

Activation by Phosphate of Yeast Phosphofructokinase*

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The activity of yeast phosphofructokinase assayed *in vitro* at physiological concentrations of known substrates and effectors is 100-fold lower than the glycolytic flux observed *in vivo*. Phosphate synergistically with AMP activates the enzyme to a level within the range of the physiological needs. The activation by phosphate is pH-dependent: the activation is 100-fold at pH 6.4 while no effect is observed at pH 7.5. The activation by AMP, phosphate, or both together is primarily due to changes in the affinity of the enzyme for fructose-6-P. Under conditions similar to those prevailing in glycolysing yeast (pH 6.4, 1 mM ATP, 10 mM NH_4^+) the apparent affinity constant for fructose-6-P ($S_{0.5}$) decreases from 3 to 1.4 mM upon addition of 1 mM AMP or 10 mM phosphate; if both activators are present together, $S_{0.5}$ is further decreased to 0.2 mM. In all cases the cooperativity toward fructose-6-P remains unchanged. These results are consistent with a model for phosphofructokinase where two conformations, with different affinities for fructose-6-P and ATP, will present the same affinity for AMP and phosphate. AMP would diminish the affinity for ATP at the regulatory site and phosphate would increase the affinity for fructose-6-P. The results obtained indicate that the activity of phosphofructokinase in the shift glycolysis-gluconeogenesis is mainly regulated by changes in the concentration of fructose-6-P.

The central role of phosphofructokinase in the carbohydrate metabolism of different organisms including yeast is clearly established (for a review see Refs. 1 and 2). However in studies with the enzyme from yeast it seems to have been overlooked that when phosphofructokinase is assayed at the concentrations of substrates and known effectors (fructose-6-P, AMP, ATP, and citrate (1)) thought to prevail in the cell (3), the

activity found fails to account for the rate of glucose utilization by the yeast. The measured activity reaches about 0.2 $\mu\text{mol/min/g}$ of yeast while the glucose consumption attains about 20 $\mu\text{mol/min/g}$ of yeast.¹ Since most of the glucose consumed is channeled through glycolysis (4), the measured activity is much lower than expected. A possible explanation would be that the local concentrations of metabolites around phosphofructokinase are different from those measured in the whole cell. Since phosphofructokinase is a cytoplasmic enzyme such differences do not appear very likely except perhaps for ATP that could be concentrated in the mitochondria. With this reserve the following hypotheses appear as most plausible to explain the observed differences. (a) The enzyme presents a behavior *in vitro* that is different of that prevailing *in vivo* (5-7), (b) an unknown activator is not present in the assay, (c) a pathway exists that goes from glucose-6-P to triose phosphate without the involvement of phosphofructokinase. This last hypothesis has not been considered in the present work.

The first hypothesis was tested measuring the activity of the enzyme *in situ* as described by Serrano *et al.* (8), the second by looking for compounds which could activate the enzyme. Phosphate appeared as a good candidate since it activates animal phosphofructokinases (5, 9) and is a requirement for the enzyme from *Rhodotorula glutinis* (10). Our results show that hypothesis *b* seems to be the correct one.

We have found that physiological concentrations of phosphate activate the phosphofructokinase from *Saccharomyces cerevisiae*. This activation is further increased by the presence of AMP and this increase is not only due to the activatory effect of the added nucleotide. In the presence of AMP and phosphate the activity of phosphofructokinase found is well within the range of the physiological needs. A possible mechanism for the activation is suggested.

EXPERIMENTAL PROCEDURES

Growth of Yeast, Toluenuization, and Preparation of Cell-free Extracts—*Saccharomyces cerevisiae* CJM 13 (originally provided as *S. cerevisiae* 1714-24 A by Professor D. C. Hawthorne, Washington University) was used. The organism was grown in the minimal medium of Olson and Johnson (11) substituting NaCl (0.25 g/liter) for the original sodium citrate and adjusting the initial pH of the medium to 5.5 by addition of 5 M NaOH. As carbon sources, glucose or ethanol at a final concentration of 2% were used. Cells were harvested by centrifugation and washed twice with distilled water. Toluenuization for *in situ* assays was performed essentially as described by Serrano *et al.* (8). Cell-free extracts were obtained grinding the yeasts with 3 times their weight of alumina and extracting them with 5 volumes of 40 mM 2-(*N*-morpholino)ethanesulfonic acid

* Preliminary accounts of these results have been presented at the 10th Meeting of the Federation of European Biochemical Societies (Paris, 1975) and at the 10th International Congress of Biochemistry (Hamburg, 1976). The results presented are part of the doctoral thesis of M. B. This paper is dedicated to Professor A. Sols on his 60th birthday. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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¹ C. Gancedo, unpublished results.

(Mes), 0.1 M KCl, 1 mM MgCl₂, and 5 mM 2-mercaptoethanol, pH 6.4. After centrifugation for 10 min at 27,000 × *g*, the clear supernatant was used for enzyme assays.

Partial Purification of Phosphofructokinase—Phosphofructokinase was purified to free it from adenylate kinase and ATPases present in the crude extract. The purification involved protamine sulfate treatment, ammonium sulfate fractionation between 35 and 55% saturation, passage through a Sephadex G-200 column and concentration on an Amicon XM-100 membrane. The purification was 10-fold with 45% recovery. The unwanted contaminant activities of the purified enzyme were less than 0.05%.

Assay of Phosphofructokinase—The standard assay was adapted from that described by Racker (12) with aldolase, triose phosphate isomerase, and glycerophosphate dehydrogenase. The composition of the assay mixture was as follows: 40 mM 2-(*N*-morpholino)ethanesulfonic acid (Mes), 0.1 M KCl, 5 mM MgCl₂, 2.5 mM 2-mercaptoethanol, 0.2 mM NADH, 1 mM ATP, 10 mM NH₄Cl, and 0.5 unit of each of the auxiliary enzymes. The pH of the mixture was 6.4. As substrate a mixture of glucose-6-P and fructose-6-P in the proportion 3:1 was used at an end concentration of 0.3 mM fructose-6-P unless otherwise stated. The auxiliary enzymes were dialyzed to free them from ammonium sulfate.

Other Methods—Metabolites were determined by a rapid sampling method as described by Sáez and Lagunas (13).

Protein was assayed according to the method of Lowry *et al.* (14) after precipitation with 5% trichloroacetic acid. Bovine serum albumin was used as standard.

In the case of the *in situ* assays it was assumed that 1 g of toluenized yeast affords 50 mg of extractable protein (8).

Biochemicals were from Sigma or Boehringer. Other reagents were of analytical reagent grade.

RESULTS

Effect of Phosphate on Activity of Phosphofructokinase—To assay phosphofructokinase under physiological conditions the concentration of several metabolites in yeast was measured. The results of these determinations are presented in Table I. As far as data are available, they agree with those reported in the literature (3). An internal pH of 6.4 for the yeast was accepted (15).

When phosphofructokinase was assayed at pH 6.4 at concentrations of substrates and effectors similar to that found in the cell growing on glucose (0.3 mM fructose-6-P, 1 mM ATP, 0.3 mM ADP, 0.1 mM AMP, 10 mM NH₄⁺), the activity observed was only 0.2 unit/g of fresh yeast. The same low activity was obtained when the enzyme was assayed *in situ*, a procedure in which dilution of the protein is prevented (8). Citrate was not included in the assay mixture since preliminary experiments had shown that 0.5 mM citrate, the concentration present in

glucose growing cells, had no effect on the activity of phosphofructokinase.

Addition of phosphate to the above assay mixture caused a great activation as it may be seen in Fig. 1. The activating effect was observed both *in situ* and *in vitro*. The action of phosphate was mimicked by arsenate but not by sulfate, pyrophosphate, or glycerophosphate. Activation by phosphate was also seen in phosphofructokinase from yeast grown on ethanol.

Fig. 2 shows the effect of phosphate on the activity of purified yeast phosphofructokinase at different values of pH. In the presence of phosphate the optimum pH of the enzyme is displaced toward a more acidic region. The effect of phosphate is strongly dependent on the pH of the assay: about 100-fold activation at pH 6.4 and slight inhibition at pH 7.5.

Characteristics of Activation by Phosphate—Omission of AMP and ADP from the reaction mixture resulted in a much lower degree of activation by phosphate. To determine whether the phosphate activation is dependent on the presence of AMP, or ADP, or both, a purified preparation of phosphofructokinase free of adenylate kinase and ATPase was used. AMP alone activated about 10 times, a maximum effect being reached at 2 mM; ADP alone had no effect. Phosphate alone activated only 2-fold as can be seen in the *inset* of Fig. 3. The same figure shows the effect of increasing amounts of phosphate added in the presence of AMP or ADP. Phosphate plus ADP can increase activity by a factor of 40 while phosphate plus AMP activate up to 300-fold. The activation observed in the presence of AMP and phosphate is greater than the product of the activation observed with AMP or phosphate alone.

Since the concentration of fructose-6-P in the experiments described above (0.3 mM) was not saturating, it appeared of interest to determine whether the activation was due to an increase in the maximal velocity of the enzyme or to an increased affinity for fructose-6-P. The results presented in Fig. 4 show that the *V*_{max} increases only about 2-fold when AMP, phosphate, or both are present. On the other hand the half-saturation value (*S*_{0.5}) is decreased by AMP or by phosphate, and the presence of both effectors together lowers the *S*_{0.5} value for fructose-6-P by an order of magnitude. This

TABLE I

Concentrations of different metabolites in growing yeast

The yeast was grown as described under "Experimental Procedures" with glucose or ethanol as carbon source. While in the exponential phase of growth, the organisms were treated as described under Ref. 13 for extraction of metabolites. Metabolites were determined by conventional spectrophotometric methods. The values given are the mean of at least four determinations ± 1 S. E.

Metabolite	Yeast growing on	
	Glucose	Ethanol
	mM	
Glucose-6-P	0.9 ± 0.2	<0.1
Fructose-6-P	0.23 ± 0.08	<0.1
Fructose-1,6-P ₂	3 ± 1	<0.1
ATP	1.5 ± 0.1	1.3 ± 0.1
ADP	0.41 ± 0.06	0.44 ± 0.07
AMP	0.10 ± 0.01	0.10 ± 0.02
Citrate	0.5 ± 0.3	2.0 ± 0.2
Phosphate	9 ± 1	8.2 ± 0.5
NH ₄ ⁺	10 ± 4	12 ± 1

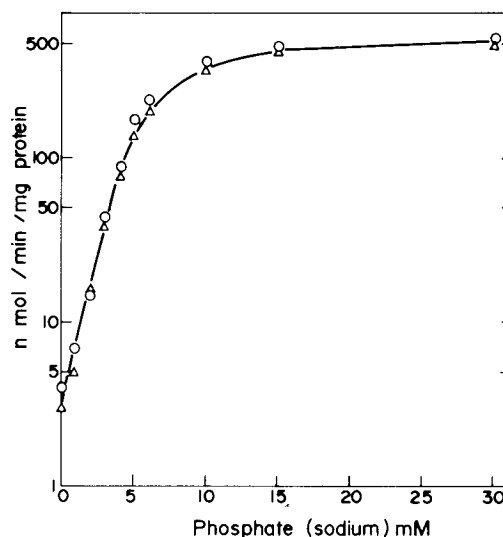


Fig. 1. Effect of phosphate on the activity of yeast phosphofructokinase. Activity was assayed as described under "Experimental Procedures" in the presence of 0.1 mM AMP and 0.3 mM ADP. Phosphate was varied as indicated on the abscissa ○—○, cell-free extracts; △—△, toluenized cells (*in situ* assay).

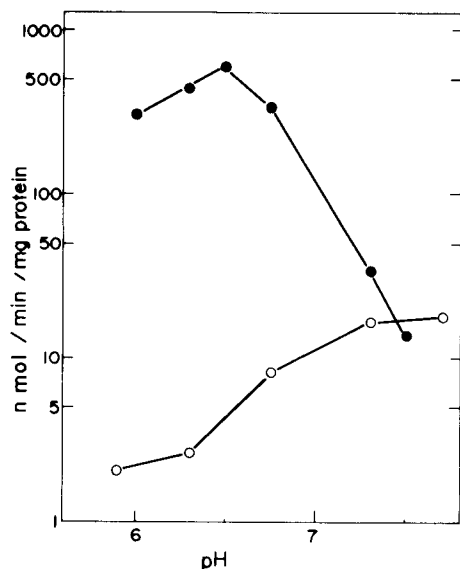


FIG. 2. Effect of pH on the activity of phosphofructokinase. A preparation purified as described under "Experimental Procedures" was used. The enzyme was assayed as described under "Experimental Procedures" with a 50 mM mixture of 3-(*N* morpholino)propanesulfonic acid and 1,4-piperazinediethanesulfonic acid substituting the usual buffer; 0.1 mM AMP and 0.3 mM ADP were also added. ●—●, with 10 mM phosphate added; ○—○, without phosphate added.

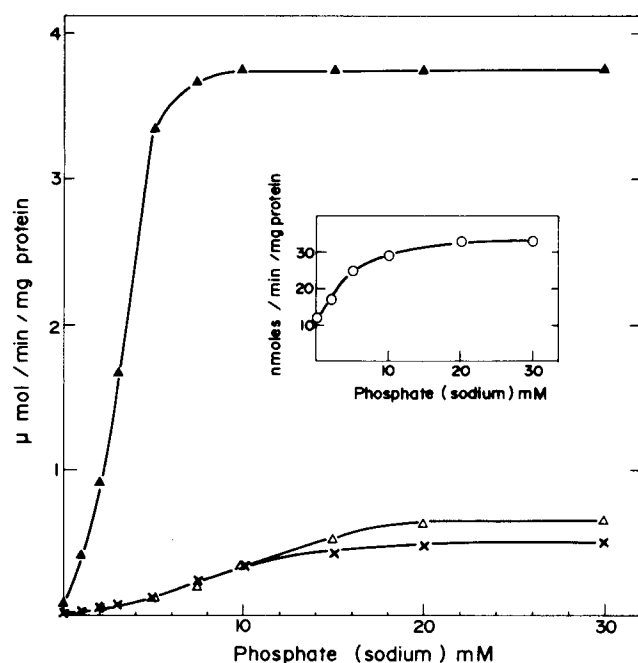


FIG. 3. Influence of added phosphate on the activity of yeast phosphofructokinase in the presence of AMP or ADP. A purified preparation was used. The enzyme was assayed as described under "Experimental Procedures" with the additions indicated. ○—○, no additions; △—△, 0.1 mM AMP; ×—×, 1 mM ADP; ▲—▲, 1 mM AMP.

decrease occurs without marked changes in the sigmoidicity of the curve as evidenced by the Hill coefficient (n_H).

AMP has been reported to activate yeast phosphofructokinase by relieving ATP inhibition (16) and that seems also to be the case for phosphate in a number of animal tissues (17). The activation by AMP and phosphate of the yeast enzyme was

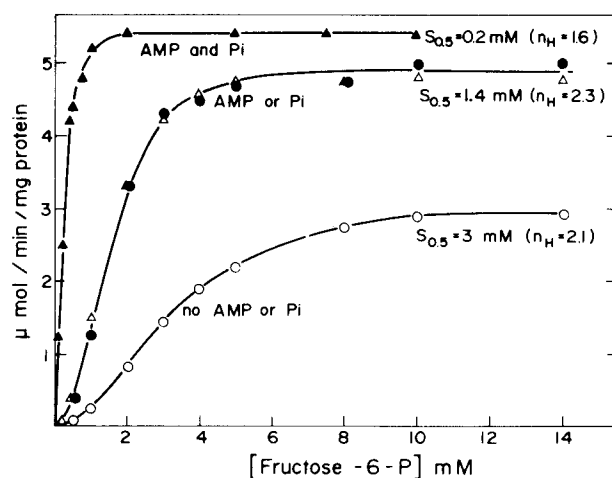


FIG. 4. Activity of yeast phosphofructokinase with changing fructose-6-P concentrations. A purified preparation was used and assayed as described under "Experimental Procedures" with the following additions: ○—○, no additions; △—△, 1 mM AMP; ●—●, 10 mM phosphate (P_i); ▲—▲, 1 mM AMP and 10 mM phosphate.

studied at different concentrations of ATP. The results presented in Fig. 5 show that phosphate alone has no effect on ATP inhibition, and that 5 mM AMP only slightly relieves ATP inhibition but that no inhibition by ATP is observed in the presence of both AMP and phosphate. From examination of the curves it appears that phosphate added to AMP not only completely relieves ATP inhibition but produces an additional activation of about 5-fold.

If GTP is used instead of ATP, the inhibition by excess nucleotide is much less marked (18) and can be counteracted by AMP alone. In this case phosphate also increased activity about 6-fold when present in addition to AMP (data not shown).

DISCUSSION

At physiological concentrations of substrates and other known effectors, phosphate activates yeast phosphofructokinase about 100-fold. The phosphofructokinase activity measured in these conditions is sufficient to account for the rate of glucose utilization by intact yeast. That such an effect of phosphate has been overlooked may be due to the fact that activation is dependent on the presence of AMP and on the pH of the assay. The allosteric properties of yeast phosphofructokinase have been mostly studied at pH 7.5 or higher where ATP inhibition is most marked. This, and the absence of AMP in the assay mixture may explain the report that phosphate has no effect on yeast phosphofructokinase (16) or is inhibitory (19). Although it has been stated that ATP is only slightly inhibitory, or not at all, below pH 6.5 (19, 20) we consistently found a marked inhibition by ATP at this pH with our enzyme preparations even when using the assay conditions employed by these authors. It is possible that the discrepancies between different results are due to some modification of phosphofructokinase during the purification procedure.

Phosphate and AMP, alone or in combination do not greatly affect the Hill coefficient for fructose-6-P. Our results confirm and extend the observation that the Hill coefficient for the *Saccharomyces cerevisiae* enzyme is independent of the concentration of ATP and of the presence or absence of AMP (21). However, in the case of the phosphofructokinase from *Saccharomyces carlsbergensis* a strong influence of AMP and ADP on

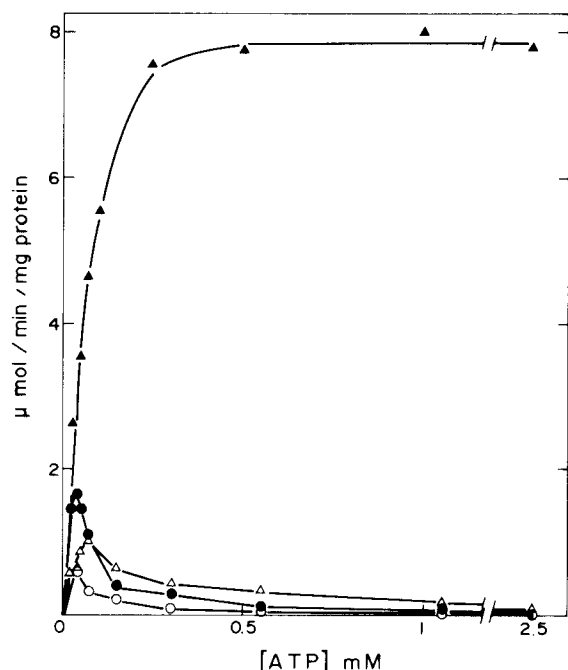


Fig. 5. Effect of the ATP concentration on the activity of yeast phosphofructokinase. A purified preparation was assayed as described under "Experimental Procedures" except that Mg^{2+} was always kept 1 mM in excess over ATP. Additions were made as follows: ○—○, no additions; ●—●, 10 mM phosphate; △—△, 5 mM AMP; ▲—▲, 10 mM phosphate and 5 mM AMP.

the cooperativity of the enzyme toward fructose-6-P has been reported (22).

Phosphate activates phosphofructokinase from animal origin by changing the kinetics toward fructose-6-P from sigmoidal to hyperbolic (23). This has been interpreted as being consistent with the existence of an equilibrium between two conformations of the enzyme with different affinity for fructose-6-P.

Phosphate would displace the equilibrium toward the conformation with greater affinity. In yeast where the Hill coefficient remains constant a different hypothesis has to be advanced.

Phosphate alone produces a 2-fold activation independent of the concentration of ATP. On the other hand AMP activates mainly by removing the inhibition by ATP. The behavior of the enzyme may be accounted for accepting the existence of two conformations R and T of yeast phosphofructokinase (24) with the following properties: R present high affinity for fructose-6-P and low for ATP as an inhibitor and T behaves the opposite (24). AMP and phosphate would present the same affinity for both conformations of the enzyme. AMP would act diminishing the affinity for ATP at the regulatory site and phosphate increasing the affinity for fructose-6-P. In addition, in the presence of both the increase in affinity for fructose-6-P would be greatly enhanced. This model is consistent with the fact that the sigmoidicity toward fructose-6-P is conserved in the presence of AMP and phosphate. The synergic effect of phosphate and AMP cannot be explained by an increase in the affinity of the enzyme for one of the compounds in the presence of the other since even at saturation neither phosphate nor AMP alone produce an activation comparable with that observed when both compounds act together. This observation also discards the possibility that AMP and phosphate have the same binding site on the enzyme. The situation differs from

that observed in animal tissues where the effects of AMP and phosphate are no more than additive (5, 23). The number of different phosphofructokinase conformations in equilibrium with each other could still be greater than discussed. In particular the sigmoidal kinetics of the activation by phosphate suggests the existence of two forms of enzyme·AMP complex with different affinity for phosphate.

It has been stated that yeast phosphofructokinase is a pure K system (24). However our results are more consistent with the idea that this enzyme is a mixed K-V system. Even at a saturating concentration of fructose-6-P, AMP or phosphate increases the rate of reaction 2-fold. In this case, their effects are not additive, the activation found with both effectors together is not markedly different from that found for each alone.

Although ADP cannot replace AMP for relieving ATP inhibition, phosphate with ADP activates also, although less than with AMP.

Many effectors have been suggested to control the activity of yeast phosphofructokinase in different metabolic situations. The shift glycolysis-gluconeogenesis is particularly interesting in view of the sophisticated mechanisms used to shut off gluconeogenic enzymes (25, 26). An analysis of the changes of the effectors in these conditions revealed that only citrate, fructose-1,6- P_2 , and fructose-6-P change markedly. However, the increase in citrate is not likely to play a crucial role in the regulation of yeast phosphofructokinase since at 2 mM citrate the decrease on phosphofructokinase activity is only about 30% (27). Fructose-1,6- P_2 has been shown to activate yeast phosphofructokinase up to 2-fold (27, 28), therefore the sharp drop in fructose-1,6- P_2 concentration would only cut activity by one-half. Since the rate of fructose-6-P phosphorylation in gluconeogenesis should decrease at least to one-twentieth of its glycolysis rate in order to have a net flux from fructose-1,6- P_2 to fructose-6-P the effect of the changes in citrate and fructose-1,6- P_2 concentrations are insufficient by far. In contrast, the drop in concentration of fructose-6-P in the shift glycolysis-gluconeogenesis would decrease the activity of phosphofructokinase by a factor of 10. Therefore in this case it appears that the activity of phosphofructokinase is basically regulated by the availability of substrate.

Phosphofructokinase from cells grown on ethanol is as sensitive to activation by phosphate as the enzyme from glucose-grown yeast. This fact together with the finding that levels of phosphate do not change significantly in the shift glycolysis-gluconeogenesis indicates that although necessary for activity in glycolysis phosphate does not control the activity of phosphofructokinase in this shift. However in situations where changes in the concentrations of phosphate occur like in the shift aerobiosis-anaerobiosis (Pasteur effect) (29) this compound may play a direct role in the regulation of phosphofructokinase activity.

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