 24 hr at 80°C) and thus resembles PFK from another extreme thermophile, *Flavobacterium thermophilum* [18].

ATP behaves as a normal substrate with a  $K_M$  of  $2.5 \cdot 10^{-5}$  M. The  $K_M$  for the second substrate, F-6P ( $3 \cdot 10^{-4}$  M) on the other hand is subject to a 5–10-fold variation, depending upon the concentration of phosphoenolpyruvate (PEP), which in the range of  $10^{-4}$  to  $10^{-3}$  M is an inhibitor, and of ADP which in the same range of concentration is a potent activator of the enzyme. PFK activity in *T. aquaticus* is thus subject to regulation by the intracellular concentrations of PEP and ADP. In this respect it resembles other prokaryotic PFKs [18, 19] but differs from the muscle and yeast enzymes which are subject to allosteric regulation by ATP [1].

### 2.3. Purification and crystallisation of *B. stearotherophilus* PFK

PFK from *B. stearotherophilus* was obtained pure by a method similar to that already described for preparing pure *T. aquaticus* PFK from DEAE-

Sephadex fraction (c). Cells were grown, disrupted and extracted according to published methods [10, 11] and the PFK-rich fraction eluted from DEAE-Sephadex (6100 units PFK with a spec. act. of 2.9 u/mg total protein) was applied to a column of AMP-Sepharose (1–2 mg settled vol. /mg PFK) equilibrated with Buffer A. The column was washed with several column volumes of Buffer A containing 400 mM NaCl and the PFK activity was eluted with the same buffer with the addition of 2 mM ATP and 5 mM  $Mg^{++}$ . The PFK prepared in this way possessed a specific activity of  $105 \pm 5$  Ju/mg and was in all respects identical to enzyme prepared earlier (albeit with considerably more difficulty and in lower yield [3]; E. Kolb and J. I. Harris, unpublished results) by methods not involving the use of AMP-Sepharose. It has a mol. wt of 130 000 and comprises four identical subunits. The complete amino acid sequence of the subunit comprising some 300 residues has been determined and will be published elsewhere (E. Kolb and J. I. Harris, 1975).

Crystals (1 mm  $\times$  1 mm  $\times$  0.3 mm) were obtained by dialysis against 2 M sodium phosphate, pH 6.0, (fig. 2) and a preliminary X-ray crystallographic study of these crystals is being undertaken.

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