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SOME KINETIC PROPERTIES OF YEAST PHOSPHOFRUCTOKINASE DESENSITIZED TO INHIBITION BY ADENOSINE TRIPHOSPHATE

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SUMMARY

Yeast phosphofructokinase (ATP:D-fructose-6-phosphate 1-phosphotransferase, EC 2.7.1.11) desensitized to ATP inhibition by the method of Afting *et al.* ((1971) *Arch. Biochem. Biophys.* 143, 587) exhibits normal Michaelis kinetics as judged by a double reciprocal plot or by a Hill plot with an apparent K_m of 0.095 mM for fructose 6-phosphate which is about three times less than the minimum value observed for the untreated enzyme. The desensitized phosphofructokinase is neither inhibited to any great extent by citrate, nor activated by AMP. However, the desensitized phosphofructokinase is still activated by NH_4^+. The desensitized phosphofructokinase can be reconverted to the ATP-sensitive phosphofructokinase by preincubating the enzyme at 28 °C with 3 mM ATP, 10 mM citrate, or both at pH 6.8 or 7.5. These results may be interpreted in terms of the model for allosteric enzymes proposed by Monod *et al.* ((1965) *J. Mol. Biol.* 12, 88) where ATP-sensitive phosphofructokinase which predominates in the absence of ligands is converted to the desensitized phosphofructokinase form in a synergistic manner by preincubation of the enzyme with the positive effectors and salts of fluoride and magnesium.

INTRODUCTION

Yeast phosphofructokinase (ATP:D-fructose-6-phosphate 1-phosphotransferase, EC 2.7.1.11) is inhibited by excess ATP[1,2] and deinhibited by AMP and fructose 6-phosphate[2]. It was observed that its sensitivity to ATP inhibition is decreased by the partial purification of the enzyme according to the method of Ramaiah[3] or Boehringer and Co. (see Freyer *et al.*[4]) while after purification by the procedure described by Lindell and Stellwagen[5] or by Atzpodien and Bode[6] it is fully sensitive to inhibition by excess ATP. The ATP inhibitable yeast phosphofructokinase (ATP-sensitive phosphofructokinase) can be desensitized to ATP inhibition either by trypsin treatment[7,8], or by preincubation of the enzyme in the presence of NH_4^+, ADP, fructose 6-phosphate, Mg^{2+} and F^- (ref. 9). The slope of Hill plot of ATP-sensitive yeast phosphofructokinase was 2 to 2.2 in the presence or absence of AMP, either with ATP,

GTP, or ITP as phosphate donor[10], suggesting more than one fructose 6-phosphate interacting site on the enzyme.

In this communication, data are presented to indicate that yeast phosphofructokinase desensitized to ATP inhibition by the method of Afting *et al.*[9]. exhibits normal Michaelis kinetics as judged by a double reciprocal plot or by a Hill plot with an apparent K_m of 0.095 mM for fructose 6-phosphate which is about three times less than the minimum value observed for the untreated enzyme[5]. The desensitized phosphofructokinase is neither inhibited to any great extent by citrate, nor activated by AMP. However, it is still activated by NH_4^+.

These results may be interpreted in terms of the model for allosteric enzymes proposed by Monod *et al.*[11] where the ATP-sensitive phosphofructokinase which predominates in the absence of ligands is converted to the desensitized phosphofructokinase form in a synergistic manner, by preincubation of the enzyme with the positive effectors and salts of fluoride and magnesium.

On this basis, AMP may bind both forms of the enzyme equally well but competes with ATP for the ATP-inhibitory site on the ATP-sensitive enzyme which is consistant with previous observations on this enzyme[2,12].

MATERIALS AND METHODS

Tris, imidazole, fructose 6-phosphate, fructose 1,6-diphosphate, ATP, ADP, AMP, NADH, fructose diphosphate aldolase (EC 4.1.2.13), triose phosphate isomerase (EC 5.3.1.1), and α-glycerophosphate dehydrogenase (EC 1.1.1.8) were obtained from Sigma Chemical Company, U.S.A. All other reagents used were of analytical reagent grade. The yeast phosphofructokinase was partially purified from commercial dried baker's yeast (Indian Yeast Company), essentially according to the method of Lindell and Stellwagen[5] with a slight modification. The dried yeast cells were broken according to the method of Ferguson and Rudney[13] except that 0.6 ml of toluene was added for a paste made with 1 g of dried yeast and 2 ml of distilled water. The enzyme at Step 3 of the purification procedure of Lindell and Stellwagen[5], which had a specific activity of about 2.0 was used in the experiments described below. Enzyme activity was assayed as described earlier[2] using a Perkin–Elmer spectrophotometer attached to a Hitachi–Perkin–Elmer recorder. Unless otherwise mentioned, the assay mixture in a total volume of 1.0 ml contained the following, at final concentrations: 50 mM Tris–HCl, pH 7.5, 3.0 mM $MgCl_2$, 2.5 mM 2-mercaptoethanol, 0.15-mM NADH, and an excess of fructose diphosphate aldolase, triose phosphate isomerase and α-glycerophosphate dehydrogenase freed of $(NH_4)_2SO_4$ by dialysis overnight against 0.01 M Tris–HCl, pH 8.0. ATP and fructose 6-phosphate were added at concentrations mentioned under each legend. The reaction was initiated by adding the enzyme, and the rate is linear with enzyme concentrations. The reaction rate was linear for the first 2 min at least at a concentration of 1 mM or less of fructose 6-phosphate while at a higher concentration of fructose 6-phosphate, acceleration of the rate was noticed in the desensitized enzyme. This was not due to coupling enzymes being insufficient since they are present to give about 10 times the maximum rate obtained at higher fructose 6-phosphate concentrations. The rate acceleration at higher fructose 6-phosphate concentration may perhaps be due to incomplete conversion of the ATP-

sensitive phosphofructokinase to the desensitized enzyme. The rate in the first 2 min was taken as the initial rate of enzymatic reaction.

RESULTS AND DISCUSSION

The preincubation mixture ("conversion mixture") for conversion of the enzyme to the desensitized phosphofructokinase contained at final concentrations 50 mM imidazole–HCl buffer, pH 6.8, 0.07 mM fructose 6-phosphate, 0.1 mM ADP, 10 mM $(NH_4)_2SO_4$, 1 mM $MgCl_2$, 10 mM NaF and 5 mg protein of the enzyme preparation in a total volume of 1 ml. The conversion reaction was carried out for 15 min at room temperature (21–22 °C). The ratio of activity at 0.05 mM ATP to the activity at 1 mM ATP was about 0.4 for the desensitized phosphofructokinase which confirms the observation of Afting *et al.*[9] as compared to about 6 before preincubation of the enzyme. The desensitized enzyme thus made was stable for at least 10 days when stored at −70 °C.

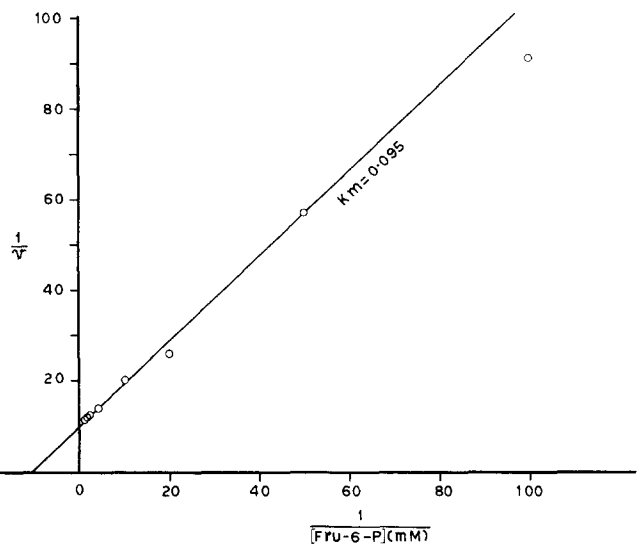

Fig. 1. The activity of desensitized phosphofructokinase at various concentrations of fructose 6-phosphate was plotted according to Lineweaver and Burk[14]. ATP was present at 1 mM and the other conditions of the assay were as described in Materials and Methods. The desensitized enzyme was prepared according to the method of Afting *et al.*[9].

The velocity of reaction of desensitized enzyme at various concentrations of fructose 6-phosphate at a constant concentration of 1 mM ATP (Fig. 1) when plotted according to Lineweaver and Burk[14] and to Hill[15] (Fig. 2) gave straight lines. The Hill plot slope was 0.91 and the apparent K_m for fructose 6-phosphate was calculated to be 0.095 to 0.10 mM from these plots. This value is about three times less than the minimum value observed for the ATP-sensitive enzyme at a noninhibitory concentration of ATP[5] and about 26 times less than that at an inhibitory concentration of 1 mM ATP (Fig. 2). The desensitized enzyme was not activated by the presence of 1 mM AMP and the apparent K_m value for fructose 6-phosphate was about

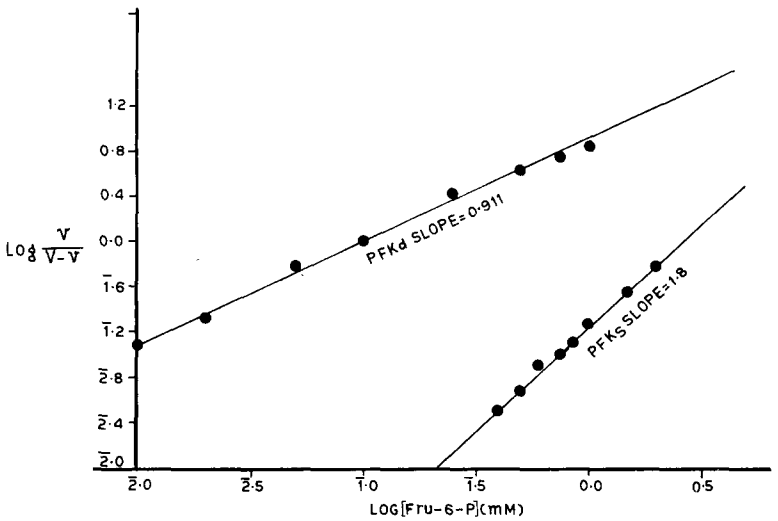

Fig. 2. The activity of desensitized and ATP-sensitive phosphofructokinase at various concentrations of fructose 6-phosphate was plotted according to Hill[15]. The maximum velocity of the desensitized enzyme was obtained from Fig. 1. The maximum velocity of the ATP-sensitive enzyme was obtained from a double-reciprocal plot of its activity at 1 mM ATP and varying concentrations of fructose 6-phosphate in the presence of 100-fold diluted "conversion mixture" in order to compare with the assay conditions of the desensitized enzyme, and extrapolating to infinite concentration of fructose 6-phosphate using points corresponding to high concentration of fructose 6-phosphate only. PFKd = desensitized, PFKs = ATP-sensitive phosphofructokinase.

the same as in its absence as obtained by double reciprocal plot. 10 mM citrate brought about 30% inhibition of the desensitized phosphofructokinase at low or high concentration of ATP and a fixed concentration of 0.25 mM fructose 6-phosphate, but the inhibition was almost abolished by increasing the concentration of $MgCl_2$ from 3 mM to 7.5 mM.

The desensitized enzyme was activated by NH_4^+ as shown in Table I. NH_4^+ activates the desensitized enzyme to about the same extent either at low or high concentration of fructose 6-phosphate, but it stimulates the ATP-sensitive phosphofructokinase 14-fold at a low concentration of fructose 6-phosphate while at a high

TABLE I

EFFECT OF AMMONIUM IONS ON THE ACTIVITY OF ATP-SENSITIVE AND DESENSITIZED PHOSPHOFRUCTOKINASE

The desensitized phosphofructokinase was prepared according to the method of Afting *et al.*[9]. The other conditions of assay were the same as described in Materials and Methods.

Phosphofructokinase used	*NH_4Cl (mM)*	*Activity at 0.25 mM Fru-6-P + 1 mM ATP (ΔA_{340}/min)*	*Stimulation by NH_4Cl relative to control*	*Activity at 10 mM Fru-6-P + 1 mM ATP (ΔA_{340}/min)*	*Stimulation by NH_4Cl relative to control*
ATP-sensitive	0.0	0.001	1.0	0.049	1.0
	50.0	0.014	14.0	0.115	2.35
Dezensitized	0.0	0.025	1.0	0.04	1.0
	50.0	0.053	2.1	0.068	1.7

concentration of fructose 6-phosphate its extent of activation is about the same as for the desensitized enzyme. The effects of NH_4^+ on the ATP-sensitive enzyme at low and high fructose 6-phosphate concentration are in agreement with the observations of Mavis and Stellwagen[16].

The desensitized enzyme can be reconverted to the ATP-sensitive enzyme by preincubating it for 15 min at 28 °C with 3 mM ATP, 10 mM citrate, or both at pH 6.8 or 7.5.

These results suggest that yeast phosphofructokinase may exist in two interconvertible forms, the ATP-sensitive and the desensitized, the former having inhibitory sites for ATP and citrate and low affinity for fructose 6-phosphate, with the latter having a comparatively high affinity for fructose 6-phosphate and NH_4^+. This may be depicted as follows:

ATP-sensitive phosphofructokinase has inhibitory sites for ATP and citrate and low affinity for Fru-6-*P*	NH_4^+, Fru-6-*P*, ADP, F^-, Mg^{2+}, 21 °C ⟶ ⟵ ATP, citrate, or both, 28 °C	desensitized phosphofructokinase has high affinity for Fru-6-*P* and NH_4^+.

On this basis AMP may bind to both forms equally well but competes with the ATP inhibitory site on the ATP-sensitive enzyme, without itself having any effect on catalysis.

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