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ALLOSTERIC AND NON-ALLOSTERIC PHOSPHOFRUCTOKINASES FROM LACTOBACILLI

PURIFICATION AND PROPERTIES OF PHOSPHOFRUCTOKINASES FROM *L. PLANTARUM* AND *L. ACIDOPHILUS*

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Summary

Phosphofructokinase (ATP : D-fructose-6-phosphate 1 phosphotransferase, EC 2.7.1.11) from two different lactobacilli, *Lactobacillus plantarum* and *Lactobacillus acidophilus* were isolated and purified. Both enzymes have a molecular weight of 154 000 and consist of four subunits of identical size. Antisera from sheep immunized against the purified phosphofructokinase from *L. plantarum* showed immunologic cross reaction with the enzyme from *L. acidophilus*. In spite of the close molecular relationship indicated by the immunologic cross reaction, the kinetic behaviour of the two enzymes was strikingly different. Phosphofructokinase from *L. plantarum* showed pure Michaelis-Menten behaviour. Phosphofructokinase from *L. acidophilus*, however, showed sigmoidal substrate saturation curves for fructose 6-phosphate in the presence of slightly alkaline pH and high ATP concentrations; it was activated by fructose 1,6-biphosphate and inhibited by ADP. The results indicate that even enzymes which are structurally very similar may differ greatly with respect to their kinetic and regulatory properties and suggest that allosteric and non-allosteric phosphofructokinases have the same origin in evolution.

Introduction

Phosphofructokinase (ATP : D-fructose-6-phosphate 1-phosphotransferase, EC 2.7.1.11) plays a major role in the regulation of glycolysis in most organisms as is underlined by the pronounced allosteric properties of phosphofructo-

Abbreviations: SDS: sodium dodecyl sulfate; *L.*: *lactobacillus.*

kinases from most sources. Phosphofructokinases exhibiting allosteric behaviour have been reported in animal tissues [1—4], some plants [5], yeast [6,7], and bacteria [8—10]. Some bacterial phosphofructokinases, however, are devoid of allosteric properties [11,12]. This opens the question as to whether there is a fundamental difference between allosteric and non-allosteric phosphofructokinases and whether they belong to different lines of evolution, or, alternatively, whether allosteric properties may result from relatively small differentiation processes of enzymes.

The aim of this study was to contribute arguments by comparative kinetic and immunological studies of phosphofructokinases from closely related organisms. The investigation was begun using phosphofructokinase from *L. plantarum*. This enzyme was purified and the finding of a previous report [12] that the enzyme was not allosteric was confirmed. Antibodies against the pure enzyme were used as a probe of relationship of phosphofructokinases from lactobacilli. Phosphofructokinase from *L. acidophilus* was proved to show immunologic cross reaction with a non-allosteric enzyme and to exhibit pronounced allosteric behaviour.

Materials and Methods

Materials

Auxiliary enzymes and substrates for enzyme assays were supplied by Boehringer Mannheim, Germany. Unless otherwise stated, the other chemicals and constituents of the growth media were from Merck Darmstadt, Germany. Lyophilized cultures of *L. plantarum* and *L. acidophilus* were gifts from the Department of Microbiology of the Bundesanstalt fur Milchforschung, Kiel, Germany. Phosphofructokinase from rabbit muscle was prepared as described previously [13].

Assays

Two different assay systems were used for the determination of phosphofructokinase activity. Unless otherwise stated, assay system A contained triethanolamine · HCl (pH 7.6) 50 mM, $MgCl_2$ 4.0 mM, ATP 4.0 mM, fructose 6-phosphate 4.0 mM, NADH 0.5 mM, fructose 1,6-bisphosphate aldolase 60 μg/ml, triosephosphate isomerase 5 μg/ml, and glycerol 1-phosphate dehydrogenase 40 μg/ml. Temperature was maintained at 25° C. This assay system was also used for the determination of activities designated as $V_{\text{reference}}$.

Some kinetic studies were performed with assay system B containing triethanolamine · HCl (pH 7.6) 50 mM, $MgCl_2$ 4.0 mM, KCl 50 mM, ATP 4.0 mM, fructose 6-phosphate 4.0 mM, phospho*enol*pyruvate 1.0 mM, NADH 0.5 mM, pyruvate kinase 30 μg/ml, and lactate dehydrogenase 30 μg/ml.

Deviations from the compositions given here are described in the text.

Protein concentrations were determined according to Lowry et al. [14].

Cultivation of bacteria

The lactobacilli were grown on a selective medium [15]. 6 flasks each containing 0.2 l of the medium were inoculated by the addition of a suspension of the lyophilized bacteria. When the pH had fallen to 4, 30 ml of the cultures

were transferred to each of 38 flasks containing 800 ml of the growth medium. The bacteria were kept at 38° C and harvested when the pH had dropped to 4. About 170 g of lactobacilli were obtained from 30 l medium by centrifugation.

Purification of phosphofructokinase from L. plantarum

170 g of wet bacteria were suspended in 1200 ml of 50 mM sodium phosphate buffer (pH 7.6) and were disrupted by a Manton Gaullin 15 M - 8 TA homogenisator at 700 atm. for 25 min. After centrifugation, the precipitate was suspended in another 1200 ml of extraction buffer and homogenized as before.

The crude extract was brought to pH 4.0 by the addition of 50% acetic acid. After 10 min stirring, the precipitate containing phosphofructokinase was collected by centrifugation. The sediment was dissolved in 0.2 M sodium phosphate buffer (pH 6.5) ("acid precipitate").

Precipitation with protamine sulfate. 0.015 ml of a 10% solution of protamine sulfate per unit of phosphofructokinase were added to the redissolved acid precipitate. The precipitate formed was spun down and discarded. The supernatant was dialyzed against 10 mM sodium phosphate buffer (pH 6.5). The precipitate formed during 12 h dialysis contained all the phosphofructokinase. The precipitate was separated by centrifugation (16 000 × *g*, 20 min) and redissolved in 0.5 M NaCl + 0.1 M sodium phosphate (pH 6.5) and cleared by centrifugation ("protamine sulfate precipitate").

CM-Sephadex treatment. In order to remove residual protamine sulfate, a 0.6-fold volume of CM-Sephadex (Pharmacia, Uppsala, Sweden) soaked in H_2O was added to the supernatant. The mixture was stirred for 20 min and then filtered on a Buchner funnel. The filtrate containing the phosphofructokinase was dialyzed against 0.01 M sodium phosphate buffer (pH 7.2).

Chromatography on Cibacron Blue-Sephadex. The dialyzed filtrate was applied to a column (14 cm in diameter) filled with Sephadex G-200 coupled to Cibacron Blue 3G-A (Ciba-Geigy AG, Basel, Switzerland) according to the method of Kopperschläger et al. [16]. A minimum of 0.3 ml of the gel per equivalent of 1 μmol/min phosphofructokinase activity were required. After 2 h incubation of the mixture, the column was washed with a 15-fold volume of 0.01 M sodium phosphate buffer. Phosphofructokinase was specifically eluted by the addition of 5 mM ATP.

Anion exchange chromatography. The eluate of the Cibacron Blue chromatography was applied to a column (diameter 5 cm, length 60 cm) of DEAE-Sephadex A 50 (Pharmacia, Uppsala, Sweden) which was equilibrated with 0.03 M sodium phosphate buffer (pH 7.0). Phosphofructokinase was eluted by a linear gradient of 0 to 1.5 M NaCl dissolved in 0.03 M sodium phosphate. The fractions containing phosphofructokinase were concentrated by ultrafiltration on an Amicon model 202 apparatus.

Purification of phosphofructokinase from L. acidophilus

Most steps of the purification procedure of phosphofructokinase from *L. acidophilus* were adopted from that developed for the enzyme from *L. plantarum*. Some important modifications, however, had to be introduced primarily due to the nonspecific elution of the *L. acidophilus* enzyme from the Cibacron Blue column. The relatively low purification during this step was

overcome only by repeated anion exchange chromatographies and by the introduction of an electrofocusing step. In general, phosphofructokinase from *L. acidophilus* turned out to be more labile during the purification procedure than the *L. plantarum* enzyme.

Protamine sulfate treatment. The acid precipitate prepared in the same way as described for *L. plantarum* phosphofructokinase was dissolved in 0.2 M sodium phosphate buffer (pH 6.5). 1 mg protamine sulfate in a 10% solution was added per 1 μmol/min phosphofructokinase activity. The precipitate formed contained only 50% of total phosphofructokinase activity. Therefore, the enzyme was solubilized by the addition of solid NaCl to a final concentration of 0.88 M. The remaining precipitate was spun down (16 000 × *g*, 10 min). The supernatant was dialyzed against 0.01 M sodium phosphate buffer (pH 6.5) for 12 h.

Chromatography on Cibacron Blue-Sephadex. Phosphofructokinase from *L. acidophilus* binds to Sephadex coupled to Cibacron Blue much more strongly than the enzyme from *L. plantarum*. After absorption in the gel and washing with an excess of 0.02 M sodium phosphate buffer (pH 7.6), the elution was performed with a solution containing 0.02 M sodium phosphate, 2.0 M $(NH_4)_2SO_4$, and 10 mM ATP.

Isoelectric focusing. Due to the rigorous conditions necessary for the elution of *L. acidophilus* phosphofructokinase from Cibacron Blue-Sephadex, the total amount of protein in the eluate was much higher than in the purification procedure for *L. plantarum* phosphofructokinase. This interfered also with the effectiveness of the anion exchange chromatography on DEAE-Sephadex which was performed in the same way as described above. The enzyme preparation was not homogeneous even after repetition of the DEAE-Sephadex chromatography. Therefore, the eluate fractions containing phosphofructokinase were dialyzed against distilled water for 12 h and subjected to isoelectric focusing with 1% Ampholine, pH 4—6, on the LKB 8102 column (content 440 ml). The current became constant after 2 days (600 V) and phosphofructokinase was concentrated in the fractions of pH 4.9 to 5.1.

Evaluation of kinetic constants

The kinetic constants were evaluated by non-linear fitting of the experimental data to the exponential form of the Hill equation:

$$v = V \cdot s^{n_H} / (s_{0.5}{}^{n_H} + s^{n_H}) ,$$

where s is the concentration of the substrate varied, n_H is the interaction coefficient and $s_{0.5}$ corresponds to the substrate concentration for which half maximum velocity of the enzyme reaction was obtained.

The parameters V, $s_{0.5}$, and n_H were calculated by the method of least squares so that the function was optimally approximated by the given arguments. The calculations were performed on the TR 440 digital computer (Computer Gesellschaft Konstanz) of this university.

Results

Purification of phosphofructokinase from L. plantarum and L. acidophilus

The purification procedure for phosphofructokinase from *L. plantarum* is

summarized in Table I. The enzyme preparation obtained had a specific activity of 92 μmol substrate turnover per min and per mg of protein. The recovery of initial activity was 32%. The enzyme gave a single symmetrical peak in the analytical ultracentrifuge and a single band in SDS electrophoresis (cf. Fig. 2a). Phosphofructokinase from *L. acidophilus* was less stabile than the enzyme from *L. plantarum*. This is evident from the low yield of 3% pure enzyme obtained by the purification procedure summarized in Table II. Although the specific activity reached only 56.5 μmol/min · mg protein, the enzyme turned out to be homogeneous on SDS electrophoresis (Fig. 2a).

Molecular and immunologic properties of phosphofructokinases from L. plantarum and L. acidophilus

Molecular weight. The molecular weights of the native phosphofructokinases from *L. plantarum* and *L. acidophilus* were determined by sedimentation equilibrium analysis in the analytical ultracentrifuge [17]. The plot of the natural logarithms of absorbances against the square of radius is shown in Fig. 1. The molecular weight was 154 000 ± 5000 ($n = 4$) for the enzyme from *L. plantarum* and 154 000 ± 5000 ($n = 3$) for phosphofructokinase from *L. acidophilus*. A partial specific volume of 0.75 ml/g was used for the calculations.

Subunit structure. Photographs of SDS electrophoreses of phosphofructokinases from *L. plantarum* and *L. acidophilus* after staining with Coomassie Blue are presented in Fig. 2. Both enzymes show a single band of identical relative mobility. An electrophoresis of phosphofructokinase from rabbit muscle was included for comparison. The molecular weights of the phosphofructokinase subunits from lactobacilli were determined as 38 000 ± 1000 by the method of Weber and Osborn [18] using bovine serum albumin, aldolase and triosephosphate dehydrogenase from rabbit muscle, and cytochrome *c* as references (cf. Fig. 2a).

These experiments confirmed that the native phosphofructokinases of the molecular weight 154 000 are tetramers containing subunits of identical molecular weight.

Immunologic properties. Phosphofructokinase from *L. acidophilus* is

TABLE I

PURIFICATION OF PHOSPHOFRUCTOKINASE FROM *L. PLANTARUM*

	Volume (ml)	Activity (μmol/min)	Protein (mg)	Specific activity $\left(\frac{\mu mol}{min \cdot mg}\right)$	Purification factor (-fold)	Recovery (%)
Extract	2500	6100	13260	0.46	—	100
Acid precipitate	1100	5775	7804	0.74	1.6	94
Protamine sulfate precipitate	925	4900	5757	1.03	2.23	80
CM-Sephadex	764	4584	3986	1.15	2.5	75
Cibacron Blue chromatography	4000	3500	1094	3.2	6.95	57
DEAE-cellulose chromatography	140	1964	21.3	92.2	200	32

TABLE II

PURIFICATION OF PHOSPHOFRUCTOKINASE FROM *L. ACIDOPHILUS*

Extr	Volume (ml)	Activity (μmol/min)	Protein (mg)	Specific activity $\left(\frac{\mu mol}{min \cdot ml}\right)$	Purification factor (-fold)	Recovery (%)
Extract	2500	7500	16300	0.46	—	100
Acid precipitate	890	5879	8989	0.65	1.4	78
Protamine sulfate treatment	1260	5550	4032	1.38	2.2	74
Cibracon Blue chromatography	7995	4060	2478	1.64	4.2	54
DEAE-cellulose chromatography I	430	1990	129	15.4	7.3	26
DEAE-cellulose chromatography II	380	1178	38	31.0	67.0	15
Isoelectric focusing	50	225	4	56.3	121	3

inhibited and precipitated by antibodies from the sera of sheep immunized against purified phosphofructokinase from *L. plantarum*.

Fig. 3 shows the photograph of an immunodouble diffusion test. The antibodies, which were obtained from the sera by repeated Na_2SO_4 precipitation [19], were placed in the center well. Both enzymes from lactobacilli gave precipitation bands and formed spurs indicating partial antigenic identity [20].

Kinetic properties

pH-dependence of catalytic activity. Phosphofructokinase from *L. plantarum*

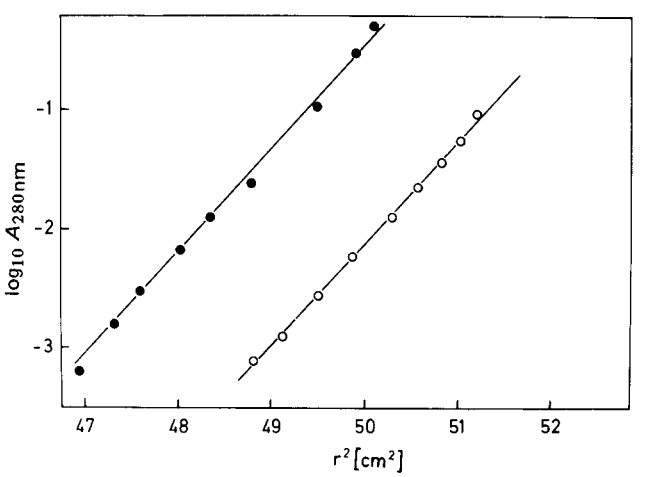

Fig. 1. Sedimentation equilibrium centrifugation of phosphofructokinase from *L. plantarum* (●———●) and *L. acidophilus* (○———○). Measurements were performed in 0.66 M sodium phosphate buffer, pH 7.6, at 20°C with the Spinco Model E analytical ultracentrifuge equipped with an automatic split-beam scanning absorption optical system. The protein was sedimented in one sector of a 12 mm cell; the second sector contained the solvent. Sedimentation equilibrium at 10 000 rev./min attained after 50 h. The concentration of the protein was measured at 280 nm; the initial protein concentrations were 0.4 mg/ml for the *L. plantarum* enzyme and 0.15 mg/ml for phosphofructokinase from *L. acidophilus*.

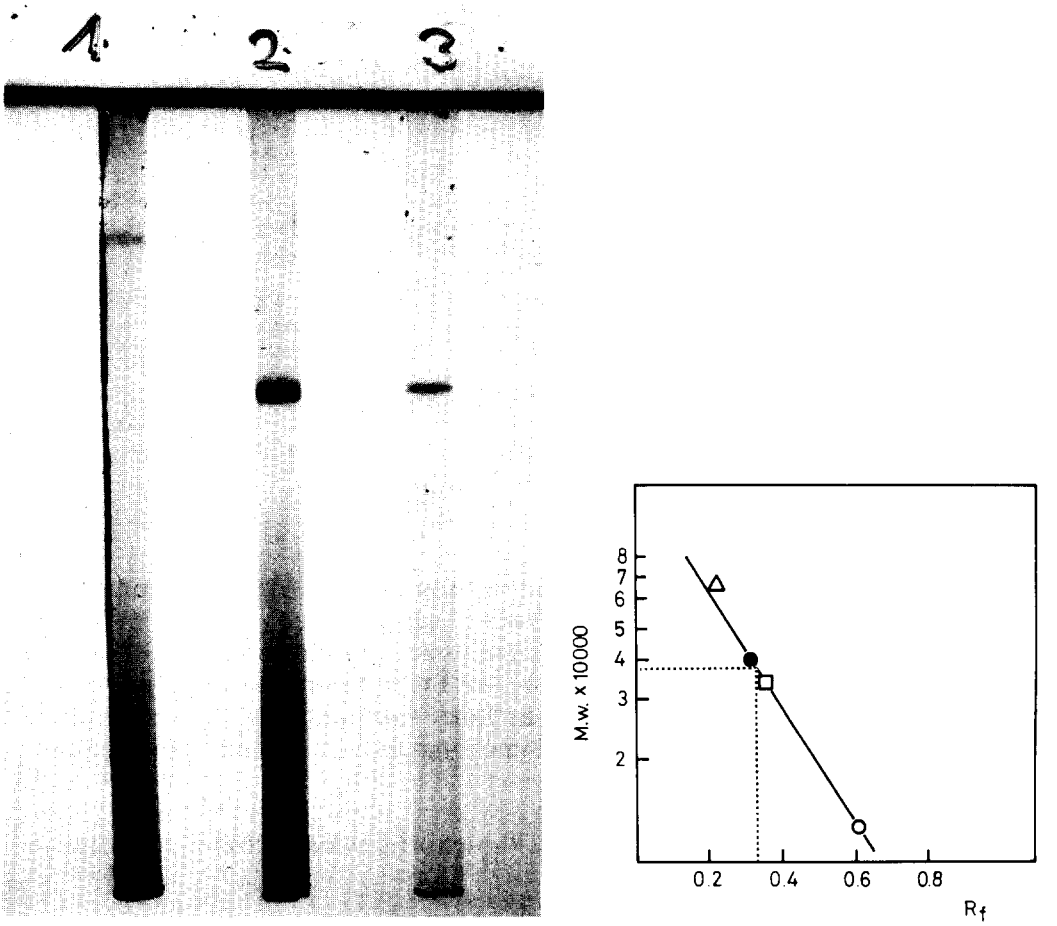

Fig. 2. SDS-electrophoreses of phosphofructokinases. The experiments were performed on 7.5% polyacrylamide gels in 0.1 M phosphate buffer, pH 7.3, containing 0.1% SDS (8 mA/tube). a: Phosphofructokinase from rabbit skeletal muscle (1), *L. plantarum* (2), and *L. acidophilus* (3) after staining with Coomassie Blue. b: Molecular weight (M.w.) estimations of phosphofructokinase from lactobacilli in SDS gels. The R_F values were referred to the migration of pyronin G. The following proteins were used as references: bovine serum albumin, mol. wt. 68 000 (△), rabbit muscle aldolase, mol. wt. 40 000 (●), rabbit muscle glyceraldehydephosphate dehydrogenase, mol. wt. 35 000 (□), and cytochrome *c*, mol. wt. 13 000 (○).

shows broad pH optima in the range of pH 7.0—7.5 in the presence of high (5.0 mM) and low (0.1 mM) fructose 6-phosphate concentrations. The curves of pH dependence of enzyme activity in the presence of different substrate concentrations were almost parallel. On the other hand, phosphofructokinase from *L. acidophilus* showed a shift of the pH optimum from pH 6.0 in the presence of 0.1 mM fructose 6-phosphate to pH 6.5 in the presence of 5.0 mM fructose 6-phosphate.

Saturation of phosphofructokinases with fructose 6-phosphate. In another series of experiments, saturation curves of phosphofructokinase from *L. plantarum* for fructose 6-phosphate were obtained at different pH values (Fig. 4a). The saturation curves were hyperbolic and the data fitted well to straight

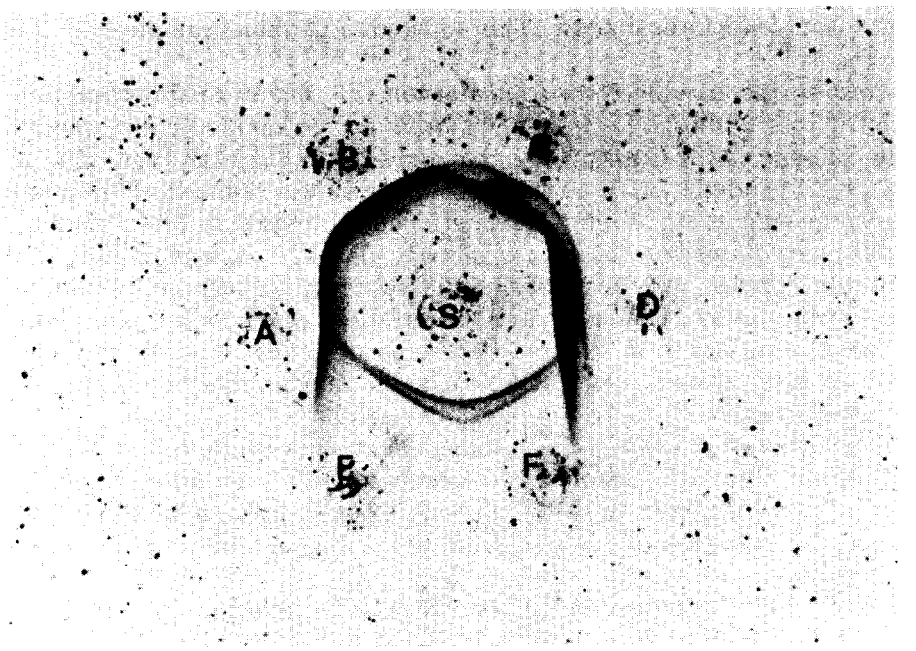

Fig. 3. Immunodouble diffusion test of sheep antiserum against phosphofructokinase from *L. plantarum* with different concentrations of the homologous antigen (A—D), and phosphofructokinase from *L. acidophilus* (E, F). The antiserum was placed in the center well (S). The immunoplates were incubated for 24 h at 4°C, extensively washed with phosphate buffer, dried and stained with Lichtgrun [20].

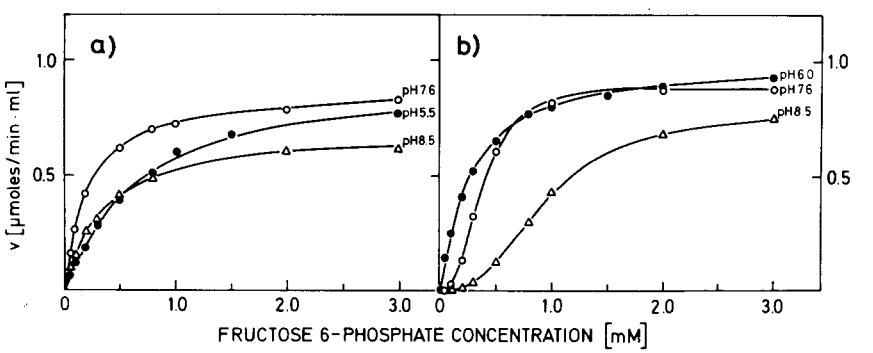

Fig. 4. Fructose 6-phosphate saturation of phosphofructokinases from *L. plantarum* (Fig. 4a) and *L. acidophilus* (Fig. 4b) in the presence of different pH values. The experiments were performed in 0.1 M phosphate buffer in the presence of 1.0 mM ATP with a modification of assay system A. The pH was controlled at the end of each assay. The curves were drawn using the parameters obtained by the fitting of the experimental data to the Hill equation (see Methods). The following values of the kinetic parameters were used:

	pH	V (μmol/min · ml)	$S_{0.5}$ (mM)	n_H	K_m (mM)
L. plantarum	5.5	0.93	0.65	1.1	0.77
	7.6	0.89	0.22	1.0	0.22
	8.5	0.69	0.36	1.1	0.39
L. acidophilus	6.0	0.99	0.27	1.1	
	7.6	0.88	0.37	1.8	
	8.5	0.78	0.96	2.5	

The K_m values of the *L. plantarum* phosphofructokinase were obtained by fitting of the experimental data to the Michaelis-Menten equation ($n_H = 1$).

lines in the double-reciprocal plots. The apparent K_m values are given in the legend to Fig. 4.

In contrast to the enzyme from *L. plantarum*, the shapes of the saturation curves for fructose 6-phosphate from *L. acidophilus* are markedly changed by pH. In the presence of 1.0 mM ATP, the curves were distinctly sigmoidal at pH 8.5 ($n_H = 2.5$) and pH 7.6 ($n_H = 1.8$). Sigmoidicity was reduced at lower pH values, as can be seen in Fig. 4b; the Hill coefficient decreased to 1.1 at pH 6.0.

Dependence of phosphofructokinase activity on ATP concentration. The ATP saturation curves of phosphofructokinase from *L. plantarum* and *L. acidophilus* in the presence of constant concentrations of fructose 6-phosphate and Mg^{2+} are shown in Fig. 5. The reaction rates are referrred to the activities measured with assay system A (see Materials and Methods). In accordance with the high concentration of fructose 6-phosphate required for saturation of the *L. acidophilus* enzyme, the $v/v_{Reference}$ values reach a maximum of 0.4. Whereas the saturation curve of phosphofructokinase from *L. plantarum* is purely hyperbolic, the analogous curve of the enzyme from *L. acidophilus* reaches an optimum in the presence of about 1 mM ATP; higher concentrations are slightly inhibitory.

Influence of ATP concentration on the fructose 6-phosphate saturation of phosphofructokinase from L. acidophilus. Fig. 6 shows fructose 6-phosphate saturation curves of phosphofructokinase from *L. acidophilus* at pH 8.5 in the presence of different ATP concentrations. The maximum velocity V and also the values of the Hill coefficient n_H rise considerably with increase of ATP concentration from 0.1 mM to 4.0 mM. The increase of cooperativity is also demonstrated by the Hill plots shown in Fig. 6b.

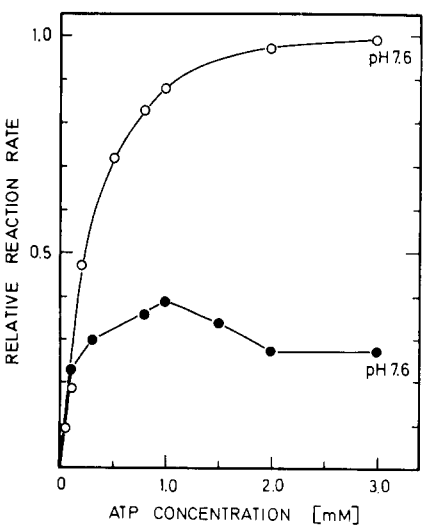

Fig. 5. ATP saturation of phosphofructokinase at pH 7.6 in the presence of 1.0 mM fructose 6-phosphate. The tests were performed with modifications of assay system A and the activities measured in the presence of varying ATP concentrations were referred to $v_{Reference}$ as measured as described in Methods. ○——○, phosphofructokinase from *L. plantarum*; $v_{Reference}$ = 0.79 μmol/min · ml. ●——●, phosphofructokinase from *L. acidophilus*; $v_{Reference}$ = 0.72 μmol/min · ml.

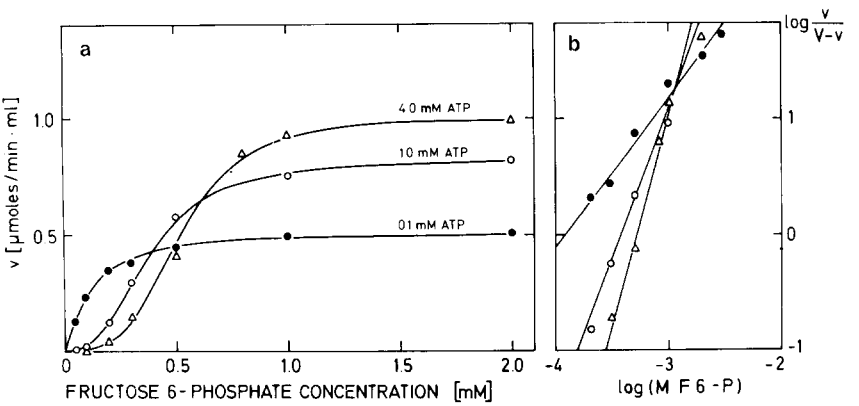

Fig. 6. a: Fructose 6-phosphate saturation of phosphofructokinase from *L. acidophilus* in the presence of 0.1 (●———●), 1.0 (○———○), and 4.0 (△———△) mM ATP. Measurements were performed at pH 8.5 using a modified assay system A. The curves were drawn using the parameters (as listed below) obtained by fitting of the Hill equation to the experimental data:

ATP (mM)	V (µmol/min · ml)	$S_{0.5}$ (mM)	n_H
0.1	0.52	0.12	1.3
1.0	0.83	0.38	2.6
4.0	1.01	0.52	3.6

b: Hill plots of the fructose 6-phosphate saturation data. Symbols are the same as listed above.

Nucleotide specificity of phosphofructokinase from L. acidophilus. ATP as phosphate donor in phosphofructokinase reaction can be replaced by GTP and ITP. Fig. 7 shows saturation curves for fructose 6-phosphate in the presence of 1.0 mM nucleotide triphosphate. In the presence of GTP and ITP, the saturation curves show considerable negative cooperativity (n_H = 0.7). The catalytic activity is lower when ATP is replaced by GTP or ITP even in the presence of 3.0 mM fructose 6-phosphate; yet, V extrapolated to infinite fructose 6-phosphate concentrations is not much different from that obtained with ATP.

Activators. In contrast to phosphofructokinase from *L. plantarum*, which is not activated by fructose 1,6-bisphosphate, the enzyme from *L. acidophilus* is most potently activated by this metabolite. Saturation curves for fructose 6-phosphate in the presence and absence of fructose 1,6-bisphosphate are presented in Fig. 7a. The measurements were performed at pH 8.5; the ATP concentration was 4.0 mM. Fructose 1,6-bisphosphate leads to a marked increase of the maximum velocity V and reduces the interaction coefficient n_H as is confirmed by the Hill plots shown in Fig. 7b. Glucose 1,6-bisphosphate, which can replace fructose 1,6-bisphosphate as activator of phosphofructokinase from several mammalian tissues [4], had no effect on the bacterial enzymes.

The only activator of the *L. plantarum* enzyme were NH_4^+ ions. 200 mM NH_4Cl increased V by about 20% in the presence of 2.0 mM ATP. This effect is less pronounced as that observed by Doelle [12] in crude homogenates. K^+ ions were slightly inhibitory. Slight activation by NH_4^+ and K^+ ions occurred also in the case of phosphofructokinase from *L. acidophilus*. Whereas V was only slightly affected, the cooperativity coefficient was reduced from 3.3 to 2.2

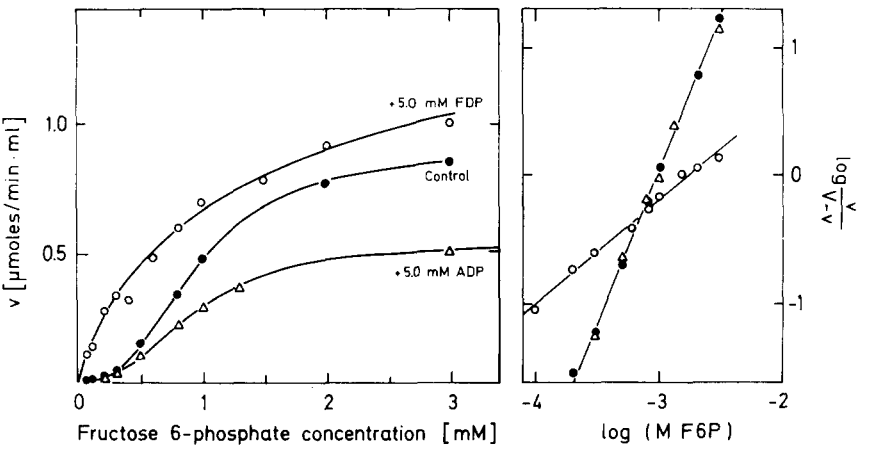

Fig. 7. a: Fructose 6-phosphate saturation of phosphofructokinase from *L. acidophilus* in the presence of 5.0 mM fructose 1,6-bisphosphate (○———○) or 5.0 mM ADP (△———△) and without effector (●———●). Measurements were performed in the presence of 4.0 mM ATP at pH 8.5 using either assay system A (in the presence of ADP and control) or assay system B (in the presence of fructose 1,6-bisphosphate). Controls as measured in both assay systems did not show significant differences. The curves are plots of the Hill equation using the following parameters:

	V (µmol/min · ml)	$S_{0.2}$ (mM)	n_H
Control	0.90	0.96	2.5
+5.0 mM Fru-1, 6-P_2	1.73	1.78	0.8
+5.0 mM ADP	0.54	0.97	2.4

b: Hill plot of some of the data presented in Fig. 7a. (The data lying outside the graph as shown here were omitted).

when the pH of the assay medium was 8.0 (ATP concentration 1.0 mM).

Inhibitors. Phosphofructokinase from *L. acidophilus* was slightly inhibited by high concentrations of its substrate ATP. Citrate, phospho*enol*pyruvate and 5'-AMP had no effect in the presence of 1.0 mM ATP. The most effective inhibitor of the enzyme was ADP as is demonstrated by Fig. 7. In the presence of 5.0 mM ADP, the maximum velocity of phosphofructokinase reaction dropped to about one half of that extrapolated from the curve measured in the absence of the inhibitor, whereas the cooperativity was not significantly affected. Phosphofructokinase from *L. plantarum* was not markedly influenced by similar concentrations of ADP, although higher concentrations of the nucleotide diphosphate were responsible for product inhibition.

Discussion

Allosteric behaviour of enzymes is best explained by conformational changes of proteins independent of the assumptions underlying the different models designed for quantitative explanation of non-hyperbolic ligand binding [21,22]. The fundamental difference between allosteric and non-allosteric enzymes, therefore, lies in the faculty of changing ligand affinity by structural transitions accompanying ligand binding. This property must be conditioned by the structure of the proteins, i.e. subunit composition and primary structure of the

peptide chains. As a result of this study, it is obvious that marked differences of regulatory properties may occur between enzymes of closely related protein structure.

Phosphofructokinase from *L. plantarum* is a tetrameric enzyme which obeys Michaelis-Menten kinetics under all circumstances. The only activators detected were NH_4^+ ions; an allosteric inhibitor was not found. The Michaelis-Menten character of the kinetics was not influenced by changes of the pH.

Phosphofructokinase from *L. acidophilus* has the same molecular weight and quaternary structure as the enzyme from *L. plantarum*. The molecular relationship between both enzymes is confirmed by immunologic cross reaction with antisera. The kinetic properties of phosphofructokinase from *L. acidophilus*, however, are very different. The enzyme shows pronounced cooperatively of fructose 6-phosphate saturation over a wide range of ATP concentrations and pH values. It is markedly activated by fructose 1,6-biphosphate. In the presence of the activator, the positive cooperatively of fructose 6-phosphate saturation curves turned to negative cooperativity. Negative cooperativity was also found when ATP as phosphate donor was replaced by GTP or ITP. The kinetic behaviour of the enzyme is therefore not sufficiently explained by the model of Monod et al. [21] as was suggested for *Escherichia coli* phosphofructokinase [23]. The grade of cooperativity of fructose 6-phosphate saturation is affected by the pH of the assay medium and increases at alkaline pH. Thus in the presence of acid pH values, the kinetic behaviour of *L. acidophilus* phosphofructokinase approaches the kinetics of the *L. plantarum* enzyme. With respect to this finding it would be of interest whether the intracellular pH of lactobacilli is influenced by the pH of the surrounding medium. Acid environment may be regarded as normal for most members of this group of bacteria. Yet, *L. acidophilus* is also found as an intestinal microorganism. Under these conditions, i.e. neutral or slightly alkaline pH, phosphofructokinase would show allosteric properties similar to those known for *E. coli* phosphofructokinase [23,24].

Phosphofructokinase from *L. acidophilus* is slightly inhibited by ATP and the cooperativity of fructose 6-phosphate saturation is influenced by increasing the ATP concentration from 0.1 to 4.0 mM. As with the phosphofructokinases of other organisms [3,4,25,26] an appropriate structure and concentration of the nucleotide triphosphate seems to be a prerequisite for cooperativity of fructose 6-phosphate saturation.

Immunological cross-reaction between two different enzymes suggests that regions exist in both proteins which are similar not only in their amino acid composition but also with respect to the spatial arrangement of the peptide chain [27]. Antigenic determinants are preferably located in the superficial regions of a protein molecule; the amino acid residues from these regions tend to be more rapidly exchanged during evolution than the amino acids of the core [28]. Antibodies therefore, are a sensitive probe for structural homologies within those parts of protein molecules that are primarily subject to evolutionary modifications [29,30]. As a consequence, immunologic cross reaction between phosphofructokinases from *L. plantarum* and *L. acidophilus* suggests that the enzymes differ only in a limited number of amino acid positions. The faculty for conformational transitions in response to ligand binding that distinguishes the allosteric phosphofructokinase of *L. acidophilus* from the

enzyme of *L. plantarum* must, therefore, be due to only minor differences in the structure of the peptide chains. Such changes may be produced by small evolutionary events.

It is of interest that the pressure of selection has generated different expressions of the kinetics of a regulatory key enzyme in closely related organisms which exist under similar life conditions. The pathways of energy metabolism in *L. plantarum* and *L. acidophilus* are also very similar; both bacteria ferment glucose to DL-lactate. The principles of regulation of this pathway, however, are most probably different. The comparison of metabolic regulation in these organisms will therefore provide an interesting model for the study of the regulatory role of phosphofructokinase in glycolysis.

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