PURIFICATION, CRYSTALLIZATION AND PROPERTIES OF ACTIVATED SHEEP HEART PHOSPHOFRUCTOKINASE*

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Previous investigations indicated that partially purified phosphofructokinase (PFK) from Fasciola hepatica (Mansour and Mansour, 1962) and from the guinea pig heart (Mansour, 1963) can be activated by adenylic nucleotides through an effect on assay levels of PFK as well as through an effect on concentrated inactive form of the enzyme (Mansour, 1965). Activation of assay levels of PFK from other sources has been reported by several workers (Passonneau and Lowry, 1962; Vinuela et al., 1963; Atkinson et al., 1965). In the present investigation, PFK from the sheep heart was found to be largely inactive and is present in the insoluble fraction of the cell. Procedure for activation, purification and crystallization of the enzyme as well as some of its properties will be reported. Purification of PFK was facilitated after the finding that a combination of fructose-1,6-P_2, an adenylic nucleotide and mercaptoethanol can maintain the stability of the enzyme (Wakid and Mansour, 1965).

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EXPERIMENTAL AND DISCUSSION

Homogenates from sheep hearts received from the slaughter house had low PFK activity (Table I). When the residue of these homogenates was incubated with ATP and $MgSO_4$, high PFK activity was observed in the supernatant fluid of the incubate (Table I). The supernatant fluid of the original homogenate was not activated when incubated with the same ATP/$MgSO_4$ mixture. AMP and ADP can replace ATP in the activating-solubilizing mixture. $MgSO_4$ in high concentrations (0.1 M) in the absence of ATP was also effective in solubilizing and

TABLE I. Extraction of Phosphofructokinase from the Heart

Cell Fraction	PFK (units/gm heart)		
	Sheep	Fresh guinea pig	Aged* guinea pig
A. Homogenate	2.9	31.8	14.5
B. Supernatant fluid (24,000 X g)	.1	37.2	16.1
C. Residue	.3	3.8	3.9
D. Supernatant fluid of incubated residue with ATP/$MgSO_4$	21.9	.8	5.6

*Half hour after sacrifice.

Homogenates were prepared by homogenizing 1 gm of tissue in 4 ml of 0.01 M Tris-Cl buffer pH 8.0 in .002 M EDTA. Residue was isolated (24,000 X g, 20 min) and suspended in the same homogenizing solution and then sedimented again by centrifugation. It was resuspended in a solution containing 0.05 M $MgSO_4$, 0.005 M mercaptoethanol, 0.01 M Tris-Cl (pH 8.0) and 5×10^{-4} M ATP. The suspension was stirred in a bath at 37° for 20 min and then centrifuged at 24,000 X g for 30 min at 0°. The supernatant fluid of this incubate was collected (fraction D). PFK activity was assayed spectrophotometrically at 22° as was described before (Mansour, 1963).

activating the enzyme. The process of activation and solubilization was temperature and time dependent. The supernatant

fluid after activation had a high specific activity and was used as a starting material for further enzyme purification (Step I, Table II).

An attempt was made to find whether the unusual properties of sheep heart PFK described above might be related to changes which occur as a result of the delay between killing the animal and the preparation of the homogenates for assay. The results summarized in Table I show that PFK activity in homogenates from aged guinea pigs was less by a factor of 2 than those from freshly killed guinea pigs. Furthermore, residue from hearts of aged animals which was incubated with ATP and $MgSO_4$ yielded activated soluble PFK. On the other hand, residue from hearts of freshly killed guinea pigs incubated in the same mixture did not yield significant enzyme activity.

Activated PFK extract from the sheep heart was then precipitated with ethanol between 8 and 13% (5800 X g, 10 min) by a procedure similar to that of Ling, Byrne and Lardy (1955). PFK in this fraction was not fully active. Addition of 10^{-4} M ATP to this fraction resulted in 50-100% increase in enzyme activity (Step II). PFK was extracted from the sedimentable material of the ethanol fraction with a solution of 0.02 M $MgSO_4$; 0.01 M Tris-Cl (pH 8.6), 0.005 M 2-mercaptoethanol, 10^{-4} M ATP and 10^{-5} M fructose-1,6-P_2 (Step III). The extract was then applied to a DEAE cellulose column. PFK activity was eluted as a single peak with a linear gradient between 0.5 M and 0.6 M Tris-Cl buffer containing ATP, fructose-1,6-P_2 and 2-mercaptoethanol as stabilizers in the concentrations indicated above. PFK was then precipitated from the eluate between 0.42 and 0.6 saturation of $(NH_4)_2SO_4$ (Step V). Enzyme in this step had a specific activity which was about

TABLE II. Purification of Sheep Heart Phosphofructokinase

Step	Fraction	Total PFK (units)	Protein mg/ml	Specific activity unit/mg
I	Activated heart extract	5050	2.28	1.8
II	Ethanol precipitate:			
	Without ATP	2270		
	With ATP	3970	8.50	7.8
III	Extract of ethanol precipitate	3490	1.65	36.8
IV	DEAE cellulose eluate	2900	0.45	89.0
V	$(NH_4)_2SO_4$ 0.42-0.6 saturation	2900	11.50	126.0

PFK activity was assayed spectrophotometrically at 22° (pH 8.2) as was described before (Mansour, 1963). A unit of enzyme is expressed as μmoles of fructose-1,6-P_2 formed per min.

126 units/mg protein (Step V). Representative data of this procedure with 300 gm of heart is summarized in Table II.

The purified enzyme moved in the electrophoretic field on the horizontal starch electrophoresis (Smithies, 1959) as a single diffuse patch at the very slow rate of 1 mm/hr in a current of 6 V/cm at pH 8.4.

For crystallization, the concentrated enzyme (12-15 mg/ml) was left at 0° for several days in the presence of $(NH_4)_2SO_4$ (.3 saturation). Minute hexagonal crystals started to precipitate after one day (Fig. 1). The specific activity of these crystals never exceeded the specific activity of the $(NH_4)_2SO_4$ fraction.

Ultracentrifugal analysis in Spinco Model E of the crystalline enzyme revealed a Schlieren pattern with an asym-

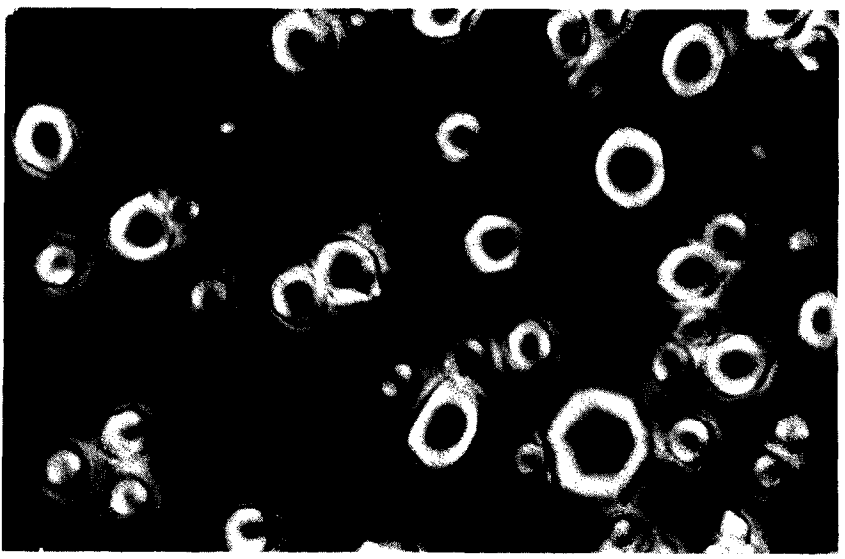
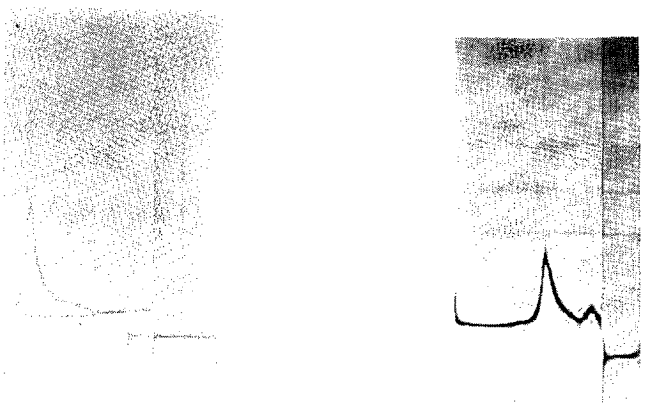


Fig. 1. Crystals of sheep heart phosphofructokinase(750x).

metric peak with an S_{20w} of about 38.3 (Fig. 2) characteristic of a monomer-polymer system in rapid equilibrium (Schachman, 1959). The pattern was characterized by a sharp boundary at the bottom and a partially skewed edge trailing towards the top of the cell. Enzyme isolated before the process of crystallization which had approximately the same specific activity had a Schlieren pattern which consisted to two main components--a fast moving component which comprised 80-90% of the area and had an S_{20w} of about 41.0 S, and a slow moving component with an S_{20w} value which ranged from 8 S to 11 S (Fig. 2). The two components, when separated through the use of Yphantis-Waugh moving partition cell, had enzyme activity. The presence of 2 M NaCl under these conditions resulted in a Schlieren pattern with a single peak of 16.6 S. Removal of NaCl by dialysis resulted in restoration of the original Schlieren patterns with the double peaks. S_{20w} determinations



A B

Fig. 2. Ultracentrifuge patterns of PFK in 0.05 M K-PO_4 pH 8.0, 0.005 M mercaptoethanol, 10^{-4} M ATP and 10^{-5} M fructose-1,6-P_2, speed - 44,770 r.p.m. at 0°. Sedimentation from right to left, (A) crystallized PFK protein concentration 0.98%, specific activity was 115, bar angle 65°, time 46 min, (B) PFK before crystallization 0.612%, specific activity was 117, run was in Yphantis-Waugh moving partition cell, bar angle 60°, time 22 min.

in sucrose gradients of PFK at concentrations which varied from 1 mg per 4.8 ml gradient to 0.48 μg revealed that the sedimentation coefficient is dependent on the concentration of the enzyme. The lowest sedimentation coefficient obtained with the sucrose gradient experiments was 15.2 S (6 μg or less) and the highest was 26.4 S (1.06 mg protein). A series of concentrations which ranged from 14 mg/ml to 2.4 mg/ml did not significantly affect the S_{20w} values when tested in the Model E ultracentrifuge.

These experiments suggest that purified heart PFK is present in the form of aggregates of several sizes. The aggregated enzyme dissociates to smaller polymers when diluted or in the presence of high concentrations of NaCl. The

relationship between the degree of aggregation of the enzyme and its catalytic activity cannot be ascertained from the results reported here. This is because the catalytic activity was always tested after several thousand-fold dilution. Presumably the enzyme at that low concentration is in the monomeric form.

REFERENCES

Atkinson, D. E. and Walton, G. M. (1965). J. Biol. Chem., 240, 757.

Ling, K. H., Byrne, W. L. and Lardy, H. (1955). In "Methods in Enzymology", eds. S. P. Colowick and N. O. Kaplan, Vol. I, 306, Academic Press, New York.

Mansour, T. E. and Mansour, J. M. (1962). J. Biol. Chem., 237, 629.

Mansour, T. E. (1963). J. Biol. Chem., 238, 2285.

Mansour, T. E. (1965). J. Biol. Chem., 240, 2165.

Passonneau, J. V. and Lowry, O. H. (1962). Biochem. Biophys. Research Communs., 7, 10.

Schachman, H. K. (1959). In "Ultracentrifugation in Biochemistry", 152, Academic Press, New York.

Smithies, O. (1959). Biochem. J., 71, 585.

Vinuela, E., Salas, M. L. and Sols, A. (1963). Biochem. Biophys. Research Communs., 12, 140.

Wakid, N. and Mansour, T. E. (1965). Mol. Pharmacol., Vol. I, No. 1 (In press).