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PROPERTIES OF YEAST PHOSPHOFRUCTOKINASE

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SUMMARY

The rate of conversion of Fru-6-*P* to Fru-1,6-P_2 catalyzed by phosphofructokinase (ATP:D-Fru-6-*P* 1-phosphotransferase, EC 2.7.1.11) is regulated by the inhibition of its activity by a high concentration of one of its substrates (ATP) and the relief of this inhibition by various effector metabolites. AMP specifically reverses the ATP inhibition of phosphofructokinase of yeast. In this communication, data are presented on the partial purification of yeast phosphofructokinase and its properties. The yeast phosphofructokinase prepared by the method described here was less sensitive to ATP inhibition than was the enzyme in the crude fraction. The enzyme, though stable at a concentration range of 8–10 mg protein per ml, lost half of its activity on dilution to about 0.8–1.0 mg protein per ml and on storing at 0°. The phosphofructokinase activity remaining after such dilution and storage appeared to be almost completely insensitive to ATP inhibition and AMP activation.

This loss of phosphofructokinase activity and of its sensitivity to ATP inhibition as well as AMP activation could be prevented by diluting the enzyme and storing it in the presence of ATP, AMP or Fru-6-*P* but not in the presence of $MgCl_2$ or NH_4Cl.

INTRODUCTION

The rate of conversion of Fru-6-*P* to Fru-1,6-P_2, catalyzed by phosphofructokinase (ATP: D-Fru-6-*P* 1-phosphotransferase, EC 2.7.1.11), appears to control the overall rate of conversion of Glc-6-*P* to pyruvate *via* the Embden–Meyerhof pathway in a variety of mammalian tissues[1,2], yeast[3,4], bacteria[1,5] and other organisms[1,6]. The salient features of phosphofructokinase regulation are the inhibition of enzyme activity by high concentrations of one substrate, ATP, and the relief of this inhibition by various effector metabolites[7]. In the case of yeast phosphofructokinase the ATP inhibition is specifically reversed by AMP and not by other mononucleotides[4]. In this communication data are presented demonstrating that the yeast phosphofructokinase partially purified by the method described here is less sensitive to ATP inhibition than is the enzyme in the crude fraction. On dilution and storage the enzyme loses

half of its activity and becomes almost completely insensitive to ATP inhibition and AMP activation. These effects can be prevented by diluting the enzyme in the presence of ATP, AMP or Fru-6-*P*.

EXPERIMENTAL PROCEDURE

Materials. Fru-6-*P*, nucleotides and enzymes were obtained from Sigma Chemical Co., U.S.A. Fru-6-*P* was labeled as 80% pure and the nucleotides as 95–97% pure. Alumina C_γ gel and hydroxylapatite were obtained from the Sigma Chemical Co., U.S.A. All other chemicals used were of Analar Reagent grade.

Assay of phosphofructokinase. Phosphofructokinase was assayed by coupling with excesses of aldolase, triosephosphate isomerase, and α-glycerophosphate dehydrogenase and following the rate of oxidation of NADH at 340 mμ. Unless otherwise mentioned, the assay mixture of 1.0 ml contained 33 mM Tris–HCl (pH 7.5), 3.3 mM $MgCl_2$, 2.3 mM reduced glutathione, 0.1 mM NADH and ATP as well as Fru-6-*P* at the concentrations mentioned under each legend to the figures. A unit of phosphofructokinase activity corresponds to the production of 1 μmole of Fru-1,6-P_2 per min. Catalase was estimated spectrophotometrically as described by CHANCE AND HERBERT[13]. Protein was estimated spectrophotometrically[10].

RESULTS

Partial purification of yeast phosphofructokinase

Step 1: Extraction. All procedures were carried out at temperatures of 0–4°. About 100 g of frozen yeast were suspended in 50 ml of 0.05 M phosphate buffer (pH 7.5) and homogenized for 30 min in a Virtis homogenizer with 150 g of glass beads at 65–70 speed (on the speed dial). This procedure was repeated to homogenize about 4 kg of frozen yeast. The resulting solution was centrifuged at 27 000 $\times g$ for 60 min in a Servall centrifuge. The supernatant was further centrifuged at 80 000 $\times g$ in a Spinco centrifuge for 90 min. The precipitate was discarded and the supernatant had phosphofructokinase with a specific activity of 0.06.

Step 2: Alumina C_γ gel treatment. To the supernatant (680 ml) was added alumina C_γ gel (solid gel was used) to provide 1 mg gel per 9 mg protein and the mixture was stirred for 30 min in the cold. The gel was collected by centrifugation and the supernatant was discarded. The gel was washed with 900 ml of distilled water and stirred. Phosphofructokinase was eluted from the gel by stirring it with 0.05 M phosphate buffer of pH 7.5 (3 $\times$ 600 ml). The specific activity of phosphofructokinase in this fraction was about 0.3, and 70% of the adsorbed enzyme was eluted.

Step 3: $(NH_4)_2SO_4$ precipitation. The alumina C_γ gel eluent was treated with $(NH_4)_2SO_4$ at 30% satn. (23.6 g/100 ml), stirred for 20 min and centrifuged. The precipitate had a negligible phosphofructokinase content and was discarded. 130 g of $(NH_4)_2SO_4$ were added to the supernatant (2030 ml) to provide 40% satn. and the combination was stirred for 30 min. The precipitate collected by centrifugation was dissolved in 30 ml of 0.05 M phosphate buffer (pH 7.5). This solution contained phosphofructokinase with a specific activity of 0.7 to 1.0.

Step 4: Alumina C_γ gel treatment. The phosphofructokinase fraction from the

above step was treated with alumina C_γ gel at a ratio of 1 mg gel per 6 mg protein and the mixture stirred. The supernatant collected after centrifugation contained phosphofructokinase with a specific activity of about 1.25. This was treated with $(NH_4)_2SO_4$ at 30% satn. (23.6 g/100 ml), stirred for 30 min and centrifuged. The precipitate was dissolved in 10 ml of 0.05 M phosphate buffer (pH 7.5). This solution had phosphofructokinase with a specific activity of 2.0.

Step 5: Hydroxylapatite treatment. The phosphofructokinase solution from the preceding step was treated with hydroxylapatite (1 mg wet weight for each 20–25 mg protein) and stirred for 20 min and 97–98% of the phosphofructokinase was adsorbed. The hydroxylapatite was washed with distilled water followed by 0.0625 M phosphate buffer (pH 7.9). Phosphofructokinase was finally eluted with 0.125 M phosphate buffer (pH 7.9) in three successive steps, which in all extracted about 50% of the adsorbed enzyme, and had a specific activity of 3–3.75. This was treated with $(NH_4)_2SO_4$ at 40% satn. (30 g/100 ml) and the precipitate was collected by centrifugation. The precipitate was dissolved in 5 ml of 0.05 M phosphate buffer (pH 7.5). The solution contained phosphofructokinase with a specific activity of 3.5–4.0.

Step 6: Heat treatment. The phosphofructokinase solution from the above step was immersed in a beaker containing hot water at 55–60° for 0.05 min and then at 50–51° for 5 min. About all phosphofructokinase remained in the supernatant after centrifugation, with a specific activity of 8–9.0. The same extent of purification of phosphofructokinase could be obtained from dried yeast even though, as compared to frozen yeast, it has a 4 times greater specific activity of the enzyme to start with. Attempts at further purification by DEAE-cellulose, CM-cellulose chromatography were not successful.

Sucrose gradient analysis of partially purified phosphofructokinase

Phosphofructokinase is extremely unstable in very dilute solutions. When 6.4 units of phosphofructokinase were applied on a discontinuous sucrose gradient with 10–20–40% sucrose in 0.05 M phosphate buffer (pH 7.5) at 30 000 rev./min in a Spinco centrifuge for 16 h, only a very small fraction, less than 0.1%, of the applied phosphofructokinase could be recovered. The same procedure was repeated in the presence of 3 mM Fru-6-*P* in the discontinuous gradient with catalase as the internal standard. About 70% of the phosphofructokinase could be recovered. The above experiment was repeated once and identical results were obtained. 20 fractions of 30 drops each were collected by puncturing the bottom of the container with a needle with a hole. The peak of activity of the phosphofructokinase appears at the 9th tube from the bottom, whereas the peak of activity of the catalase appears at the 13th tube. These results are shown in Fig. 1.

The peak of phosphofructokinase activity was symmetrical except for a little trailing in the end. With 11.3 as the sedimentation constant for catalase, the value of $s_{20,w}$ for phosphofructokinase is found to be 17.4, which is in close agreement with the value obtained by LINDELL AND STELLWAGEN[8] for the homogeneous preparation of phosphofructokinase of yeast. The molecular weight of phosphofructokinase was estimated to be 447 000 when calculated as described by MARTIN AND AMES[14].

Effect of NH_4^+

The stimulation of phosphofructokinase by NH_4^+ with ATP as phosphate donor

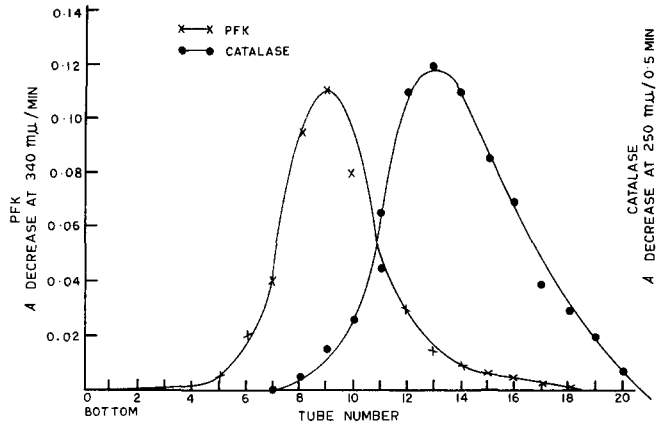

Fig. 1. Sedimentation pattern of phosphofructokinase in a 10–20–40% sucrose gradient, containing 3 mM Fru-6-*P*. 0.8 mg of protein in 0.1 ml was applied on the top of the gradient, and after 16 h of centrifugation at 30 000 rev./min at 4°, the gradient was fractionated and analysed. Activity of phosphofructokinase (PFK) is expressed as absorbance decrease at 340 mμ per min per 0.1 ml of 30-drop fractions collected. Catalase was assayed as absorbance decrease at 250 mμ per 0.5 min per 0.1 ml of the 30-drop fractions.

in the presence and absence of AMP at different Fru-6-*P* concentrations is shown in Fig. 2. The stimulation of phosphofructokinase activity by NH_4^+ is about the same at different concentrations of Fru-6-*P*; this is unlike the activation of the enzyme by AMP which has maximal effect at low concentrations of the substrate. When NH_4^+ and AMP are present together, they act synergistically, *i.e.* the percent increase in the activity of the enzyme in the presence of NH_4^+ and AMP together is greater than the sum of the percent increases brought about by NH_4^+ and AMP individually. A similar synergistic action of the effectors on increasing the phosphofructokinase activity of rabbit tissues has been observed[15,11].

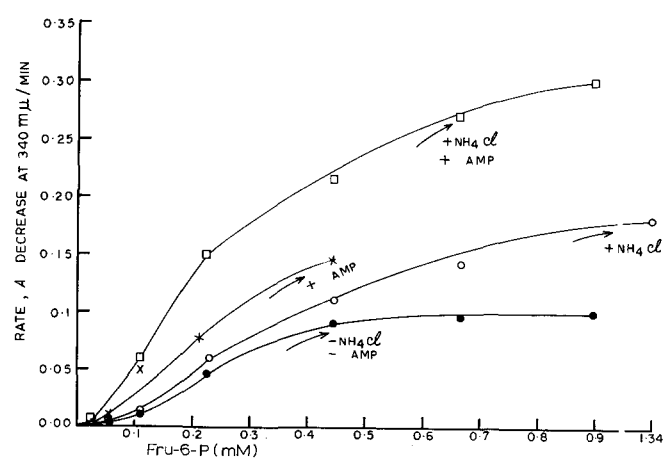

Fig. 2. Effect of NH_4^+ in the presence and absence of AMP on the activity of phosphofructokinase at different concentrations of Fru-6-*P*, with ATP at 0.16 mM as phosphate donor. Rest as described under EXPERIMENTAL PROCEDURE. The auxiliary enzymes were freed from $(NH_4)_2SO_4$. $(NH_4)_2SO_4$ and AMT were added at a concentration of 1.8 mM and 0.16 mM, respectively.

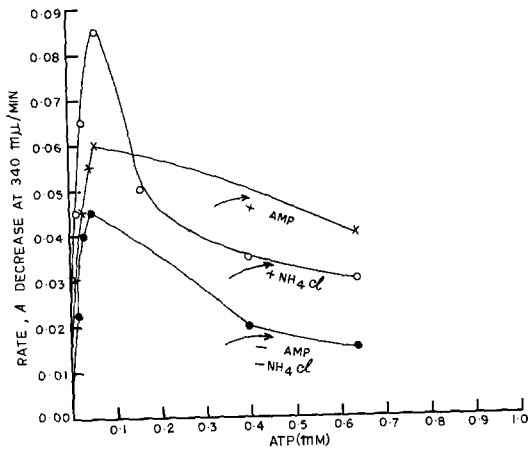

Fig. 3. Effect of NH_4^+ on the activity of phosphofructokinase at different ATP concentrations. Fru-6-*P* was added at 0.224 mM. Rest as described under EXPERIMENTAL PROCEDURE. The auxiliary enzymes were freed from $(NH_4)_2SO_4$. $(NH_4)_2SO_4$ and AMP were added at a concentration of 1.8 mM and 0.16 mM, respectively.

To see whether NH_4^+ similarly to AMP reverses the inhibition of phosphofructokinase activity by an excess concentration of ATP, the activity of phosphofructokinase was determined in the presence and absence of NH_4^+ at 0.224 mM concentration of Fru-6-*P* and various concentrations of ATP and the results are presented in Fig. 3. It can be seen that although the maximum velocity obtained in the presence

TABLE I

PURIFICATION OF YEAST PHOSPHOFRUCTOKINASE

Fraction	*Total Vol. (ml)*	*Activity (units/ml)*	*Total units*	*Protein (mg/ml)*	*Units/mg protein*
Initial fraction	—	—	—	115	0.025
Spinco supernatant of 30 000 rev./min for 90 min	680	4.125	2805	75	0.055
Alumina C_γ gel, 1 mg gel per 9 mg protein. The solid gel was eluted with 0.05 M phosphate buffer, pH 7.5	1800	1.0	1800	3.7	0.27
40% $(NH_4)_2SO_4$ ppt. of 30% $(NH_4)_2SO_4$ supernatant	27.0	32.0	864	32	1.0
Alumina C_γ gel, 1 mg gel per 6 mg protein -ve adsorption	28.0	30.0	847	24	1.26
30% $(NH_4)_2SO_4$ ppt.	9.0	94.0	846	50	1.88
Hydroxylapatite treatment	76.0	3.02	230	0.95	3.2
40% $(NH_4)_2SO_4$ ppt.	5.2	34.8	181	9.4	3.7
Heat-treated 50–51° for 5 min	—	—	—	—	8.0–9.0

TABEL II

ATP INHIBITION OF PHOSPHOFRUCTOKINASE AT DIFFERENT STAGES OF ITS PURIFICATION

Comparison of effects of different concentrations of ATP on the activity of yeast phosphofructokinase at different stages of the purification procedure. Fru-6-*P* was added at 0.28 mM. Rest as described under EXPERIMENTAL PROCEDURE. Activity is expressed as absorbance decrease at 340 mμ per min.

ATP (mM)	*Crude enzyme*		*40% $(NH_4)_2SO_4$ ppt. in 0.05 M phosphate buffer*		*The maximally purified enzyme*	
	Activity	*Inhibition (%)*	*Activity*	*Inhibition (%)*	*Activity*	*Inhibition (%)*
0.0						
0.016	0.02		0.035		0.04	
0.04	0.05		0.06		0.06	
0.08	0.05		0.07		0.07	
0.4	0.015	70	0.035	50	0.055	21
0.8			0.03	57	0.04	43
1.6	0.0	100	0.02	71	0.03	57

of NH_4^+ is about twice the velocity obtained in its absence, the percent inhibition by an excess concentration of ATP is about the same in the presence or absence of NH_4^+.

The yeast phosphofructokinase partially purified by the procedure given (Table I) did not appear to be as sensitive to inhibition by ATP as the yeast phosphofructokinase in the crude fraction. A comparison of the effects of varying concentrations of ATP on phosphofructokinase at different stages of purification is shown in Table II.

Stability of phosphofructokinase

The yeast phosphofructokinase obtained by the above procedure was fairly stable when stored frozen at 8–12 mg protein per ml. Repeated freezing and thawing caused a loss of only about 20% of its original activity. But if the enzyme was diluted to 0.8–1.0 mg protein per ml with 0.05 M phosphate buffer (pH 7.5), stored, frozen for about 14 h and then assayed after thawing, it lost 50% of its original activity. The

TABLE III

LACK OF ATP INHIBITION ON PHOSPHOFRUCTOKINASE AS A RESULT OF ITS DILUTION AND STORAGE

Lack of effect of ATP inhibition on the rate of yeast phosphofructokinase when diluted to a concentration of 0.8 mg protein per ml, frozen overnight and assayed the next day after thawing in the presence of 1.8 mM concentration of NH_4Cl, at different Fru-6-*P* concentrations. Activity of phosphofructokinase is expressed as absorbance decrease at 340 mμ per min.

ATP (mM)	*Activity of phosphofructokinase*		
	Fru-6-P concn:		
	0.112 mM	*0.28 mM*	*0.56 mM*
0.04	0.0125	0.04	0.085
0.2	0.0125	0.04	0.085
0.8	0.0125	0.04	0.080

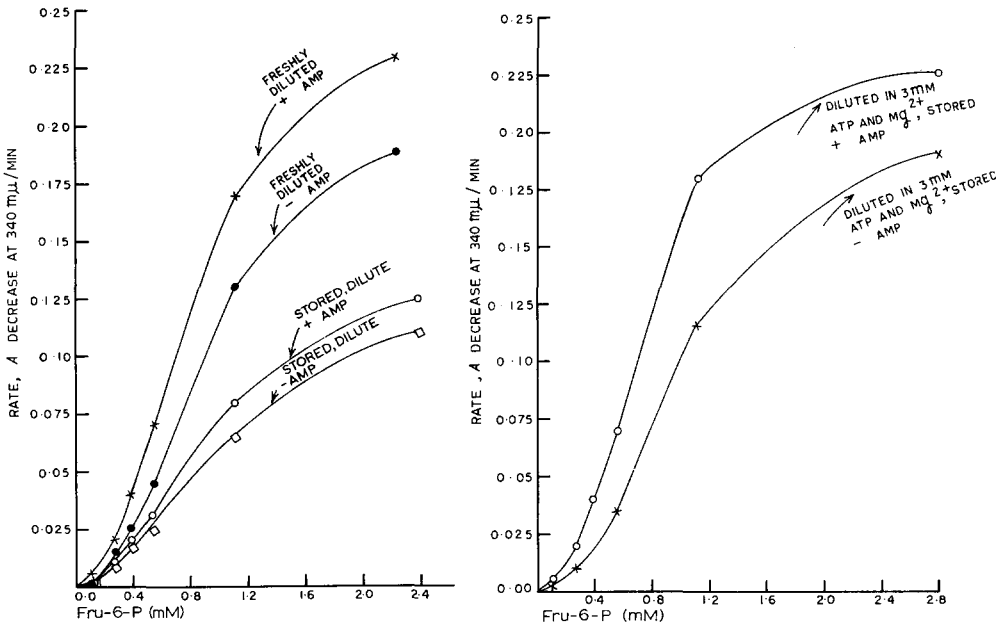

Fig. 4. Comparison of the effect of AMP (0.16 mM) on the activity of phosphofructokinase freshly diluted to give 0.8 mg protein per ml with the same after freezing overnight and thawing, at different Fru-6-*P* concentrations with ATP at 0.2 mM. Rest as described under EXPERIMENTAL PROCEDURE.

Fig. 5. Effect of AMP (0.16 mM) on the activity of phosphofructokinase diluted with 0.05 M phosphate buffer (pH 7.5) containing 3 mM Mg^{2+} and ATP, stored frozen for 14 h and assayed after thawing, at different Fru-6-*P* concentrations, with ATP at 0.2 mM.

loss of enzyme activity was not simply due to the change in the physical state of the solution, since no loss of activity was observed when assayed immediately after the freezing and thawing of freshly diluted enzyme. The loss of enzyme activity due to dilution of stock phosphofructokinase to give a solution of 0.8–1.0 mg protein per ml was a gradual process. The enzyme, when diluted and kept in ice for 5 h, lost about 40% of its activity, 60% when kept for 9 h.

The phosphofructokinase after diluting, freezing for 14 h and thawing showed different kinetic properties. It was neither inhibited by inhibiting concentrations of ATP nor activated to any great extent by AMP. These results are shown in Table III and Fig. 4.

The loss of enzyme activity by dilution and storage and changes in its kinetic properties could be prevented by diluting the enzyme with 0.05 M phosphate buffer (pH 7.5) containing 3 mM ATP, ATP and Mg^{2+}, Fru-6-*P* or AMP. Mg^{2+} or NH_4^+ added as $MgCl_2$ or NH_4Cl to a concentration of 3 mM in the dilution medium did not protect the enzyme from either loss of enzyme activity or sensitivity to ATP inhibition (Table IV and Fig. 5). The AMP- or ATP-treated enzymes showed properties similar to Fru-6-*P*-treated enzyme in relation to total enzyme activity and to inhibition by ATP as depicted in Table IV. AMP- or Fru-6-*P*-treated enzyme retained the sensitivity to AMP activation similar to ATP–Mg^{2+}-treated enzymes as shown in Fig. 5.

TABLE IV

PREVENTION OF LOSS OF ACTIVITY AND ATP SENSITIVITY OF PHOSPHOFRUCTOKINASE BY THE PRESENCE OF FRU-6-*P* IN THE DILUTION FLUID

Comparison of the effects of different concentrations of ATP on the activity of yeast phosphofructokinase freshly diluted with 0.05 M buffer (pH 7.5), containing nothing else or a 3 mM concentration $MgCl_2$, NH_4Cl or Fru-6-*P*, and the same enzymes stored overnight frozen, thawed, left for 5 h and then assayed at 0.28 mM Fru-6-*P*. Rest as described under EXPERIMENTAL PROCEDURE. Activity of phosphofructokinase is expressed as absorbance decrease at 340 mμ per min.

ATP (mM)	*Untreated phosphofructokinase activity*		*Mg^{2+}-treated phosphofructokinase activity*		*NH_4Cl-treated phosphofructokinase activity*		*Fru-6-P-treated phosphofructokinase activity*	
	Freshly diluted	*Stored, frozen and thawed*	*Freshly diluted*	*Stored, frozen and thawed*	*Freshly diluted*	*Stored, frozen and thawed*	*Freshly diluted*	*Stored, frozen and thawed*
0.0	0.0	0						
0.08	0.08	0.02	0.08	0.025	0.08	0.025	0.10	0.10
0.8	0.04	—	0.04	—	0.04	—	0.05	—
2.4	0.03	0.02	0.03	0.02	0.025	0.02	0.03	0.03
V at 1.7 mM Fru-6-*P*, 0.08 mM ATP	0.14	0.06	0.15	0.07	0.14	0.07	0.18	0.15

To see whether the changes in the properties of the enzyme as a result of dilution and storage are brought about by conformational alterations of the enzyme protein, part of the data of Figs. 4 and 5 was used to get Hill coefficients by the HILL[16] plot. The slopes of the lines or Hill coefficients (Figs. 6 and 7) are functions of the number of substrate binding sites on the enzyme and the strength of interaction between them, if one assumes that the sigmoidicity of the Fru-6-*P* saturation curve is due to more than one Fru-6-*P* binding site on the enzyme. The slope of the lines for freshly diluted

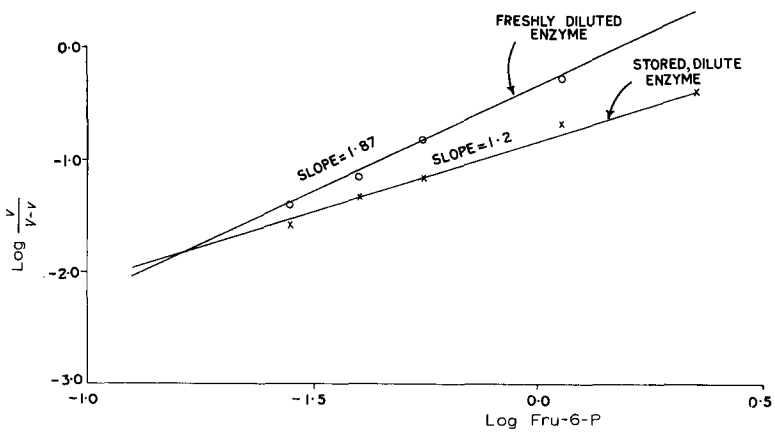

Fig. 6. HILL representation of the data of the freshly diluted and stored, diluted enzyme without AMP of Fig. 4. The maximum velocity of the reaction has been obtained for each curve by plotting the data according to Lineweaver and Burk and extrapolating to infinite concentrations of Fru-6-*P*, using only the points corresponding to high substrate concentration.

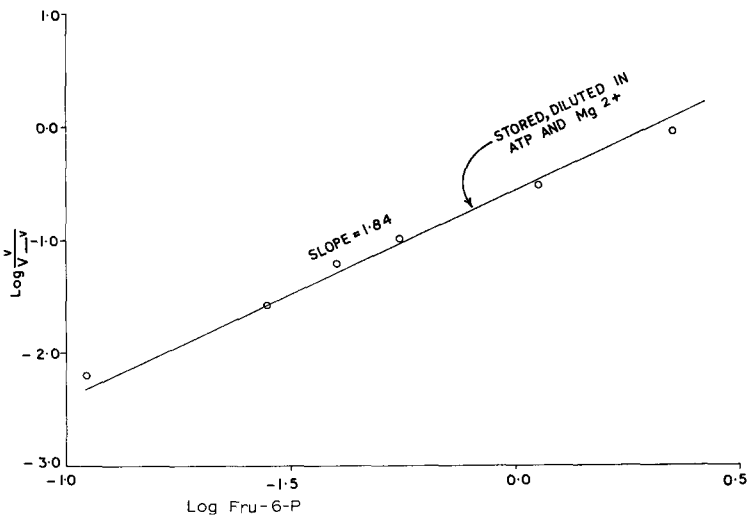

Fig. 7. HILL representation of the data of the stored enzyme diluted in 3 mM ATP–Mg^{2+} without AMP of Fig. 5. The maximum velocity of the reaction has been obtained by plotting the data according to Lineweaver and Burk and extrapolating to infinite concentrations of Fru-6-*P*, using only the points corresponding to high substrate concentration.

enzyme and for stored, diluted in ATP–Mg^{2+} enzyme is about same (Figs. 6 and 7), while the slope of the line for the stored, dilute enzyme is 1.2. ATKINSON *et al.*[17] observed a slope of 2.2 for a 25–45% $(NH_4)_2SO_4$ fraction of phosphofructokinase from the crude yeast cell preparation. The decrease in slope as a result of dilution and storage (Fig. 6) suggests that perhaps some conformational change of the enzyme protein is responsible for the observed kinetic properties of the stored, dilute enzyme. Such conformational change is prevented by storing the enzyme in 3 mM ATP and Mg^{2+} (Fig. 7).

DISCUSSION

The phosphofructokinase from a variety of tissues was activated by NH_4^+ and K^+ (refs. 7, 11). NH_4^+ acted as a deinhibitor of ATP inhibition of phosphofructokinase from muscle and heart of rabbit[11]. As shown in Fig. 3, NH_4^+ added as NH_4Cl mainly increased *V* but did not reverse inhibition of phosphofructokinase by ATP to any extent, as the percent of inhibition by an inhibitory concentration of ATP in the presence of NH_4^+ is about the same as in its absence. This effect of NH_4^+ was similar to its effects on the phosphofructokinase of rabbit liver[7]. NH_4^+ did not affect the apparent Michaelis constants of Fru-6-*P* or ATP (Figs. 2 and 3). K^+ did not have any effect on yeast phosphofructokinase; this is in agreement with the observation of SOLS AND SALAS[12]. The effect of NH_4^+ on yeast phosphofructokinase is independent of the effect of AMP (Fig. 2), indicating that it may have distinct binding sites on the enzyme. SOLS AND SALAS[12] reported that NH_4^+ did not increase *V* but reversed inhibition of phosphofructokinase by ATP. The reason for this discrepancy, however, is not clear. The physiological importance of the stimulation of yeast phosphofructokinase by NH_4^+ is not well understood. It is known that yeast and other

microorganisms take up NH_4^+ at a high rate during the log phase of their growth. This increased uptake of NH_4^+ during the log phase may increase the supply of amino nitrogen not only to meet the higher rate of protein biosynthesis but also to increase the rate of energy generation by activating the rate-controlling enzyme, phosphofructokinase, in the energy-yielding glycolytic sequence.

The decreasing sensitivity of yeast phosphofructokinase to ATP inhibition as observed with increasing purification of the enzyme (Table II) and the lack of ATP inhibition and decreased AMP activation of phosphofructokinase remaining after dilution and storage (Table III and Fig. 4) may be due to any of the following reasons.

The ATP inhibition site on the enzyme may be modified during purification resulting in a lower affinity for ATP, and after dilution and storage the enzyme may not bind ATP at all. Since it was suggested by the kinetic data that AMP also binds at the ATP inhibitory site[4], any modification of this binding site would also lead to a loss of an AMP effect. The modification of binding sites may result from conformational alterations of the enzyme structure. This idea is supported by the observation that the Hill coefficient for the stored, dilute enzyme is significantly lower than the Hill coefficient obtained for freshly diluted enzyme or for stored enzyme, diluted in 3 mM ATP and Mg^{2+} (Figs. 6 and 7).

The other possibility is that yeast phosphofructokinase may exist as an aggregate of subunits which only can be inhibited by ATP and activated by AMP. Dilution of the phosphofructokinase solution may lead to disaggregation and consequently to a loss of sensitivity to ATP inhibition and AMP activation. Dilution of the enzyme in the presence of ATP, AMP or Fru-6-*P* may, therefore, preserve the phosphofructokinase in its native state and thus retain its sensitivity to ATP and AMP.

Another possibility is that yeast has two distinct species of phosphofructokinase, only one of which is inhibited by ATP and activated by AMP. In the crude yeast extract the phosphofructokinase unaffected by ATP or AMP constitutes a small fraction of the total phosphofructokinase but is enriched during the process of purification. On this basis, one may conclude that the ATP-sensitive phosphofructokinase is the one of the two species that is lost by dilution and storage in the absence of stabilizers, and thus the resulting enzyme preparation does not respond to ATP and AMP.

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