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TWO *ESCHERICHIA COLI* FRUCTOSE-6-PHOSPHATE KINASES

PREPARATIVE PURIFICATION, OLIGOMERIC STRUCTURE AND IMMUNOLOGICAL STUDIES

D. KOTLARZ and H. BUC

Département de Biologie Moleculaire, Institut Pasteur, 25, rue du Dr. Roux, 75015 Paris (France)

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Summary

Two isoenzymes of fructose-6-phosphate kinase (ATP : D-fructose-6-phosphate 1-phosphotransferase, EC 2.7.1.11) are present in *Escherichia coli* K12. One isoenzyme is allosterically inhibited by phospho*enol*pyruvate and activated by nucleoside diphosphates, and is a tetramer composed of four subunits of molecular weight 35 000. A simple method for the purification of this enzyme is reported. Equilibrium dialysis indicates that there are four ATP sites and four GDP sites per tetramer.

The second isoenzyme is present in low quantity in wild type bacteria. This enzyme is devoid of allosteric properties. A complete method of purification is described. Determination of its molecular weight under native and denaturing conditions indicates that this protein is a dimer composed of two subunits of molecular weight 36 000. Antisera have been produced against both isoenzymes. The antiserum against one isoenzyme does not cross-react with the other.

Discrepancies between our results and those of other workers are discussed.

Introduction

Two phosphofructokinase isoenzymes (ATP : D-fructose-6-phosphate 1-phosphotransferase, EC 2.7.1.11) have been detected in *E. coli* K12 [1]. The major species, PFK_1, is an oligomeric enzyme composed of four subunits of 35 000 ± 2000 daltons [2]. From kinetic studies, it appears that this enzyme is activated by fructose 6 phosphate and nucleoside diphosphates and allosterically inhibited by phospho*enol*pyruvate [3]. This behaviour can be quantitatively accounted by the Monod-Wyman-Changeux model assuming that four sites are present for each ligand on the oligomer [3].

Bacteria mutated at the pfkA locus lose this activity [1,4]. Biosynthesis of phosphofructokinase$_1$ is derepressed in glucose media and under anaerobic conditions [5].

The second enzyme, phosphofructokinase$_2$, represents less than 5% of the total fructose-6-phosphate kinase activity in the wild type *E. coli* strain. Its biosynthesis is increased 10-fold in mutants suppressed at a locus pfkB, unlinked to pfkA [1,6]. This enzyme exhibits no allosteric characteristics. No derepression is observed in the wild type strain when the nature of the carbon source or the oxygen pressure is changed during the growth [5].

We decided to purify fully both phosphofructokinase$_1$ and phosphofructokinase$_2$ from well-characterized strains, to check whether or not their molecular weights were affected by the growth conditions and to ascertain their oligomeric structures. Large quantities of pure phosphofructokinase$_1$ were required to perform these physico-chemical studies as well as to ascertain if the number of effector sites was equal or not to the number of subunits as predicted from the kinetic studies. It was therefore necessary to scale up and simplify the previously reported preparation of this enzyme [5]. Furthermore, to find whether these two species were immunologically related, we prepared antisera against both of them and looked for their partial identity.

Material and Methods

Strains

For the preparation of phosphofructokinase$_1$ the strain DF 1000 (B_1^-, Hfr C), which will be designated as A_0B_0, has been used. Inactive protein phosphofructokinase$_1$ has been isolated from isogenic strains, A_1B_0, carrying a punctual mutation at the pfkA locus (AM_1 or AM_2). Phosphofructokinase$_2$ has been purified from the strain A_0B_1 (*F*$^-$, *edd*-1$^-$, *galk*$^-$, *his*$^-$, *pfkB*$_1$, *pyr D*$^-$, str^R, *tyr*$^-$ *A*). All these strains were gifts from Dr. Fraenkel.

Media for growth of the cultures under aerobic or anaerobic conditions have been described in reference [5]. Concentrations of dissolved oxygen under these conditions are 7 and 0 ppm, respectively. Whenever large quantities of bacteria are required for enzyme purification, cells were aerobically grown at 37° C in minimum salt medium 63 supplemented with 10 g/l of bactotryptone (Difco) and 4 g/l of yeast extract (Difco). Though the concentration of dissolved oxygen has not been precisely measured in this case, its corresponds to an "excess state", as defined by Reichelt and Doelle (input partial pressure of oxygen equal to 60 mm of Hg) [7]. Cells are harvested in the middle of the exponential phase of the growth and eventually stored frozen at −20° C.

Assay of enzymatic activities

These were performed as described in ref. 5 unless otherwise specified. It is worthwhile to point out that phosphofructokinase$_1$ requires 0.5 mM KCl or 2 mM NH_4^+ for maximal activity (v effect). 10 mM KCl has been routinely added to the assay medium.

1. Purification of phosphofructokinase$_1$. 100 g of bacteria (wet weight) were ground in the presence of 200 g of alumina in the cold room. After complete homogenisation, 500 ml of TAEM buffer were added to the slurry (TAEM :

Tris base 50 mM, EDTA 1 mM, 2-mercaptoethanol 7 mM adjusted to pH 7.5 with acetic acid). After centrifugation for 150 min at 78 000 × *g*, the supernatant was collected.

Step 1: A Sepharose Dextran Blue gel was synthesized according to the method of Ryan and Vestling [8]. A column corresponding to 20 ml of packed Sepharose Dextran Blue was prepared in the coldroom. The crude extract was layered on top of the column and adsorbed at a rate of 60 ml/hour. The column was washed with the TAEM buffer until no absorbance at 280 nm was recorded in the eluate. The eluting buffer was then supplemented with 200 μM of ATP and 2 mM of $MgCl_2$ and elution was performed at the same rate as before. Phosphofructokinase activity was detected in the eluate. The peak (approx. 30 ml) is pooled and concentrated under pressure on a Diaflo membrane PM 10 (Amicon corporation) to a volume of 2 ml.

Step 2: Fructose 6-phosphate (10 mM), magnesium acetate (10 mM) and KCl (10 mM) were added to the solution which was heated for 10 minutes at 65° C in a water bath. Heat-denatured contaminating protein flocculated and was eliminated by centrifugation at 10 000 rev./min for 10 min at 4° C.

2. Purification of phosphofructokinase$_1$. The same procedure was used but only step 1 is performed. The mutated protein was detected in the eluate by its cross-reactivity against phosphofructokinase$_1$ antiserum on Ouchterlony plates (see below).

3. Purification of phosphofructokinase$_2$. All the operations were performed at 4° C. Crude extract from strain A_0B_1 was obtained as described for phosphofructokinase$_1$ preparation. Nucleic acids were precipitated by addition of streptomycin sulfate (3 g per 100 ml of crude extract) and discarded after centrifugation at 10 000 rev./min for 20 min.

Step 1: Ammonium Sulfate fractionation. The streptomycin sulfate supernatant was brought to 45% of saturation in ammonium sulfate by slowly adding 150 g of ammonium sulfate to 500 ml of the solution. The precipitate, which contained more than 90% of all the PFK_1 activity, was removed after centrifugation. The supernatant contained the second activity. To 500 ml of this solution, 65 g of ammonium sulfate were added, a concentration which precipitated the whole phosphofructokinase$_2$ activity. (Ammonium sulfate concentration =65% of saturation).

Step 2: Hydrophobic chromatography. An *n*-propyl Sepharose column was prepared according to the method of Er-El et al. [9]. The protein solution was adsorbed on an 80-ml column which had been previously equilibrated against a TAEM buffer containing 1.2 M of $(NH_4)_2SO_4$. The column was first rinsed with the same buffer: 70% of the proteins were eluted. When the absorbance at 280 nm reached zero, a linear gradient of $(NH_4)_2SO_4$ in TAEM, decreasing from 1.2 M to 0 M, was applied. Phosphofructokinase$_2$ activity was eluted at 0.3 M $(NH_4)_2SO_4$. Fractions corresponding to the phosphofructokinase$_2$ activity were pooled together and salted out by addition of 430 g of $(NH_4)_2SO_4$ per l of solution. After centrifugation, the precipitate was dissolved in 3 ml of TAEM buffer and dialysed for 2 h against this buffer.

Step 3: DEAE cellulose chromatography. This fraction was applied to a 50 ml column of DEAE cellulose (Whatman DE 52) equilibrated in TAEM buffer. After extensive washing with this buffer, elution was performed by a linear

gradient of NaCl between 0.05 and 0.3 M. Phosphofructokinase activity was eluted at an ionic strength of 0.26 M.

Step 4: Heat denaturation. After addition of Fru-6-*P* (10 mM) and $MgCl_2$ (10 mM), the active fraction was heated for 10 min at 65° C. After centrifugation, the supernatant was concentrated under pressure on a Diaflo membrane PM 10 until the volume reaches 1.5 ml.

Step 5: glycerol gradient centrifugation. 300-μl aliquots of the sample were layered on linear glycerol gradients of 12 ml each prepared in cellulose nitrate centrifuge tubes (composition: TAEM buffer containing 100 mM NaCl, glycerol concentration ranging from 30 to 5%). After centrifugation at 39 000 rev./min for 24 h at 4° C in a swinging bucket rotor SW 41 (Beckman Ltd), the centrifuge tubes were punctured at the bottom and 30 fractions were collected, phosphofructokinase$_2$ activity was detected at a position which corresponds to a sedimentation constant, $s_{w,20}$ of 4.5 ± 0.2 Svedberg units. Fractions are assayed by SDS polyacrylamide gel electrophoresis. All the activity was recovered in a single peak but only 25% of the activity was found in fractions showing a single band in SDS gel electrophoresis.

4. Molecular weight determinations. Estimation of molecular weight of the denatured protein was performed by polyacrylamide SDS gel electrophoresis according to the method of Laemmli [10]. The following standards have been used: bovine serum albumin (68 000), glutamate dehydrogenase from calf liver (56 000), rabbit muscle aldolase (40 000), rabbit muscle myokinase (23 000). Molecular weight determination of native phosphofructokinase$_1$ was performed by sedimentation equilibrium according to the method of Yphantis [11]. The protein sample was dialysed against Tris · HCl, 100 mM, $MgCl_2$ 1 mM, 2-mercaptoethanol 5 mM pH 8.2 supplemented with the effectors listed in Table II when required. The final protein concentration was 300 μg/ml. Equilibrium was attained after 36 h of centrifugation at 16 000 rev./min. (temperature 10° C). Profiles were scanned at 280 nm.

Combined use of gel filtration and sedimentation velocity (for a description of the method cf. ref. 12): Sedimentation coefficients were measured at 4° C, in 5—20% sucrose gradients at different protein concentrations. Protein bands were detected by recording both the absorbance at 280 nm, and the enzymatic activity (the two methods give the same results). The following standards have been used to calibrate the gradients: bovine liver catalase ($s_{20,w}$ = 11.3) and rabbit muscle glycogen phosphorylase *b* (8.9), aldolase (7.5), creatine kinase (4.85). Values reported below are corrected for temperature and viscosity effects.

Gel filtration over Sephadex G-100 was performed according to the method of Andrews [13]. The elution volumes, v_i, found for phosphofructokinases and for protein standards of known Stokes radius, allows one to define the distribution coefficient K_{av} of each protein according to the relationship:

$$v_i = v_0 + (v_b - v_0) K_{av}. \quad (1)$$

v_0: void volume, defined by the position of β-galactosidase. v_b: bed volume, defined by the position of tritiated glucose. The relationship proposed by Laurent and Killander [14] between K_{av} and the Stokes radius, a, and verified

by Siegel and Monty [15] was used:

$$K_{av} = \exp - \alpha(a + \beta)^2 \quad (2)$$

Here α and β are two constants. We used cytochrome *c*, ovalbumin myokinase creatin kinase and aldolase to check this relation. K_{av} values found for phosphofructokinase$_1$ and phosphofructokinase$_2$ allow calculation of the corresponding Stokes radii.

Molecular weight was calculated according to:

$$M_r = \frac{6\pi\eta\, aNs^0}{(1 - \overline{v}\rho)} \quad (3)$$

η: viscosity of the medium; N: Avogadro number; s°: sedimentation coefficient.

5. Stoichiometry of GDP- and ATP-binding sites on phosphofructokinase$_1$. These experiments have been performed by equilibrium dialysis, according to the method described in ref. 16. Purity of radioactive and non-radioactive nucleotides was checked by chromatography on thin layer PEI cellulose plates in 1 M LiCl according to the method of Randerath and Randerath [17]. The two main problems which had, up to now, circumvented a precise assessment of the stoichiometry were the removal of tightly bound nucleotides and the accurate determination of the extinction coefficient of the protein. Removal of ATP was obtained by an extensive dialysis against TAEM buffer supplemented with 1 M NaCl. Extinction coefficient was determined by four different methods discussed in the legend of Table IV. Some of these methods required an accurate amino-acid analysis of phosphofructokinase$_1$. (given in Table III).

6. Immunological techniques. Antibodies against a given isozyme were prepared as follows: 60—75 μg of the purified protein were emulsified in complete Freund's adjuvant and injected hypodermically into the back of a rabbit (three rabbits were simultaneously treated). These injections were repeated every two weeks for 8 weeks. Ten days after the last injection, the blood was collected and allowed to clot for one day at a room temperature. 0.1% NaN_3 was added to the serum. In some cases, the sera were precipitated with ammonium sulfate (35% saturation). Before use, sera were dialyzed against 50 mM Tris/acetate, 150 mM NaCl, 0.1% NaN_3 pH 7.5 (buffer A).

Precipitation in liquid medium. Increasing quantities of antigen were added to a given volume of serum. The final volume was kept constant by addition of buffer A. Samples were agitated for two hours and centrifuged. Enzymic activity was measured in the supernatant. The presence of excess antigen or excess antibody in the supernatant was revealed on Ouchterlony plates. In competition assays between two antigens, the competitor was always added before the homologous antigen. Controls were always performed with serum coming from non immunized rabbits.

Immobilization of antibodies on Sepharose 4B was performed as described in Givol et al. [24]. Immobilized anti-phosphofructokinase$_1$ and anti-PFK$_2$ sera were kept at 4° C as a suspension in buffer A.

Results

1. Purification of the two fructose-6-phosphate kinases

As indicated in Table I, the Dextran Blue column allows an easy separation

TABLE I

PURIFICATION OF *E. COLI* PHOSPHOFRUCTOKINASE

Step	Specific activity (units/mg protein)	Total activity (units)	Yield (%)
Phosphofructokinase$_1$ from A_0B_0 strain			
Crude extract	0.3	2500	100
Affinity chromatography	180	1150—1750	40—70
Heat denaturation	190	1150—1750	40—70
Phosphofructokinase$_2$ from A_0B_1 strain			
Crude extract	0.2	2400	100
Ammonium sulfate fractionation	0.5	2200	90
Hydrophobic chromatography	12	1200—1400	50—60
DEAE-cellulose	25	1000	40
Heat denaturation	52	1000	40
Glycerol gradient			
center of the band	120	250	10
wings of the band	approx. 80—90	750	30

of the two types of enzymatic activities, since PFK_2 does not bind to the column. In two steps, phosphofructokinase$_1$ can be purified to homogeneity, as judged by polyacrylamide gel electrophoresis performed under denaturing conditions. The final specific activity corresponds to the formation of 190 μmol of ADP per min and per mg of enzyme. If all the subunits are catalytically active, this figure corresponds to a turnover of 100 per second in our assay conditions [1].

Phosphofructokinase$_2$ can also be purified to homogeneity but with a poorer yield (see Table I again). Addition of 5% glycerol to the buffers is essential to prevent partial inactivation of the protein between steps 1 and 4. Such inactivation is due to limited proteolysis, since polyacrylamide gel electrophoresis in SDS reveals the appearance of a broad band corresponding to a molecular weight of 30 000. Partially purified enzyme fractions may be stored for more than 24 h, at 0° C in the presence of 10% glycerol.

In both preparations, a step of thermal denaturation of contaminating proteins is involved. It has been checked that neither the oligomeric structure nor the catalytic properties of phosphofructokinase$_1$ and phosphofructokinase$_2$ are modified at this step.

2. Molecular weight determinations relative to phosphofructokinase$_1$ (see Table II)

The molecular weight of denatured polypeptide chains of phosphofructokinase$_1$ has been found reproducibly equal to 35 000, in fair agreement with the value reported by Blangy [2]. Determination of the molecular weight of the native assembly has been performed on the enzyme extracts from aerobically grown cultures. The three independent methods yield a molecular weight estimate of 140 000 ± 12 000, without any evidence for an association-dissociation equilibrium. Native PFK_1 appears therefore to be a tetramer at pH 8.2. It has been checked that this molecular weight determination was not affected by

TABLE II

MOLECULAR WEIGHTS, SEDIMENTATION COEFFICIENTS AND STOKES RADII OF PHOSPHOFRUCTOKINASE$_1$ AND PHOSPHOFRUCTOKINASE$_2$

	M_r (native)	s^0 (Svedberg units)	a (nm)
Phosphofructokinase$_1$			
From aerobic cultures:			
M_r (su) = 35 000 ± 2000	138 000 ± 10 000 * 140 000 ± 5 000 ** 142 000 ± 7 000 ***	7.5 ± 0.2	4.3 ± 0.2
+ phospho*enol*pyruvate (20 mM)	148 000 ± 10 000 *		
+ KCl (10 mM)	141 000 ± 10 000 *		
+ GDP (1 mM)		7.5 ± 0.2	
+ ATP (1 mM)		7.5 ± 0.2	
+ Fru-6-*P*		7.4 ± 0.2	
from anaerobic cultures		7.55 ± 0.2	
from fully aerobic cultures		7.5 ± 0.2	
Phosphofructokinase$_2$			
M_r (su) = 36 000 ± 2000	69 000 ± 2 000 **	4.5 ± 0.2	3.5 ± 0.1

* Yphantis method.
** From sedimentation coefficient and Stokes radius a.
*** Approach to equilibrium.

the presence of phospho*enol*pyruvate (2 mM) or potassium ions (10 mM). ATP, fructose 6-phosphate and GDP had no effect on the sedimentation coefficient value. PFK_1 has also been purified from anaerobically grown cells or from strongly aerated cultures.[1] These preparations fully cross-react against PFK_1 antisera [5]. Their sedimentation coefficients were still equal to 7.5 S (Table II) which makes very unlikely that a significant change in molecular weight could occur as the oxygen tension of the culture is modified.

3. Spectral properties of phosphofructokinase$_1$

After removal of ATP, the ultraviolet spectrum of the protein shows a maximum at 278 nm, a minimum at 250 nm, the ratio between the absorbances at these two wavelengths being equal to 2.51. Table III gives the amino-acid analysis of the protein. Its extinction coefficient has been found to be given by:

$\epsilon(278\ \text{nm}) = 0.60 \pm 0.03\ (\text{l}, \text{g}^{-1}, \text{cm}^{-1})$

(See Table IV).

4. Stoichiometry of ATP- and GDP-binding sites

The binding of ATP at 4°C in Tris · HCl, 50 mM, $MgCl_2$, 1 mM, 2-mercaptoethanol, 7 mM is non-cooperative (K_d = 6 μM) on phosphofructokinase$_1$, and extrapolates on a Scatchard plot to four sites per tetramer. The same stoichiometry is found for GDP binding. In this case, the saturation function is cooperative (cf. Fig. 1).

5. Kinetic properties of phosphofructokinase$_1$ isolated from aerobically or anaerobically grown cells

Phosphofructokinase$_1$ derepressed by anaerobiosis shows the same kinetic

TABLE III

AMINO ACID COMPOSITION OF PHOSPHOFRUCTOKINASE$_1$

Four different times of hydrolysis in 6 M HCl (24, 48, 72 and 96 h) were performed on three independent samples. Tryptophan and cysteine were determined in separate experiments. From these data, partial specific volume and refractive index increments were calculated (cf. refs. 20 and 21). The number of residues per protomer is normalized for a molecular weight of 35 000. n.d., not determined.

	Number of residues per protomer
Lysine	12
Histidine	6
Arginine	18—19
Aspartic acid	27 ± 1
Threonine	12
Serine	10
Glutamic acid	23 ± 1
Proline	9 ± 2
Glycine	32 ± 2
Alanine	25 ± 2
Cysteine [19]	3
Valine	21 ± 1
Methionine	n.d.
Isoleucine	25 ± 1
Leucine	22 ± 1
Tyrosine	10
Phenylalanine	9—10
Tryptophan [18]	1

behavior, under our assay conditions, as the enzyme isolated from cultures grown under aerobic conditions (see Fig. 2). No significant difference is found in the modulation of the initial velocity by GDP or phospho*enol*pyruvate. The

TABLE IV

EXTINCTION COEFFICIENT OF PHOSPHOFRUCTOKINASE$_1$

The $\delta n/\delta A$ method consists in measuring the change in refractive index Δn of diluted protein solutions as a function of the increment absorbance of the protein, ΔA. As the refractive index increment can be calculated from the amino-acid analysis one gets the extinction coefficient of the protein by:

$$\frac{\Delta n}{\Delta A} = \frac{\delta n}{\delta c} \cdot \frac{1}{\epsilon \cdot 1},$$

1 being the optical depth of the cuvette (cf. ref. 21). Fluorescamin [22] allows to determine the total concentration of primary amino-groups of the protein after measure of its optical density and denaturation in 5.5 M guanidine.Knowing the number of these reactive groups per mg of protein from the amino-acid analysis, one can calculate a. (method fully described in ref. 23).

Method	Buffer	Extinction coefficient $g^{-1} \cdot 1 \cdot cm^{-1}$ at 278 nm
Folin assay	Potassium phosphate 20 mM pH 7.6	0.60 ± 0.02
$\frac{\delta n}{\delta A}$	Tris/acetate, 50 mM, pH 7.5, Magnesium acetate, 1 mM, 2-mercaptoethanol, 5 mM	0.62 ± 0.03
Fluorescamin	Triethanolamine · HCl, 100 mM	0.63 ± 0.03
Amino acid recovery	Tris/acetate, 50 mM, pH 7.5, or CO_3HNH_4, 0.1 M	0.58 ± 0.01

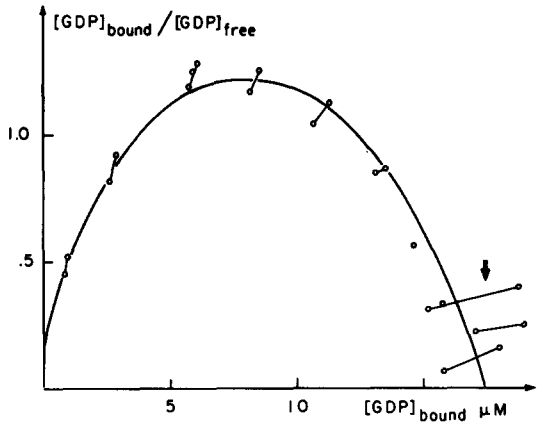

Fig. 1. Scatchard plot of the binding of GDP on phosphofructokinase$_1$. Equilibrium dialysis is performed at 4°C in Tris/acetate, 50 mM, Mg^{2+} acetate, 1 mM, 2-mercaptoethanol, 5 mM, pH 7.5. Arrow indicates a stoichiometry of 4 (total protein concentration = 620 µg/ml). The cooperative behavior can be accounted for using the Monod-Wyman-Changeux model with an isomerisation constant L_0 = 37, assuming exclusive binding of GDP (K_1 = 114 µM, K_4 = 3 µM).

degree of cooperative activation or inhibition implies that the tetramer undergoes a concerted transition [3].

6. Oligomeric structure of phosphofructokinase$_2$

As indicated in Table II, purified phosphofructokinase$_2$ appears to contain polypeptide chains of molecular weight 36 000, a value very close to the one found for phosphofructokinase$_1$. Although the difference between these two values is within experimental error, it has been repeatedly found, in electrophoresis performed on the same slab gel, that denatured phosphofructokinase$_1$

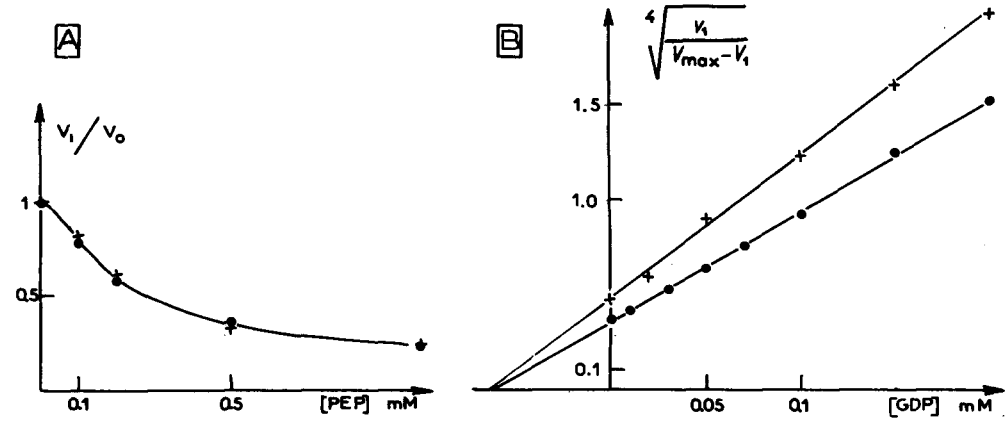

Fig. 2. (A) Inhibition by phospho*enol*pyruvate of phosphofructokinase activity in a crude extract of the strain A_0B_0 grown aerobically (·) or anaerobically (+) (the amount of phosphofructokinase$_2$ activity is negligible). Assay conditions as in ref. 1, except for the ATP concentration (0.5 mM) and for Mg^{2+} (5 mM). The ATP regenerating system is present. (B) Activation by GDP phosphofructokinase activity in a partially purified preparation (elimination of adenylate kinase activity, purification 80-fold). Strain A_0B_0 grown either aerobically (·) or anaerobically (+). Kinetic representation as in ref. 5. Assays performed as in ref. 1 except Fru-6-*P* concentration 0.5 mM.

had always a slightly higher R_F than phosphofructokinase$_2$.

The molecular weight of the native enzyme has been obtained by the combined determination of sedimentation coefficient and of a Stokes radius. Assuming a partial specific volume of 0.74, a molecular weight for the native enzyme 71 000 ± 2000 has been calculated (cf. Table II). phosphofructokinase$_2$ appears therefore to be a dimeric enzyme. No association/dissociation behavior has been detected either in sedimentation experiments (enzyme concentrations ranging between 5 and $5 \cdot 10^{-2}$ mg/ml) or in the enzymatic assay (concentrations varying between $5 \cdot 10^{-3}$ and $2 \cdot 10^{-1}$ μg/ml).

7. Search for common antigenic determinants between the two isozymes

A crude extract of the strain A_0B_1 containing a significant amount of both isozymes may be assayed by the Ouchterlony technique. Anti-phosphofructokinase$_1$ serum gives a single precipitation line against the purified phosphofructokinase$_1$ and against the crude extract; the two lines fuse, without a spur. No precipitation is observed with purified phosphofructokinase$_2$. In a parallel experiment, anti-phosphofructokinase$_2$ is assayed. The converse result is obtained: no precipitation is observed with phosphofructokinase$_1$. There is complete immunological identity between purified phosphofructokinase$_2$ and phosphofructokinase$_2$ present in the crude extracts. The two antisera are therefore completely specific for the corresponding isozyme.

The lack of immunological homology between the two proteins has been estimated more quantitatively by precipitation assays in liquid medium. A quantity Q of antiserum is required to fully precipitate a given amount of the homologous protein. Increasing quantities of the same serum are then assayed against the same amount of the other protein. No precipitation is observed when the quantity of antiserum is varied between Q and $200Q$. However, it could be argued that in the heterologous system, some non-precipitating complex was formed. This objection was excluded by the use of insolubilized antisera. Their titer was first measured against the homologous proteins. In both cases, the capacity of the antisera was estimated to be 100 units of activity per gram of wet resin. A 100-fold excess of the resin over the capacity value failed to retain any significant amount of the heterologous protein.

Since immunisations had been performed with native proteins which probably have quite different tertiary and quaternary conformations (in particular since phosphofructokinase$_1$ is known to undergo a quite concerted allosteric transition), the common sequence-specific determinants could have been masked in one native structure and exposed in the other one. This objection was discarded on the basis of the following experiment. Samples of each protein were heat-denatured for 5 min at 100° C, and treated for 2 h with 1 μg of trypsin. Polyacrylamide gel electrophoresis indicated that the resulting polypeptides are smaller than 10 000 daltons. The trypsin was then heat-denatured. Fig. 3 indicates that this material could efficiently displace the equivalent point of the parent protein assayed against the homologous antiserum, but failed to displace the equivalence zone in the heterologous assay. Both antisera recognize therefore a large number of sequence specific determinants but there is not detectable homology between the sequence specific determinants recognized by the two antisera.

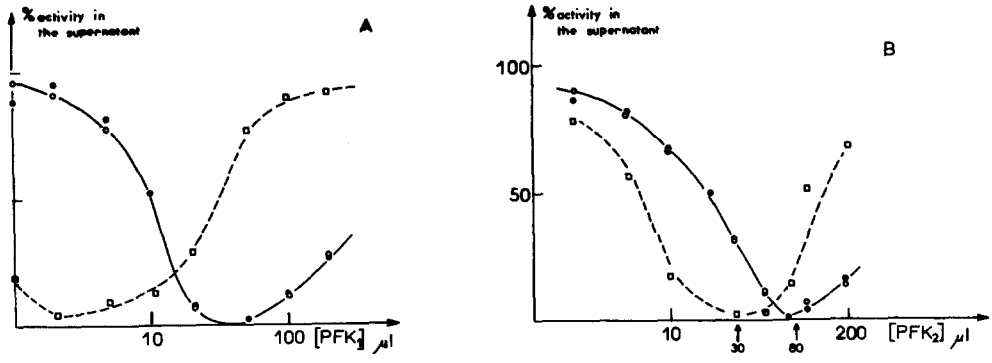

Fig. 3. Effect of the addition of trypsinised fragments of phosphofructokinase$_1$ and phosphofructokinase$_2$ on the displacement of the zone of equivalence in the precipitation of phosphofructokinase$_1$ antisera (A) or phosphofructokinase$_2$ antisera (B) by the homologous protein. (A) 10 µl of crude anti-phosphofructokinase$_1$ antiserum are incubated with increasing quantities (in µl) of phosphofructokinase$_1$ (specific activity 20 units/ml) in the presence or absence of a constant amount of trypsinised fragments. ○, native phosphofructokinase$_1$ alone □, native phosphofructokinase$_1$ + 10 µl of fragmented phosphofructokinase$_1$ (specific activity before denaturation 20 units/ml) ●, native phosphofructokinase$_1$ + 50 µl of fragmented phosphofructokinase$_2$ (specific activity before denaturation 31 units/ml). Activities are measured in the supernatants. Control experiments are performed with serum of a non-immunised rabbit in the presence of increasing amount of native phosphofructokinase$_1$. Activities found in the supernatant of this serum are taken as 100. (B) 20 µl of crude anti-phosphofructokinase$_2$ serum are assayed as described in A, with: (1) ○———○, increasing concentrations (in µl) of native phosphofructokinase$_2$ (31 units/ml). (b) □———□, native phosphofructokinase$_2$ + 20 µl of fragmented phosphofructokinase$_2$ (specific activity before denaturation 31 units/ml). (c) ▲———▲, native phosphofructokinase$_2$ + 50 µl of fragmented phosphofructokinase$_1$ (specific activity before denaturation 20 units/ml). Control experiments performed in the presence of 20 µl of serum of a non-immunised animal). Concentrations of native enzyme are plotted on a logarithmic scale.

Assays performed with both antisera against two mutated phosphofructokinase$_1$ proteins gave the expected result. They are immunologically identical with the wild-type phosphofructokinase$_1$ enzyme and show no homology with phosphofructokinase$_2$.

Discussion and Conclusions

Phosphofructokinase$_1$ contains four sites for the first substrate ATP, and four sites for the allosteric activator GDP. We have been unable to detect any significant change in the molecular weight of the tetramer in the presence of any of the known effectors of phosphofructokinase activity, or by purifying the enzyme from bacteria grown under various degrees of anaerobiosis. Moreover the kinetic behavior of the purified enzyme does not depend on the growth conditions. It shows always cooperativity indexes larger than 2.

These kinetic studies do not reveal either a dissociation of the enzyme as its concentration is lowered in the assay mixtures. The tetramer appears therefore to be the functional assembly of fructose-6-phosphate kinase 1. On the basis of a quantitative analysis of kinetic properties of this enzyme [3], it was proposed that phosphofructokinase$_1$ was undergoing a concerted transition of the type:

$$R_4 \rightleftharpoons T_4$$

the R state being stabilized by the binding of four molecules of fructose 6-

phosphate or of four molecules of GDP, the T state by the binding of four molecules of phospho*enol*pyruvate. The present biochemical evidence (ref. 2 and 16 and this study) supports this view since four molecules of activator bind to the tetramer in a cooperative manner. However, the equilibrium constant L_0 found in the catalytic assay is by far higher than the one estimated from equilibrium dialysis (for a discussion of this discrepancy, see Blangy, [16]).

Almost all the physiological observations relevant to the regulation of the Embden-Meyerhof pathway in *E. coli* by phosphofructokinase may be explained by the properties of phosphofructokinase$_1$:

(a) the enzyme biosynthesis is derepressed under anaerobiosis and in glucose media;

(b) its catalytic activity is modulated by effectors which reflect the physiological needs of the cell (ADP, fructose 6-phosphate, phospho*enol*pyruvate, NH_4^+). Inhibition at high ATP concentration and low Mg^{2+} is probably also significant [25,26].

Phosphofructokinase$_2$ differs strikingly from phosphofructokinase$_1$:

(a) its biosynthesis is not affected by a shift from aerobiosis to anaerobiosis or by a modification of the nature of the sugar onto which the bacteria (strains A_1B_0 or A_1B_1) are grown;

(b) though the molecular weight of the denatured enzyme is close to the one found for phosphofructokinase$_1$, no common immunological determinants have been detected between phosphofructokinase$_1$ and phosphofructokinase$_2$.

(c) the enzyme exists as a dimer;

(d) its catalytic activity is not affected by the known effectors of phosphofructokinase$_1$ listed above;

(e) while the optimal pH for the transphosphorylation of fructose 6-phosphate catalysed by phosphofructokinase$_1$ is 8.2, change of the maximal velocity of the reaction catalysed by phosphofructokinase$_2$ indicates two optimal pH, 6.5 and 8.5 (unpublished observation from our laboratory).

These results are in good agreement and complement those found by Babul et al. [31]. On the other hand, most of them are at variance with those reported by Doelle and coworkers [27–30].

(a) Under anaerobic conditions, they found an "ATP-sensitive" phosphofructokinase, a tetramer of 350 000 daltons. This partially purified enzyme shows, according to Thomas et al. [27] a cooperative response with respect to fructose 6-phosphate at low ATP concentration. At high ATP concentration, (or in the presence of 5′-AMP) the sigmoidal response is converted into a michaelian curve.

Though there is a large discrepancy in the molecular weight estimates, it is possible that this species is analogous to phosphofructokinase$_1$. Both enzymes are tetrameric species which tightly bind to Cibacron Blue or Sepharose-Blue Dextran columns. The apparent difference in kinetic behavior is probably an artefact, since the observations reported by Thomas et al. were done on partially purified extracts in absence of an ATP-regenerating system. For example, 5′-AMP was thought long ago to be an activator of phosphofructokinase$_1$ until it was shown that adenylate kinase was contaminating the protein extracts.

(b) Under fully adapted aerobic conditions, *E. coli* contains, according to

Doelle, two new types of fructose-6-phosphate kinase activities (while we observed only a repression of the biosynthesis of phosphofructokinase$_1$ and no change in the amount of phosphofructokinase$_2$). The minor fraction differs only slightly in its allosteric control from the anaerobic species and could be again phosphofructokinase$_1$, except that it starts to precipitate at 50% saturation in ammonium sulfate, an ionic strength where all our phosphofructokinase$_1$ activity is already insoluble.

The major fraction does not exhibit any allosteric control, binds also to a Cibacron Blue column, precipitates between 45 and 50% of the saturation in $(NH_4)_2SO_4$ and is reported to be a dimer of 68 000 daltons [29—30]. At first sight, its dimeric nature and its molecular weight would suggest it is phosphofructokinase$_2$. However, phosphofructokinase$_2$ is never derepressed under aerobiosis and does not bind to Cibacron Blue or sepharose-Blue Dextran columns. We hope that the antisera prepared in our laboratory will allow clarification of these problems.

In our opinion, the major question is whether or not, in some physiological conditions, a new control of phosphofructokinase activity appears, other than the derepression of phosphofructokinase$_1$, (for example a change in the quaternary structure of phosphofructokinase$_1$, a derepression of phosphofructokinase$_2$, or a modulation of phosphofructokinase$_1$ activity by other regulatory proteins in vivo). These possibilities are still open, since Sols et al. [26], have noticed significant qualitative differences between the properties of phosphofructokinase in permeabilized *E. coli* cells, and the in vitro behavior of phosphofructokinase$_1$.

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