BBA 66515

KINETIC CHARACTERISTICS OF PHOSPHOFRUCTOKINASE FROM *LACTOBACILLUS CASEI* VAR. *RHAMNOSUS* ATCC 7469 AND *LACTOBACILLUS PLANTARUM* ATCC 14917

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(Received August 6th, 1971)

SUMMARY

The enzyme phosphofructokinase (ATP:D-fructose 6-phosphate 1-phosphotransferase, EC 2.7.1.11) from *Lactobacillus casei* and *Lactobacillus plantarum* did not exhibit sigmoidal kinetics.

The K_m values for ATP were 0.37 mM and 0.67 mM and for Fru-6-*P* 1.43 mM and 0.15 mM, respectively.

The addition of ADP, AMP or adenosine 3′,5′-monophosphate (cyclic AMP) resulted in a general inhibition of the phosphofructokinase from *L. plantarum*, but not from *L. casei*. NH_4^+ stimulated the activity of the enzyme from both sources, but P_i did so only in the case of *L. casei*. Citrate inhibited the enzyme at physiological concentrations (2–10 mM), but lactate only to a minor extent.

The results are discussed in the light of the role of phosphofructokinase as regulatory enzyme in glucose metabolism.

INTRODUCTION

Phosphofructokinase is a key enzyme in the Embden–Meyerhof–Parnas pathway for the metabolism of glucose. In facultative anaerobic microorganisms, this enzyme appears to play a vital role as a regulatory enzyme[1] determining whether fermentation or respiration takes place. This regulatory function of phosphofructokinase has been elucidated best in mammalian tissue and yeast[2] and it was found that the kinetic behaviour of phosphofructokinase from *Escherichia coli*, *Staphylococcus aureus* and *Clostrodium perfringens*[3] is generally similar to that of the enzyme from yeasts[4], vertebrates[5] and mammals[6,7]. The mechanism of this regulation has been shown to be related to a control of the rate of the reaction depending upon the

Abbreviation: cyclic AMP, adenosine 3′,5′-monophosphate.

availability of nucleotides such as ATP, ADP, AMP or adenosine 3',5'-monophosphate (cyclic AMP)[8-10] and the synthesis of this enzyme itself[11,12].

Of the two facultative anaerobes chosen for these investigations, the growth and metabolism of one of them *Lactobacillus casei*, is known to be effected by oxygen[13-16]. As it is also known that these microorganisms have no tricarboxylic acid cycle and thus no aerobic respiration, but functional enzymes of the hexosemonophosphate pathway[13], the question arises as to how the glucose regulation functions and where the regulation of the effect of oxygen is situated.

The presented paper reports on the kinetic results of phosphofructokinase of both microorganisms showing that phosphofructokinase does not exhibit the usual picture of allosteric control of the glucose metabolism which suggests that the regulation of the oxygen effect is not connected with the Pasteur effect.

MATERIALS AND METHODS

The organisms were grown on MRS medium[17], containing 2% (w/v) glucose. Cell free extracts were prepared by sonication for 10 min at 0° using the Kerry's ultrasonic sonicator at 100 W and the respective sonication solutions as described for the investigation on enzyme stability or 0.1 M Tris–HCl buffer (pH 7.2) containing 3 mM mercaptoethanol and 0.45 mM Fru-6-*P* for the kinetic studies. The assay of phosphofructokinase activity was carried out following the oxidation of NADH at 340 nm and 30° using a Beckman DK 2A-recording spectrophotometer. The assay mixture contained in a total of 3.0 ml: 33 mM Tris–HCl buffer (pH 7.2), 0.15 mM NADH + H^+, 0.1 ml auxiliary enzymes (200 munits rabbit muscle aldolase, 200 munits α-glycerophosphate dehydrogenase and 1.13 units triose phosphate isomerase), varying concentrations of ATP–Mg (1:5) complex and Fru-6-*P*. Phosphofructokinase was proportional in the range of 0–500 μg protein. In order to study the effect of nucleotides, P_i and NH_4^+ on phosphofructokinase activity, the auxiliary enzymes used in the assay were diluted in 10 mM Tris–HCl buffer (pH 7.2) containing 2.1 mg/ml albumin and dialyzed for a minimum of 3 h at 4° against 4 l of the same buffer. Protein was determined by the biuret method.

All sugar phosphates, ADP, Tris and the auxiliary enzymes were obtained from Sigma (U.S.A.), whereas ATP and NADH was purchased from Serva (Germany) and cyclic AMP and AMP from Calbiochem.

RESULTS

Enzyme stability

Phosphofructokinase activity was easily obtainable from *L. casei* and *L. plantarum* by sonication of the cells for 10 min. The presence of 0.1 M Tris–HCl buffer (pH 7.2) gave the same activity levels as after the addition of 0.05 M 2-mercaptoethanol, 0.25 M Fru-1,6-P_2, 1 mM ATP, 0.01 M cysteine or 0.02 M reduced glutathion to the sonication solution. These compounds added singly or in combination did not influence the activity levels. The enzyme from both sources was very stable over 1 week at 4° in 0.1 M Tris–HCl buffer (pH 7.2) and the loss in activity over 1 month was approx. 40% of the original activity.

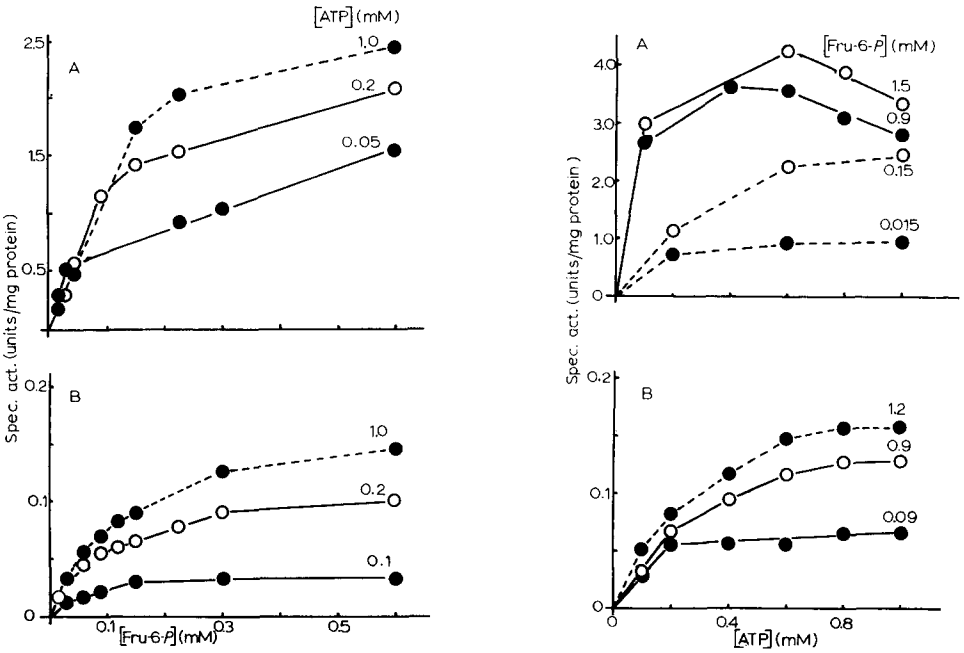

Fig. 1. Phosphofructokinase activity from *L. casei* (A) and *L. plantarum* (B) as a function of the concentration of Fru-6-*P* in the presence of ATP at varying concentrations. Each assay contained 0.1 or 0.24 mg protein, respectively.

Fig. 2. Phosphofructokinase activity from *L. casei* (A) and *L. plantarum* (B) as a function of the concentration of ATP in the presence of Fru-6-*P* at varying concentrations. Each assay cuvette contained 0.1 or 0.24 mg protein, respectively.

The effect of ATP

Due to the allosteric behaviour of phosphofructokinase in facultative anaerobic bacteria, the addition of high concentrations of ATP to low Fru-6-*P* concentrations results in sigmoidal substrate curves[1,18]. Neither the phosphofructokinase from *L. casei* nor from *L. plantarum* were inhibited by ATP in this way (Fig. 1). Using different concentrations of ATP (0.05–1.0 mM), a typical substrate saturation curve was obtained with increasing Fru-6-*P* concentrations. The enzyme from *L. casei*, however, was inhibited at high Fru-6-*P* and high ATP concentrations (Fig. 2), which was not the case with the *L. plantarum* enzyme. At low concentrations of Fru-6-*P*, the normal saturation curves were obtained, whereas at high concentrations the increase in phosphofructokinase activity reached saturation levels between 400 and 600 μM ATP and is followed by an increasing inhibition at higher ATP concentrations. At low Fru-6-*P* concentrations, high levels of ATP are rather stimulating than inhibiting, whereas ATP concentrations above 1.0 mM repress the phosphofructokinase activity indicating a substrate inhibition. The absence of ATP inhibition at low Fru-6-*P* concentrations indicates also that ATP has no influence on the apparent K_m for Fru-6-*P*. It was also noted that the activity of phosphofructokinase was more than 10-fold higher in *L. casei* compared with *L. plantarum*. This latter observation agrees with similar investigations of activity levels being carried out at present in our laboratory on Fru-1,6-P_2 aldolase.

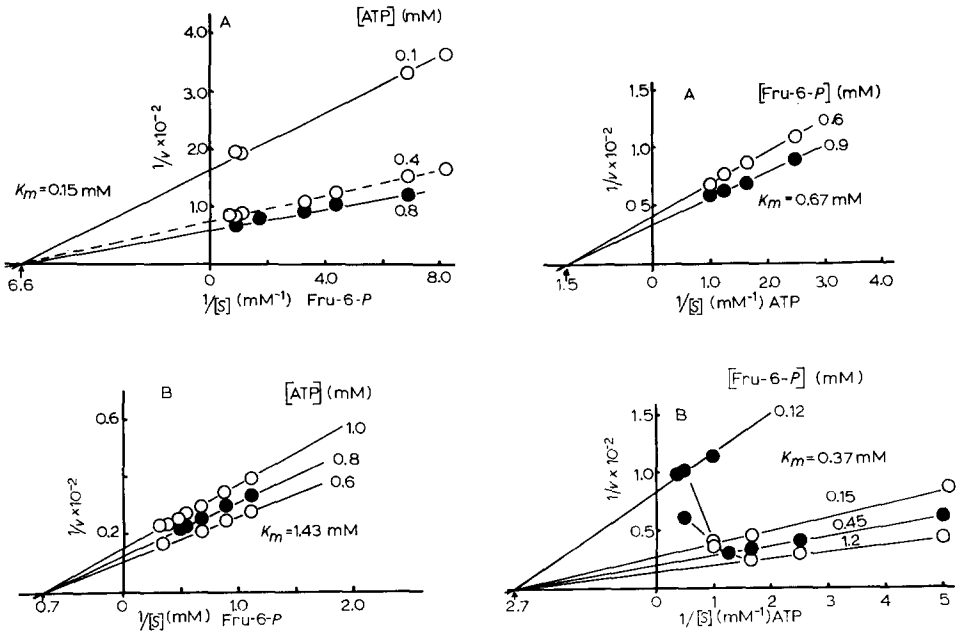

Fig. 3. Effect of Fru-6-*P* on the phosphofructokinase activity of *L. plantarum* (A) and *L. casei* (B) at various ATP concentrations. Each assay cuvette contained 0.24 or 0.1 mg protein, respectively.

Fig. 4. Effect of ATP on the phosphofructokinase activity of *L. plantarum* (A) and *L. casei* (B) at various Fru-6-*P* concentrations. Each assay cuvette contained 0.24 or 0.1 mg protein, respectively.

The apparent Michaelis constant (K_m) for Fru-6-*P* and ATP were estimated using linear plots of the initial rate data according to Lineweaver and Burk (Figs. 3 and 4). In the case of the Fru-6-*P* plot, no substrate inhibition was observed, but a significant difference in affinity toward this substrate. With a K_m of 1.43 mM, the enzyme from *L. casei* exhibited a far lower affinity compared with *L. plantarum* (0.15 mM). The plot for ATP (Fig. 4) also revealed significant differences, as the enzyme from *L. casei* showed the earlier mentioned strong substrate inhibition at high ATP concentrations. This type of inhibition was not observed with the enzyme from *L. plantarum*. The K_m values indicated the reverse to Fru-6-*P* in regard to substrate affinity. With a K_m for ATP of 0.37 mM, the phosphofructokinase from *L. casei* exhibited a higher affinity to the substrate than *L. plantarum* (0.67 mM). The differences in the affinities toward both substrates is certainly greater in the case of *L. casei* source compared with the phosphofructokinase from *L. plantarum*.

The effect of nucleotides, phosphate and NH_4^+

As all these investigations revealed that ATP does not function as an inhibitor at low Fru-6-*P* concentrations, further studies were carried out to find out whether or not the known modulators ADP, AMP, cyclic AMP, P_i and NH_4^+ have any effect on the phosphofructokinase activity. For these investigations an ATP concentration of 200 μM was chosen against varying Fru-6-*P* concentrations (15–1200 μM), as

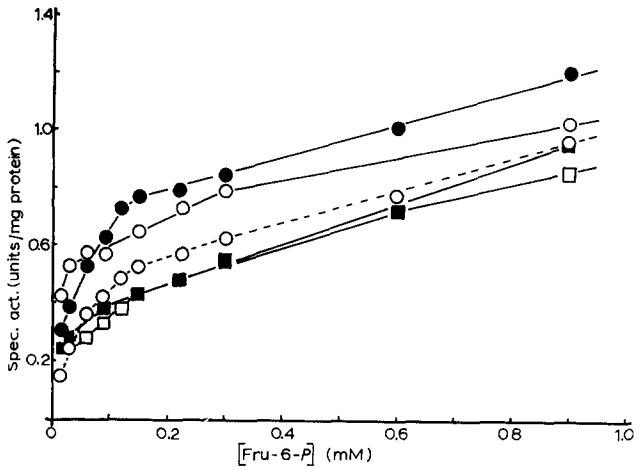

Fig. 5. Effect of various modulators on the phosphofructokinase activity of *L. casei*. Each assay cuvette contained 0.1 mg protein and dialyzed auxiliary enzymes. ■—■, 200 μM ATP; ●—●, 200 μM ATP + 10 mM $(NH_4)_2SO_4$; ○—○, 200 μM ATP + 5 mM KH_2PO_4; ○---○, 200 μM ATP + 1 mM ADP; □—□, 200 μM ATP + 5 mM AMP.

this ATP concentration causes sigmoidal substrate curves with the allosteric phosphofructokinase[19]. The results of these investigations are presented in Figs. 5 and 6. As in yeast phosphofructokinase, cyclic AMP did not have any influence on the *L. casei* phosphofructokinase and was thus identical to the curve without addition. The addition of 5 mM AMP was very similar and here again no effect could be observed. The presence of 1 mM ADP appeared to stimulate the phosphofructokinase activity slightly, but not in the way the addition of 5 mM P_i or 10 mM $(NH_4)_2SO_4$ did. Thus, the latter two additions were the only stimulators of the phosphofructokinase activity from *L. casei* and no inhibition was observed.

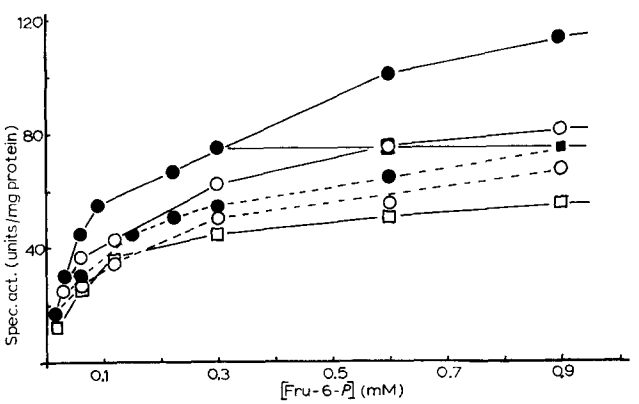

Fig. 6. Effect of various modulators on the phosphofructokinase activity of *L. plantarum*. Each assay cuvette contained 0.24 mg protein and dialyzed auxiliary enzymes. Curves: see Fig. 5 and ●---●, 200 μM ATP + 1 mM cyclic AMP.

The enzyme from *L. plantarum* showed a different behaviour (Fig. 6). The only marked stimulator appeared to be NH_4^+ under higher Fru-6-*P* concentrations, although the addition of 5 mM P_i also stimulated weakly the activity in the same concentration range. At low Fru-6-*P* concentrations, all nucleotides and P_i inhibited enzyme activity. The strongest inhibition was obtained with the addition of 5 mM AMP closely followed by cyclic AMP, ADP and P_i. Although the nucleotides had no function as modulators for inhibition of ATP, it was of interest to find that they either showed no effect or an inhibitory effect on phosphofructokinase activity. Thus, these findings are in contrast to the reactions involving an allosteric phosphofructokinase[19].

The effect of citrate and lactate

Citrate inhibits yeast phosphofructokinase within the range of physiological concentrations[2]. A similar effect was observed with the phosphofructokinase from both sources. The inhibitory effect started at a concentration of 1 mM and rapidly increased until approx. 74% of the enzyme activity was lost upon the addition of 6 mM citrate. High concentrations of Fru-6-*P* reversed this inhibitory effect. The importance of this inhibition, however, is questionable as both these Lactobacillus species do not produce citrate during their carbohydrate metabolism anaerobically or aerobically[13]. It has been reported, on the other hand, that *L. casei* is able to degrade citrate to acetoin and diacetyl under aerobic conditions, if citrate is supplied in the medium[20]. The normal glucose breakdown endproduct of the homofermenters, L-lactic acid, exhibited a very low inhibition, which was only 13% upon addition of 10 mM L-lactic acid to the assay mixture.

DISCUSSION

L. casei and *L. plantarum* are homofermentative lactic acid bacteria, which degrade glucose stoichiometrically to lactic acid under anaerobic conditions. Upon addition of oxygen, the stoichiometry changes from 2.0 to 1.0 or less and enzymes of the hexosemonophosphate pathway appear to function[13]. This change in endproduct formation is very similar to the Enterobacteriaceae, *e.g. E. coli* K 12 (ref. 11), although the actual products are different. In contrast to mammalian tissue, yeast and the Enterobacteriaceae, lactobacilli do not possess an extensive respiratory chain[14] and also no tricarboxylic acid or glyoxalate cycle during aerobic growth on glucose. This fact would make it difficult to explain the effect of oxygen on the metabolism of glucose with the standing theories of the Pasteur effect and its close relationship to phosphofructokinase as a metabolic energy regulator. It has been stated earlier, that the Pasteur effect and metabolic regulation in general probably has a different mechanism for different tissue cell types[26]. It is also likely that different bacterial groups will have different control factors for their energy metabolism[21]. As was the case with Fru-1,6-P_2 aldolase[22], phosphofructokinase of *L. casei* and *L. plantarum* was much more stable than its counterpart of *E. coli* or yeast[2]. Stabilizers such as 2-mercaptoethanol which are absolutely necessary for yeast and mammalian tissue[23,24] are not required as they do not improve the stability of the enzyme.

The lack of inhibition by ATP at low Fru-6-*P* concentrations as well as the lack of stimulation by the nucleotides ADP, AMP and cyclic AMP indicates very strongly that phosphofructokinase does not function as an allosteric protein in lactobacilli.

Whereas in yeast 100 μM ATP is sufficient for a 50% inhibition at a Fru-6-*P* concentration level of 500 μM (ref. 2), or an increase from 50 to 500 μM ATP at 75 μM Fru-6-*P* for 90% inhibition in the case of primate sperm phosphofructokinase[19], no such inhibition was observed with *L. casei* or *L. plantarum* phosphofructokinase. The stimulation of phosphofructokinase by NH_4^+ was very similar to that reported for mammalian[19] and yeast[2] enzymes. The different action of the nucleotides on the phosphofructokinase of *L. casei* and *L. plantarum* as well as the different K_m values appear to support the natural difference of these organisms as was reported earlier[25]. As phosphofructokinase does not appear to play the regulatory role as in other facultative anaerobes, further evidence will be sought to find out the regulatory mechanism responsible for the metabolic changes occurring in *L. casei* upon oxygenation.

ACKNOWLEDGEMENT

This work has been supported by Grants from the University and the Australian Research Grant Commission.

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