Factors Influencing the Stability of Heart Phosphofructokinase

NABIL WAKID[1] AND TAG E. MANSOUR

Department of Pharmacology, Stanford University School of Medicine, Palo Alto, California

(Received April 20, 1965)

SUMMARY

Phosphofructokinase from the guinea pig heart is an extremely labile enzyme. It becomes more stable in the presence of perchloric acid extracts from the liver. Fructose-1,6-P_2 has been identified in these extracts as a potent stabilizer for the enzyme. The stabilizing effects for enzyme activity of the following four groups of compounds were tested: thiol derivatives, hexose phosphates, adenylic nucleotides, and polyvalent inorganic anions. The most potent representatives of these groups are: glutathione or mercaptoethanol, fructose-1,6-P_2, ADP or ATP, and phosphate or sulfate anion. A combination of these agents, besides providing complete protection for the enzyme, causes a slight activation during incubation.

INTRODUCTION

Phosphofructokinase is the enzyme which catalyzes Reaction 1:

$$\text{fructose-6-P} + \text{ATP} \xrightarrow{Mg^{++}} \text{fructose-1,6-P}_2 + \text{ADP} \quad (1)$$

A number of reports in the literature indicate that phosphofructokinase plays an important role in the regulation of glycolysis in the cell (1–3). Two types of drugs have been shown to affect glycolysis through a specific action on the enzyme: (a) antimonial compounds, which inhibit glycolysis in *Schistosoma mansoni* (4); (b) serotonin, which stimulates glycolysis in *Fasciola hepatica* (5). Some evidence has been reported recently which suggests that insulin might affect phosphofructokinase in heart and skeletal muscle (6, 7). Attempts to purify phosphofructokinase from various sources have always been handicapped by the marked instability of the enzyme (8, 9). Recent investigations in our laboratory designed to isolate heart phosphofructokinase in a highly pure state demonstrated that the enzyme becomes extremely labile after a mild degree of purification (10). Factors that were found to contribute to the instability of the enzyme are: an acid pH, the presence of the enzyme in the diluted form, and the separation of the enzyme from certain tissue components, particularly after passage through a Sephadex G-75 column. Colowick (11) and Utter (12) suggested that a thermostable "stabilizing factor"[2] is present in the tissues and is important for maintaining the stability of phosphofructokinase. Recently, it was demonstrated that certain tissue extracts from the heart and other organs can reactivate acid-inactivated heart phosphofructokinase (13). This effect was not

[1] Participant of the Visiting Scientists Research Program of the National Academy of Sciences. Present address: Department of Biochemistry, American University of Beirut, Beirut, Lebanon.

[2] The following terms are used in this paper: "stabilization" describes the process of preventing loss of enzyme activity; "reactivation" describes the process of recovering activity of an enzyme which has previously been inactivated; "activation" describes an increase in activity of an enzyme which has not previously been inactivated.

abolished after boiling the tissue extracts. Of the tissue extracts tested liver extracts were the most potent. The question arose whether mammalian tissues contain certain components that would increase the stability of phosphofructokinase. This paper presents data relating to the identification of some of these components as well as the conditions found to be optimal for enzyme stability.

MATERIALS AND METHODS

Guinea pig heart phosphofructokinase was prepared by a procedure described before (10). The enzyme, isolated after Mg-ethanol precipitation (step 3), with a specific activity of approximately 2.3 units (see below) per milligram protein at 25° and pH 8.2 was used throughout these experiments. The enzymes aldolase, triosephosphate isomerase, α-glycerophosphate dehydrogenase, phosphoglucose isomerase, glucose-6-phosphate dehydrogenase, and phosphoglucomutase were purchased from Boehringer and Sons through the California Corporation for Biochemical Research.

Phosphofructokinase activity was measured in a series of reactions in which it was coupled with excess amounts of aldolase, triosephosphate isomerase, and glycerophosphate dehydrogenase systems for the measurement of fructose-1,6-P_2 produced. The rate of disappearance of DPNH was measured spectrophotometrically. The reaction mixture provided conditions for maximal enzyme activity as reported before (10). A unit of phosphofructokinase is the amount of enzyme that catalyzes the formation of 1 μmole of fructose-1,6-P_2 per minute. Since the enzyme used in the experiments described below was highly diluted, the activity is expressed in milliunits. Perchloric acid extracts from the guinea pig liver were prepared by homogenizing the liver in a Waring Blendor with an equal volume of water; 70% perchloric acid was then added dropwise with constant stirring until the final concentration was 4%. The protein residue was removed by centrifugation and the supernatant fluid was decanted and neutralized with 30% KOH. The potassium perchlorate residue formed was centrifuged in the cold and discarded. The supernatant fluid is referred to as the liver perchloric acid extract. The following hexose phosphates were determined enzymically through coupled enzyme reactions which involved oxidation of DPNH or reduction of TPN: glucose-1-P, glucose-6-P, fructose-6-P, fructose-1,6-P_2, and triosephosphates (14). The following analytical methods were used: the method of Gomori (15) for inorganic phosphate, the method of Somogyi (16) for reducing sugars, and the resorcinol method of Roe for ketoses (17) as modified by Higashi and Peters (18). Stability of the enzyme was measured by incubating a diluted solution of the enzyme containing about 20 milliunits (mU) of phosphofructokinase in 0.05 M Tris-chloride pH 8.0 and the tested additions. Enzyme activity was measured before incubation and at the end of the incubation time. The percentage of enzyme activity which was left after such incubation was calculated.

RESULTS

Stability of Phosphofructokinase

As illustrated in Fig. 1, a dilute solution of phosphofructokinase, when kept at 0°, shows a progressive inactivation which was nearly complete in 4–5 hr. The addition of 0.01 M glutathione and 0.01% albumin did not significantly affect enzyme stability. Inactivation was accelerated at high temperatures and at an acidic pH.

In preliminary experiments, it was found that extracts from the heart, liver, brain, and other tissues were effective in enhancing the stability of phosphofructokinase. This property was not diminished by boiling or by precipitation of the proteins by 4% perchloric acid. Thus, the stabilizing effect of these tissue extracts is not due to a nonspecific protein protective effect. The stabilizing effect of guinea pig liver perchloric acid extracts on phosphofructokinase is illustrated in Fig. 1. It can be seen that at a concentration as low as 5% (by volume), the liver extracts greatly

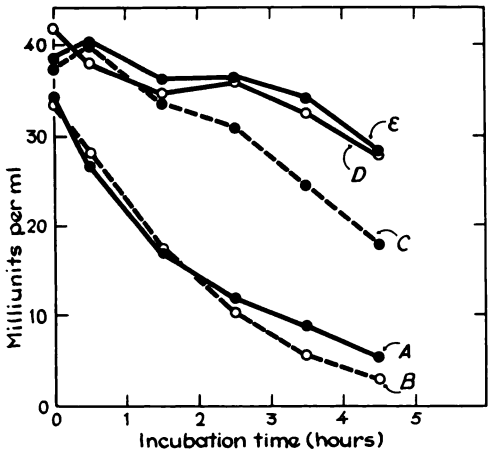

FIG. 1. *Stabilizing effect of liver perchloric acid extract on phosphofructokinase*

Each tube contained an incubation mixture (1 ml) which had a final concentration of 0.01 M Tris-HCl buffer (pH 8.0) and 34 mU of phosphofructokinase. Other additions: curve *A*, none; curve *B*, 0.01 M reduced glutathione and 0.01% bovine serum albumin; curves *C*, *D*, and *E*, respectively, 2%, 5%, and 10% (by volume) liver perchloric acid extract. All tubes were incubated at 0°. Aliquots were taken and assayed for enzyme activity at the indicated time intervals.

increased the stability of the enzyme. Experiments were carried out to test the stabilizing effect of the liver extract in the presence as well as in the absence of sulfhydryl compounds. It was found that while the liver extract could stabilize the enzyme by itself its effect was potentiated in the presence of glutathione. Glutathione without liver extracts had no significant effect.

Studies on the Nature of the Stabilizing Effect of Liver Perchloric Acid Extracts

Preliminary studies on the nature of the stabilizing factor(s) in the liver perchloric acid extract indicated that it is an anion since it was adsorbed on Dowex-1 resin and not on Dowex-50. In order to achieve a certain degree of purification of the stabilizing factor(s) in these extracts a sample was placed on a Dowex-1 resin column. All the stabilizing activity was adsorbed on this column. It was eluted with a linear gradient of ammonium formate and sodium formate. When the different fractions of the eluate were assayed for their effect on stabilizing phosphofructokinase, it was observed that the active component in these extracts was eluted as a relatively broad band. This suggested the existence of more than one stabilizing factor.

The fractions that contained stabilizing factor(s) eluted from the Dowex-1 were pooled and lyophilized to eliminate the formate. The sample was then placed on a DEAE-cellulose column for further fractionation. Linear gradient elution of this column resulted in extraction of the stabilizing factor(s) in two separate peaks which corresponded with UV-absorbing material. Analysis of the pooled eluate from the two active peaks is summarized in Table 1. It is observed that these two peaks contain ketoses which include some D-fructose-1,6-P_2, inorganic phosphate, and UV-absorbing substances. A large portion of the ketoses remained unaccounted for.

TABLE 1

Analysis of active eluate from DEAE cellulose

Two pooled active fractions eluted from the DEAE cellulose column (see text) were analyzed. Enzymic assays for hexose phosphate and trioses (dihydroxyacetone phosphate and glyceraldehyde-3-phosphate) were carried out as described in Materials and Methods. Total reducing compounds, total ketoses, and inorganic phosphate were determined nonenzymically (see Materials and Methods). Results are in μmoles per fraction (total volume for each fraction was 102 ml for A and 51 ml for B).

Components	Fractions	
	A	B
Ketoses	24.7	0.13
Total reducing compounds	2.53	—[a]
Trioses	0.25	0.05
Fructose-1,6-P_2	2.34	0.10
Fructose-6-P	0.0	0.0
Glucose-6-P	0.0	0.0
Glucose-1-P	0.0	0.0
Inorganic phosphate	3.75	0.32
E_{max} (mμ)	256	258
E_{min} (mμ)	233	238

[a] Not determined.

It was further found that the stabilizing factor(s) in the liver extract can be precipitated with $BaCl_2$ at pH 8.0. The barium salts were fractionated on a Dowex-1 formate column. Two major active peaks were obtained. Again relatively large amounts of ketoses were present in both peaks. About one-third of these could be accounted for as fructose-1,6-P_2, and the rest remain as yet unidentified.

Studies on the Stabilizing Effect of Hexose Phosphates, Adenine Nucleotides, and Inorganic Ions

The finding that the stabilizing fraction from the liver extract contained hexose phosphates, inorganic phosphate, and nucleotide-like material led to further investigation on the stabilizing properties of these agents. The protective effect of the adenylic nucleotides is summarized in Table 2. None of the adenylic nucleotides tested showed a considerable effect in stabilizing phosphofructokinase when these agents were tested alone. However, when the nucleotides were tested in the presence of a sulfhydryl compound, a significant stabilizing effect was observed. In experiments not summarized here, glutathione and mercaptoethanol were shown to be more effective than cysteine in potentiating the stabilizing action of the nucleotides. The most active stabilizing nucleotides tested were ATP and ADP (Table 2).

TABLE 2
Stabilizing effect of adenylic nucleotides

Phosphofructokinase (21 mU) was incubated in 1 ml of a solution containing 0.05 M Tris chloride pH 8.0 for 10 minutes at 30° with the indicated additions. Enzyme activity was determined at the beginning as well as at the termination of incubation time. Figures represent percentage of initial activity left in the incubation mixture after 10 min incubation.

Nucleotide (10^{-4} M)	Activity after 10 min incubation (% of initial activity)	
	Without glutathione	With glutathione 0.01 M
None	22	25
ATP	24	77
ADP	32	76
AMP	36	44
Cyclic 3′,5′-AMP	38	50

Figure 2 shows the protective effect of different hexose phosphates. Of the compounds tested, fructose-1,6-P_2 had the highest protective effect. A concentration of 10^{-5} M of the hexose phosphate provided 100% protection for the enzyme during the test period of 10 min. A significant stabilizing effect was also observed at concentrations of 10^{-7} M. For comparison the effect of ADP is also shown in Fig. 2, and it can be seen that it is less potent than fructose-1,6-P_2. The other hexose phosphates, fructose-6-P, glucose-6-P, and glucose-1-P, were less potent. The free monosaccharides had no protective effect on the enzyme (Fig. 2).

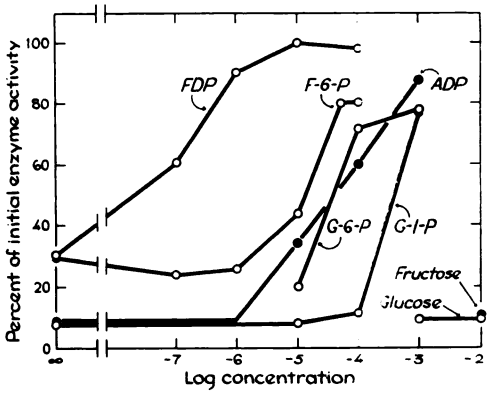

FIG. 2. *Stabilizing effect of the hexose phosphates on phosphofructokinase*

Each tube contained in 1 ml volume: 0.05 M Tris-HCl (pH 8.0), 0.005 M reduced glutathione, 15.5 mU of phosphofructokinase, and the indicated addition. Abscissa represents log concentration of the indicated hexose phosphate, and ordinate represents percentage of enzyme activity left in the mixture after 10 min incubation at 30°. The following abbreviations are used in the figure: *G-1-P*, glucose-1-P; *G-6-P*, glucose-6-P; *F-6-P*, fructose-6-P; and *FDP*, fructose-1,6-P_2.

Experiments on the protective effect of inorganic ions on phosphofructokinase are

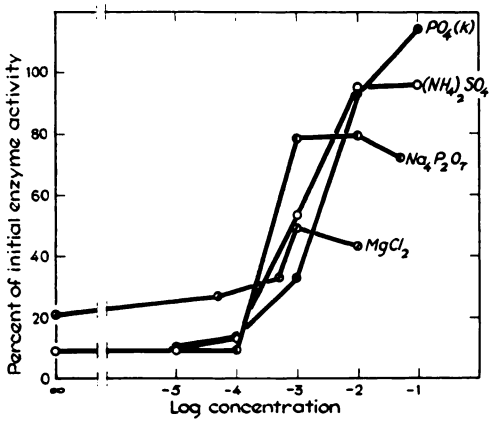

FIG. 3. *Stabilizing effect of inorganic ions on phosphofructokinase*

Each tube contained in 1 ml: 0.05 M Tris chloride, 0.005 M glutathione, 25 mU of enzyme, and the indicated concentration of inorganic ion. Abscissa represents log concentration of the indicated ion, and ordinate represents percentage of enzyme activity left in the mixture after 10 min incubation at 30°.

summarized in Fig. 3. Phosphate, sulfate, and pyrophosphate protected the enzyme at concentrations above 0.001 M whereas Mg^{++} appeared to have no significant effect.

A combination of different varieties of stabilizing agents described above could provide complete protection to the enzyme under conditions that cause nearly complete enzyme inactivation. These results are summarized in Table 3. For example, a combination of an adenylic nucleotide (ADP) and a hexose phosphate (fructose-1,6-P_2) or a polyvalent anion (PO_4^{---} or SO_4^{--}) showed an initial enzyme activity higher than that of the control and some degree of enzyme activation during the 15-min period of incubation. The high initial enzyme activity might be due to protection of the enzyme during enzyme dilution afforded by the stabilizers.

The effect of a combination of stabilizers was tested on phosphofructokinase during and after its passage through Sephadex G-75. Elution in the absence of any stabilizers recovered only 42% of the enzyme activity. Enzyme recovered through this procedure lost nearly half of its activity after standing in ice for half an hour, a result indicating that the enzyme was markedly unstable. On the other hand, when elution was effected with a mixture

TABLE 3

Stabilizing effect of adenylic nucleotides with the hexose phosphates

Phosphofructokinase was incubated in 1 ml of a solution containing 0.05 M Tris chloride pH 8.0, 0.005 M mercaptoethanol, and the indicated additions. Initial enzyme activity (0 time) as well as activity after 15 min incubation at 30° were measured. Phosphate tested was in the form of K-PO_4 buffer (pH 8.0), and sulfate was in the form of $(NH_4)_2SO_4$ adjusted with NH_4OH to pH 8.0.

Additions	Enzyme activity (mU)		Per cent of zero time activity
	0 time	15 min	
None	21.0	1.2	5.7
ADP 10^{-4} M	23.0	4.0	17.4
Fructose-1,6-P_2 10^{-5} M	28.4	19.5	68.5
K-PO_4 10^{-3} M[a]	29.0	9.3	32.0
$(NH_4)_2SO_4$ 10^{-3} M[a]	30.6	16.2	53.0
ADP 10^{-4} M + fructose-1,6-P_2 10^{-5} M	31.5	35.5	113.0
ADP 10^{-4} M + K-PO_4 10^{-3} M	29.0	33.0	114.0
ADP 10^{-4} M + $(NH_4)SO_4$ 10^{-3} M	29.0	35.0	121.0
Fructose-1,6-P_2 10^{-5} M + K-PO_4 10^{-3} M + $(NH_4)_2SO_4$ 10^{-3} M	30.0	29.0	97.0
ADP 10^{-4} M + K-PO_4 10^{-3} M + $(NH_4)_2SO_4$ 10^{-3} M	32.0	40.0	125.0

[a] The tests for these two anions were carried out in a separate experiment which was run with a different batch of enzyme from that used with the rest of the additions.

of 0.01 M PO_4(K) buffer pH 8.0, 0.001 M $MgSO_4$, 0.01 M mercaptoethanol, and 5×10^{-5} M fructose-1,6-P_2 enzyme recovery was 103%, and when the enzyme was left for 18 hours in the cold there was only a 30% loss of enzyme activity.

Attempts were made to test whether the amount of fructose-1,6-P_2 found in the DEAE-cellulose pooled fraction from perchloric liver extract could account for the protective effect of this fraction. The pooled extract from fraction A (see Table 1) was tested at different concentrations and compared with equivalent concentrations of known samples of fructose-1,6-P_2. Curves obtained for the protective effect of both samples were found to approximately coincide (Fig. 4). This indicates

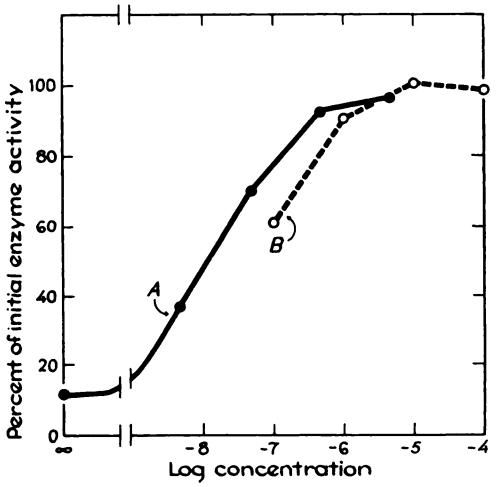

FIG. 4. *Comparison of the stabilizing effect of DEAE eluate (fraction A, Table 1) with that of fructose-1,6-P_2 on phosphofructokinase*

Each tube contained in 1 ml volume: 0.05 M Tris-HCl (pH 8), 0.005 M reduced glutathione, 20 units of phosphofructokinase; and in *A*, graded amounts fraction A (see Table 1) to contain the indicated final concentration of fructose-1,6-P_2 and in *B*, graded concentration of a standard sample of fructose-1,6-P_2. Abscissa represents log concentration of fructose-1,6-P_2, and ordinate represents percentage of enzyme activity left in the incubation mixture after 10 min incubation at 30°.

that fructose-1,6-P_2 alone may account for the major protective effect of this fraction. The protective effect in the second peak of the DEAE-cellulose eluate (fraction B, Table 1) cannot be accounted for by the fructose-1,6-P_2 present or by the nucleotide content of this fraction alone. This indicates that other factors which are as yet undetermined are contributing to the effect of liver perchloric acid extracts on phosphofructokinase stability.

Attempts to Reactivate the Enzyme

Phosphofructokinase was inactivated by incubation at 30° as described in Fig. 1. It was not possible to reactivate the enzyme by adding any of the stabilizers reported. This is in contrast to the slight activation which was observed when these agents were added before incubation had occurred (see Table 3).

DISCUSSION

The experiments reported in this paper indicate that phosphofructokinase is an extremely labile enzyme and that its stability can be enhanced by the hexose phosphates, the adenylic nucleotides, and inorganic SO_4^{--} or PO_4^{---}. The most potent stabilizer found was fructose-1,6-P_2, one of the products of the phosphofructokinase reaction. The stabilizers tested were found to be effective only in the presence of a sulfhydryl agent, a result suggesting that the catalytic activity of phosphofructokinase is dependent upon the presence of reduced sulfhydryl group(s). Recently, it was reported that heart phosphofructokinase can undergo dissociation to inactive subunits (13). Reactivation of the dissociated enzyme was enhanced by the adenylic nucleotides, the hexose phosphates, and perchloric acid extracts from different tissues. Fructose-1,6-P_2 was the most active agent. The stabilizing effect of extracts from tissues could be partly due to the presence of hexose phosphates and adenylic nucleotides in these extracts. The similarity between agents which enhance reactivation of the dissociated enzyme and those which stabilize the enzyme is indicated. This raises the possibility that inactivation of the diluted enzyme might result from

dissociation of the active enzyme to inactive subunits. Reactivation of the enzyme in the experiments described above was not successful, possibly because the enzyme was highly diluted. It was previously reported that the process of reassociation of phosphofructokinase subunits was concentration dependent (13).

The role of enzyme stabilization *in vivo* in the control of enzyme activity has recently been demonstrated by Schimke and co-workers (19) in the case of liver tryptophan pyrrolase. An increase in the pyrrolase activity in the rat liver after administration of the substrate tryptophan could be explained by a decrease in the degradation of the enzyme *in vivo*. Should this concept in enzyme regulation be applied to phosphofructokinase, it would be possible to speculate on a control mechanism based on the finding that the hexose phosphates are potent stabilizers to the enzyme. An increase in the level of these esters in the cell during anoxia or caused by insulin (6, 7) would favor stabilization of phosphofructokinase and thus might contribute to the suggested increase of activity of this enzyme. Further studies are needed to ascertain such a relationship.

ACKNOWLEDGMENT

Supported by Research Grant AI04214 from the National Institute of Allergy and Infectious Diseases, U.S. Public Health Service; Research Career Development Award GM-K3-3848 from the Division of General Medical Sciences, U.S. Public Health Service; and a grant from the Santa Clara County Heart Association.

REFERENCES

1. C. F. Cori, *Symp. Respirat. Enzymes 1942* p. 175. Univ. of Wisconsin Press, Madison, 1942.
2. V. A. Engel'hardt and N. E. Sakov, *Biokhimiya* **8**, 9 (1943).
3. F. Lynen, G. Hartmann, K. F. Netter and A. Schuegraf, *in* "Regulation of Cell Metabolism" (G. E. W. Wolstenholme, ed.), p. 256. Little, Brown, Boston, Massachusetts, 1959.
4. T. E. Mansour and E. Bueding, *Brit. J. Pharmacol.* **9**, 459 (1954).
5. T. E. Mansour and J. M. Mansour, *J. Biol. Chem.* **237**, 629 (1962).
6. E. A. Newsholme and P. J. Randle, *Biochem. J.* **80**, 655 (1961).
7. P. Ozand and H. T. Narahara, *J. Biol. Chem.* **239**, 3146 (1964).
8. J. F. Taylor, *Phosphorus Metab. Symp., Baltimore, 1951, Johns Hopkins Univ. McCollum-Pratt Inst. Contrib.* **23**, Vol. 1, p. 104. Johns Hopkins Press, Baltimore, 1951.
9. J. A. Muntz, *Arch. Biochem. Biophys.* **42**, 435 (1953).
10. T. E. Mansour, *J. Biol. Chem.* **238**, 2285 (1963).
11. S. P. Colowick, *Abstr. Am. Chem. Soc. Meeting, New York, 1947*, p. 56C.
12. M. F. Utter, *Federation Proc.* **6**, 299 (1947).
13. T. E. Mansour, *J. Biol. Chem.*, in press.
14. H. U. Bergmeyer, editor, "Methods of Enzymatic Analysis," p. 107. Academic Press, New York, 1963.
15. G. Gomori, *J. Lab. Clin. Med.* **27**, 955 (1942).
16. M. Somogyi, *J. Biol. Chem.* **160**, 69 (1945).
17. J. H. Roe, *J. Biol. Chem.* **107**, 15 (1934).
18. A. Higashi and L. Peters, *J. Lab. Clin. Med.* **35**, 475 (1950).
19. R. T. Schimke, E. W. Sweeney and C. M. Berlin, *J. Biol. Chem.* **240**, 322 (1965).