EVIDENCE FOR TWO FORMS OF FRUCTOSE DIPHOSPHATASE*

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The activity of fructose-1,6-diphosphatase (FDPase) from mammalian liver is influenced by a number of conditions both *in vivo* and *in vitro*. These include alteration of diet, administration of cortisone, and treatment with proteolytic enzymes (Pogel and McGilvery, 1952; Mokrasch and McGilvery, 1956; Mokrasch, Davidson, and McGilvery, 1956). Also, Taketa and Pogell (1963), have reported for the purified enzyme that AMP and ATP are strongly inhibitory.

We have shown previously that the diphosphatase hydrolyses FDP and sedoheptulose-1,7-diphosphate (SDP) at essentially equal rates, and that each substrate inhibits the hydrolysis of the other (Bonsignore *et al.*, 1963). Although the kinetic data imply a single site for both activities, the site appears to be complex, because FDPase activity remains after treatment with papain or urea has destroyed SDPase activity (Mangiarotti and Pontremoli, 1963).

A reciprocal control between the phosphatase and phosphofructokinase could dictate the balance between glycolysis

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and glycogenesis. However, a unified concept of control which accommodates the above observations has not been advanced. We now wish to report evidence for the existence of two enzymatically distinguishable forms of rabbit liver FDPase which previously have escaped recognition[2]. Since the conversion of one form to the other occurs readily, this observation may explain some of the changes in activity previously reported.

In appropriate preparations, the two forms are revealed by the different rates obtained with and without preincubation. In the spectrophotometric assay at pH 9.4, addition of FDPase to the complete reaction mixture resulted in a marked lag in activity (Figure 1A). With dilution in buffer prior to assay (or addition to the cuvette before FDP), an immediately linear rate was obtained. The difference in activity before and after preincubation could be amplified by changing the pH or temperature of assay. At pH 7.5 (Figure 1B), the velocity was almost zero and did not increase with time as at pH 9.4; after preincubation, the rate was nearly linear.

Table I shows the effects of preincubation measured as the release of iP. Both at pH 7.5 and at 11°, a 4- to 5-fold greater rate was obtained with preincubation. The rates at pH 9.4 and 37°, which correspond to the conditions in Figure 1A, are quite similar owing to rapid activation. Preincubation in FDP (10^{-5}M), or in SDP (2.5×10^{-3}M) completely inhibited activation. Fructose-6-P partially inhibited activation, but fructose-1-P and sedoheptulose-7-P did not.

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[2] We are indebted to Dr. C. Shaw of Tulane University, New Orleans, Louisiana, for making this observation.

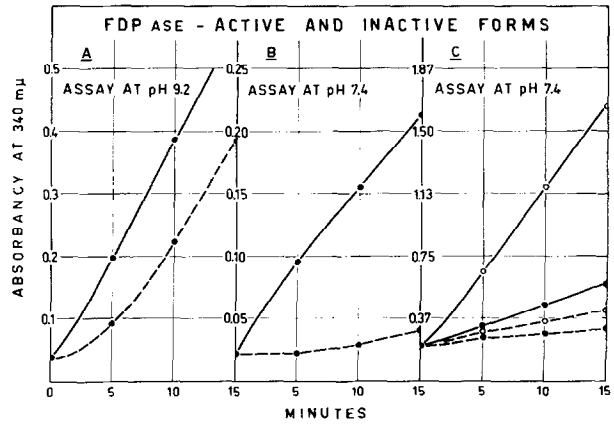

Figure 1. The effect of preincubation on FDPase activity.

Part A: The FDPase, diluted 1:5 in 0.2M glycine buffer, pH 9.4, was incubated at 20° for 5 min. (preincubated, solid line), or an equivalent amount was added to a complete assay (not preincubated, dashed line). The microcuvette contained in 0.2 ml: glycine buffer, pH 9.4 (10 μmoles); $MnCl_2$ (0.02 or 0.1 μmole); excess glucose-6-P dehydrogenase and phosphohexoisomerase; and TPN (0.1 μmole). FDPase was purified as in Table II except that after the heat step, FDPase was precipitated with 90%, and back extracted between 75% and 58% saturated $AmSO_4$. One unit reduced TPN at the rate of 5 absorbancy units per min. or released 0.8 μmole of iP per min.

Part B: Conditions were as in Part A except that triethanolamine buffer, pH 7.4 (25 μmoles), and 0.0156 unit of FDPase were used.

Part C: Conditions were as in Parts A and B except that the assay contained 2.5×10^{-3}M cysteine (open circles) and 0.0055 unit of FDPase (after acid precipitation step).

SDPase activity was only slightly increased by preincubation (Table I). Thus, in the reverse of the situation with urea and papain treatments where only SDPase activity was lost (Mangiarotti and Pontremoli, 1963), FDPase was almost inactive while SDPase remained active.

The velocity before preincubation measures the amount of FDPase already in the active form, and the velocity after preincubation measures the total amount. Such assays have shown that the phosphatase from extracts of acetone powders varied between 20% and 80% in the active form, whereas that of a

homogenate of fresh liver (in 0.85% KCl) was 50% in the active form. Also the proportions varied during purification (Table II), i.e. treatments with acid and heat, or "back extraction" with ammonium sulfate (not shown) usually increased the amount of inactive form, whereas precipitation with ammonium sulfate, dialysis, and chromatography on CM cellulose (Pogell, 1962) yielded almost entirely the active form.

TABLE I

Demonstration of active and inactive forms of FDPase

Conditions were the same as in Figure 1A and 1B except that 0.02 to 0.06 units of phosphatase were used. The complete reaction mixture was equilibrated at 11° or 37° before addition of phosphatase. After incubation for 10 minutes, the reaction was stopped by addition of reagents for determination of iP. The data are expressed as μmoles of iP liberated per unit of phosphatase per hour.

Temp.	Treatment	FDPase		SDPase	
		pH 9.4	pH 7.5	pH 9.4	pH 7.5
37°	not preincubated	16.8	1.09	29.2	5.58
	preincubated	21.8	4.20	38.4	7.30
11°	not preincubated	1.14	-	2.22	-
	preincubated	5.64	-	2.94	-

Centrifugation of inactive FDPase in a gradient of sucrose-tris (0.1M, pH 7.5) (Martin and Ames, 1961) which preserved the inactive form, gave the same sedimentation constant (S) as observed for active FDPase. In several runs, an S value of 7.2 was obtained using aldolase and catalase as standards. This corresponds to a molecular weight of 129,000 assuming sphericity of the protein. Further, the ratio of FDPase to SDPase (assayed at pH 9.4 and after preincubation) was constant throughout the peak, confirming that both activities are associated with a single protein.

TABLE II

The distribution of two forms of FDPase during purification

The procedure was a modification of that of Bonsignore et al. (1963). The percent in the active form was obtained from the ratio of rates before and after preincubation x 100.

Step	Specific Activity	Fold Purified	% Active Form*
Extract of acetone powder, 6 vols. of 0.01M phosphate, pH 8	0.16	-	47
Supernatant after pH 3.7 and return to pH 6.5	0.33	3.3	50
$AmSO_4$ ppt., 0 to 65% saturation	0.96	6.0	62
Heat - 50° for 5 minutes	4.5	28	**
Dialysis, 0.005M malonate, pH 6	4.2		100
Elution from CM cellulose, 5×10^{-4}M FDP, pH 6	70	100	100

*The same results were obtained with cysteine in the assay.
**Lower activity after preincubation.

Since -SH groups have been implicated in FDPase activity (Pogell and McGilvery, 1954), it was necessary to consider the role of -SH groups in activation by preincubation. At pH 9.4, only preincubation was needed for maximum activity. Cysteine (3×10^{-3}M), or EDTA (5×10^{-4}M) were without effect and the kinetics were as in Figure 1A. At pH 7.5, however, cysteine (or EDTA) stimulated the rate (Figure 1C), but both preincubation and cysteine were necessary for full activity. Cysteine also increased the rate for fractions not activated by preincubation.

It is concluded, therefore, that the diphosphatase can undergo reversible changes in tertiary structure, not detectable by gradient centrifugation, and unrelated to the activa-

tion by cysteine or EDTA. One form possesses both FDPase and SDPase activity, whereas the other has only SDPase activity. Most preparations are mixtures of these forms. The conversion of active to inactive FDPase, normally a rapid process is partially inhibited by FDP at pH 9.4 and 20° to 37°, but is completely inhibited by FDP or SDP at pH 7.5, or at 11°.

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