STABILIZATION OF ACTIVE FORM OF RABBIT LIVER PHOSPHOFRUCTOKINASE*

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Received February 20,1974

SUMMARY : (1) The active form of rabbit liver phosphofructokinase when preincubated in presence of F^- and effectors of the enzyme is stabilized against its conversion to less active form as a result of dilution. (2) The stabilized active form of enzyme has a Km value of 0.01 mM for fructose 6-phosphate, the same as measured in presence of all the positive effectors, and is lower, by 13 times, than the Km value of the non-stabilized control enzyme, and exhibits normal Michaelis-Menten kinetics, in contrast to the non-stabilized control enzyme which shows sigmoidal kinetics. (3) The stabilized active form of enzyme is neither inhibited by excess concentration of ATP nor activated by activators of phosphofructokinase. (4) The data thus support the proposition that the enzyme does indeed exist in two interconvertible forms with enormous difference in their affinities for fructose 6-phosphate and effectors.

INTRODUCTION: It was shown earlier that partially purified rabbit liver phosphofructokinase (ATP:D-fructose-6-phosphate I-phosphotransferase, EC 2.7.I.II) stored in presence of its effectors like NH_4^+, SO_4^{2-}, K^+, P_i and ATP exists in a form with high affinity for fructose 6-phosphate, based on the initial velocity in few seconds after starting the reaction by the addition of enzyme (1,2). The initial velocity decays with time at low fructose 6-phosphate concentration or at high ATP concentration (2). If the reaction was initiated, after incubation of enzyme for 2 min in the assay mixture in the cuvette, by addition of substrate, or by the enzyme freed of effectors by passing it through Sephadex-G-25, the enzyme exists in a form with low affinity for fructose 6-phosphate (2).

* This work constitutes a part of the Ph.D. thesis of N.S. Karadsheh, All India Institute of Medical Sciences, 1974.

In the present communication, conditions for stabilization of the active form of rabbit liver phosphofructokinase and some of its properties are presented (Some of these observations were presented at symposium (3)).

MATERIALS AND METHODS: The chemicals and enzymes and their sources are the same as described earlier (2). The rabbit liver phosphofructokinase was purified as described earlier (2). The specific activity of this preparation was 1.6 unit per mg.

Phosphofructokinase activity was determined as described earlier (2). The concentrations of fructose 6-phosphate and ATP are as given in the legends to the tables and figures. The reaction was initiated by adding the enzyme. The enzyme was applied on a small perspex spatula and immediately stirred into the assay mixture in the cuvette. The process of stirring and closing the cuvette chamber generally took 3 to 5 seconds. The rate in the first 10 seconds, after starting the reaction, was taken as the rate of reaction, unless indicated otherwise.

Stabilization mixture : 50 mM Imidazole-HCl buffer containing 10 mM NaF, 3 mM $MgCl_2$, 0.1 mM ADP and 0.1 mM fructose 6-phosphate and 70 mM $(NH_4)_2SO_4$, 1 mM ATP and 1 mM K_2HPO_4 (as carried over with the stock enzyme solution) at pH 7.5.

Dilution mixture : 100 mM tris-HCl containing 0.35 mM ATP, 4 µM fructose 1,6-diphosphate and 0.02% BSA, which was shown earlier to prevent irreversible inactivation of enzyme (1), and 70 mM $(NH_4)_2SO_4$, 1 mM ATP and 1 mM K_2HPO_4(as carried over with the stock enzyme solution) at pH 8.0.

RESULTS AND DISCUSSION: Rabbit liver phosphofructokinase stored in presence of its effectors 0.7 M $(NH_4)_2SO_4$, 10 mM ATP and 10 mM K_2HPO_4, which exists in an active form, is transformed to less active form during enzymatic assay in absence of activators and

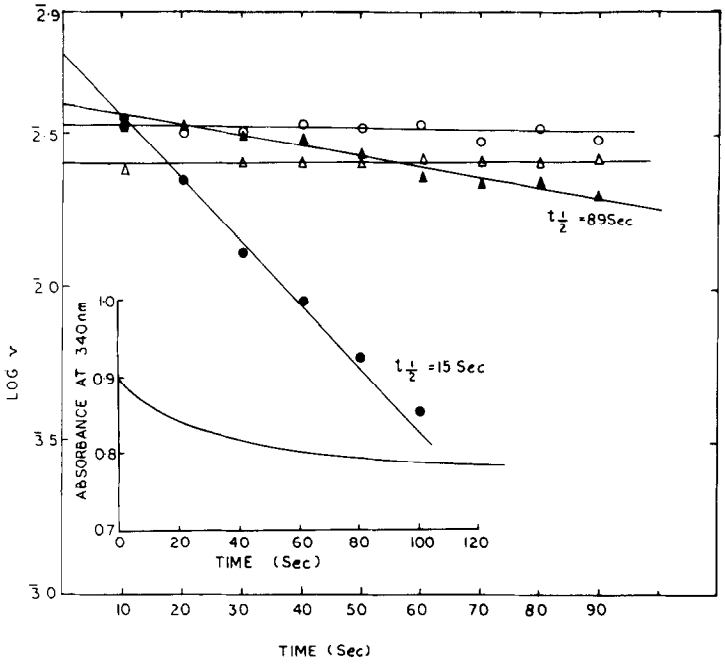

Figure 1 : Rate of deceleration of the stabilized active form and non-stabilized control phosphofructokinase at 0.1 mM fructose 6-phosphate and 0.083 or 1.0 mM ATP.

log v is the log of the rate of reaction obtained from the slope of the tangent drawn for the reaction progress curve at any time, the rate being measured as ΔA_{340}/min. The reaction progress curve for the control enzyme at 1.0 mM ATP is shown in the inset. Stock liver phosphofructokinase stored in presence of 50 mM tris-HCl, pH 7.5, 0.7 M $(NH_4)_2SO_4$, 10 mM ATP, 10 mM K_2HPO_4 and 0.33 mM EDTA, was diluted 10-fold in the "stabilization mixture" and incubated at 28-29°C for 25 min. 5 µl of this incubated mixture was used. The concentration of fructose 6-phosphate was 0.1 mM and that of ATP was 1.0 mM (O——O) or 0.083 mM (△——△).

Stock liver phosphofructokinase stored as mentioned above was diluted 10-fold in the "dilution mixture" and incubated at 28-29°C for 25 min. 5 µl of this incubated mixture was used. The concentration of fructose 6-phosphate was 0.1 mM and that of ATP was 1.0 mM (●——●) or 0.083 mM (▲——▲). All the other effectors present in the "stabilization mixture" and absent here, were added at 200-fold dilution in the assay mixture, in order to correspond with the same conditions of the assay of the stabilized active form of phosphofructokinase.

All the other conditions of the assay were the same as described in the assay procedure.

t1/2 = time required in sec for 50% conversion of active form to less active form of phosphofructokinase and was calculated as follows: t1/2 = 0.693/ka, ka = rate constant of the reaction of conversion of active form to less active form of the phosphofructokinase. This value was obtained from Fig.1 by multiplying the slopes of lines by -2.303.

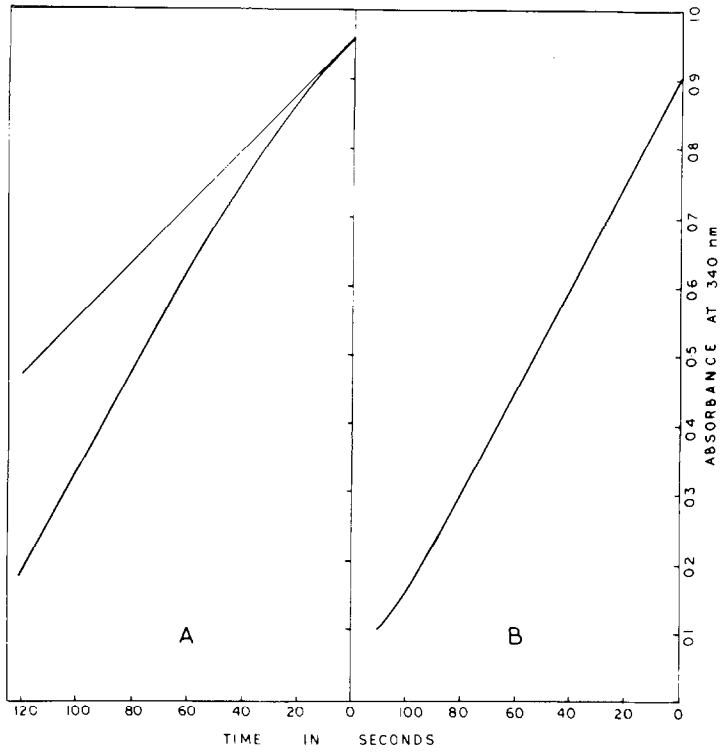

Figure 2 : Typical plot of the rate of stabilized active form and control phosphofructokinase in presence of activators.

5 µl of stabilized active form of enzyme or control enzyme, prepared as described in the legends to Fig.1, was used. The concentration of fructose 6-phosphate used was 0.1 mM and that for ATP was 1.0 mM in presence of activators, 5 mM $(NH_4)_2 SO_4$ 2.5 mM Pi and 0.185 mM 5'-AMP, in both cases. All the other conditions of assay were the same as described in the legends to Fig.1.

(A) The curved line is the actual rate of reaction of stabilized active form of enzyme. The straight line is the rate of reaction, if the rate of reaction of phosphofructokinase were to be linear with time.

(B) The linear line is the actual rate of reaction of control phosphofructokinase.

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at low concentrations of fructose 6-phosphate (2). To determine the half time of decay of enzyme activity, under various conditions, tangents were drawn from the reaction progress curves, and the velocity of reaction at any time was obtained from the slope of the tangent. The log of velocity is plotted against time as shown in Fig.1. The conversion of active form to less

TABLE I. Effect of high ATP concentration on the activity of stabilized active form and non-stabilized control rabbit liver phosphofructokinase. The stabilized active form and non-stabilized control phosphofructokinase were prepared as described in the legends to Fig.1. The rate during the period 60-90 sec was taken as the rate of reaction in all cases. The other conditions of assay were the same as described in the legends to Fig.1.

Phosphofructokinase used	Rate ΔA_{340}/min		Percentage inhibition by 1.0 mM ATP
	0.1 mM F6P* + 0.083 mM ATP	0.1 mM F6P* +1.0 mM ATP	
5 µl stabilized active form of enzyme	0.156	0.192	-
5 µl non-stabilized control enzyme	0.135	0.026	81

* F6P, fructose 6-phosphate.

active form is prevented by preincubation of enzyme at 28-29°C for 25 min in "stabilization mixture" as can be seen in Fig. 1. The non-stabilized control enzyme decays with a t1/2 of 15 sec and 89 sec at 1.0 mM and 0.083 mM ATP, respectively, while the stabilized active form of enzyme has a constant rate for at least 90 sec. However, the activity of the control enzyme as obtained by extrapolation to zero time at 1 mM ATP and 0.1 mM fructose 6-phosphate indicates that it is about 70-80% more than the activity observed for stabilized active form of enzyme (Fig.1). This suggests that all the enzyme in the control is in the active form, although it decays rapidly to the less active form during assay, while in case of the enzyme treated with the "stabilization mixture" only about half of the total enzyme appears to be in the active form and this is stabilized against its conversion to less active form, the remaining half is in the less active form unexpressed under these conditions in view of its inhibition by 1 mM ATP (Table I) and its high Km value for fructose 6-phosphate (1.0 mM) (2). This conclusion is supported

by the fact that in presence of activators, 5 mM $(NH_4)_2SO_4$, 2.5 mM P_i and 0.185 mM 5'-AMP or high fructose 6-phosphate concentration, although the initial activity of the enzyme treated with "stabilization mixture" is unaffected, its rate of reaction is accelerated to a final activity which is about twice the initial activity (Fig.2A), suggesting that the increment in rate of reaction could be due to a slow conversion of the less active form to the active form because it is not inhibited by ATP under these conditions. In case of the control enzyme, the initial activity is about twice the initial activity of the enzyme treated with the 'stabilization mixture' (Figs.1, 2A and 2B), suggesting that initially all the enzyme in the control is in the active form and its decay to less active form is prevented in presence of activators (Fig.2B) or high fructose 6-phosphate concentration, during assay.

Preliminary studies on the role of individual effectors in the "stabilization mixture" for stabilizing the active form of phosphofructokinase indicate absolute requirements for F^- and high concentration of ammonium sulphate (70 mM). The presence of ATP in the "stabilization mixture" is essential to maintain more amount of enzyme in the active form. In the absence of ATP, there is about a 50% decrease in the active form of enzyme as judged by the drop in the initial activity at 1 mM ATP and 0.1 mM fructose 6-phosphate, but the rate is stabilized against its conversion to less active form of enzyme, during the assay of the enzyme activity. Mg^{2+} does not appear to be necessary for the stabilization process. Stabilization does not occur at 0°C. The "stabilization mixture" does not stabilize the active form of enzyme if the stock enzyme were to be diluted 50-fold (1 mg protein of enzyme preparation/1 ml of "stabiliza-

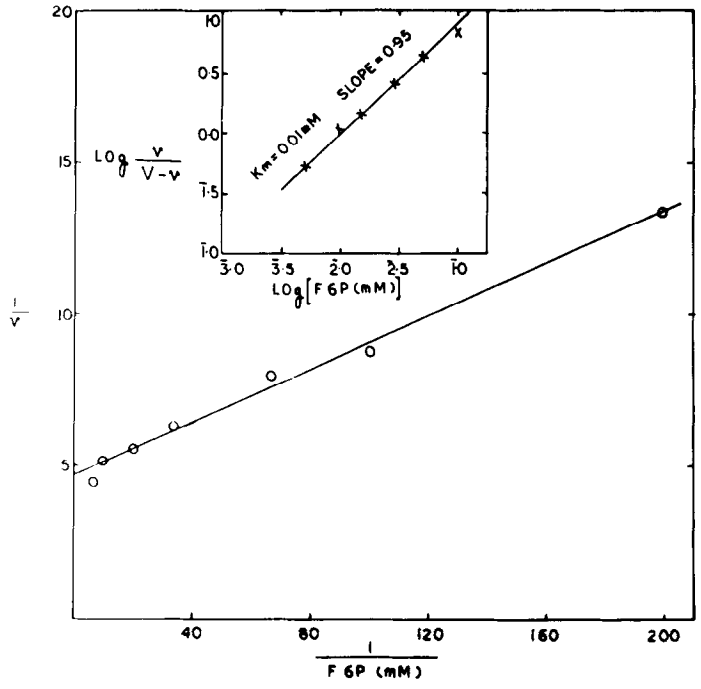

Figure 3 : The activity of the stabilized active form of phosphofructokinase at various concentrations of fructose 6-phosphate and constant concentration of 1 mM ATP was plotted according to Lineweaver and Burk and according to Hill as shown in the inset. 5 µl of stabilized active form of enzyme, prepared as described in the legends to Fig.1 was used for all the assay described here.

All the other conditions of assay were the same as described in the assay procedure. F6P = fructose 6-phosphate.

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tion mixture"), even at 28-29°C. The explanations for these results are not clear at present and are under investigations.

The stabilized active form of rabbit liver phosphofructokinase is similar to the desensitized form of yeast phosphofructokinase (4,5), in that it is not inhibited by excess concentration of ATP as shown in Table I, has very low Km value for fructose 6-phosphate, which is similar to the Km value obtained for the enzyme in presence of activators, 5 mM $(NH_4)_2SO_4$, 2.5 mM P_i and 0.185 mM 5'-AMP, and exhibits normal Michaelis - Menten kinetics. In contrast to the non-stabilized control enzyme, which has a high Km value for fructose 6-phosphate,

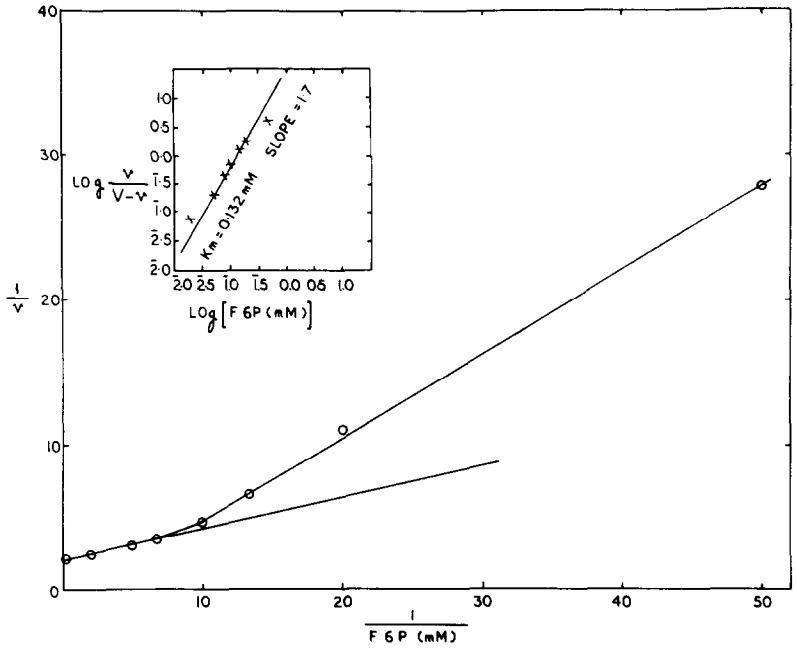

Figure 4 : The activity of non-stabilized control phosphofructokinase at various concentrations of fructose 6-phosphate and constant concentration of 1 mM ATP was plotted according to Lineweaver and Burk and according to Hill as shown in the inset. 5 µl of non-stabilized control enzyme prepared as described in the legends to Fig.1. was used for all the assays described here. All the other conditions of the assay were the same as described in the legends to Fig.1. F6P = fructose 6-phosphate.

exhibits sigmoidal kinetics and is inhibited by high concentration of ATP (Figs. 3 and 4 & Table I).

However, the desensitization process of yeast phosphofructokinase to ATP inhibition has an absolute requirements for all the effectors in the "conversion mixture" and ATP inhibits the desensitization process. The desensitization of yeast phosphofructokinase involves conversion of less active form to more active form, while in the case of liver phosphofructokinase it is stabilization of active form against its conversion to less active form. The stabilized active form of rabbit liver phosphofructokinase at excess concentration of ATP (1 mM) and very low concentrations of fructose 6-phosphate (< 0.05 mM) does decline in rate

with time, although at a far lower rate than for the nonstabilized control enzyme, suggesting that the active form of enzyme at concentrations of fructose 6-phosphate lower than 0.05 mM is unstable during assay. The non-stabilized control enzyme exhibits a Km value of 0.13 mM if the rate of reaction in the first 10 sec, after starting the reaction, was taken (Fig.4) and 0.213 mM if the rate in the period 10-40 sec, after starting the reaction, was taken as the rate of reaction (data not shown), suggesting that during assay, the proportion of less active form of enzyme increases with time of reaction, as judged by its approach towards the Km value of the less active enzyme which is 1.0 mM (2).

The data presented in this paper thus supports the proposition (2) that the enzyme exists in two interconvertible forms with enormous difference in their affinities for fructose 6-phosphate and effectors. The differences in the physical properties of the two forms, are under investigation.

ACKNOWLEDGEMENTS: This work was supported by a research grant No.BRNS/B & M/58/71 from the Department of Atomic Energy, Government of India. N.S.K. is thankful to the Council of Scientific and Industrial Research, India, for an award of Junior Research Fellowship.

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