REQUIREMENT OF SUBSTRATE FOR ADENOSINE 5'-MONOPHOSPHATE BINDING TO LIVER FRUCTOSE 1,6-DIPHOSPHATASE*

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Received February 19, 1968

It is now well established that adenosine 5'-monophosphate (AMP) is a specific, allosteric, noncompetitive inhibitor of fructose 1,6-diphosphatase (FDPase) with cooperative interactions (1). However, our initial studies on the binding of ^{14}C-AMP to rabbit liver FDPase using equilibrium dialysis, gel filtration, and density gradient ultracentrifugation failed to show any detectable binding in the absence of substrate. Further experiments proved that fructose 1,6-diphosphate (FDP) was an essential cooperative factor for AMP binding with the enzyme. This communication presents three lines of evidence in support of this conclusion.

Kinetic Studies - Kinetic measurements have shown that inhibition of the catalytic activity of the enzyme by AMP at 3^{o} was not immediate and took 15-20 min for maximum effect after the addition of FDP (1) (Fig. 1). This time lag could not be reduced by preincubating the enzyme with inhibitor for 10 min before the addition of substrate. Identical inhibition patterns were obtained at two inhibitor concentrations, whether or not the enzyme was preincubated with AMP. Similar results were found by preincubating enzyme, FDP, and

* This work was supported by grants from the National Institutes of Health (GM-12888) and the National Science Foundation (GB-2441).

** Research Career Development Awardee of the United States Public Health Service, No. GM-K3-3033.

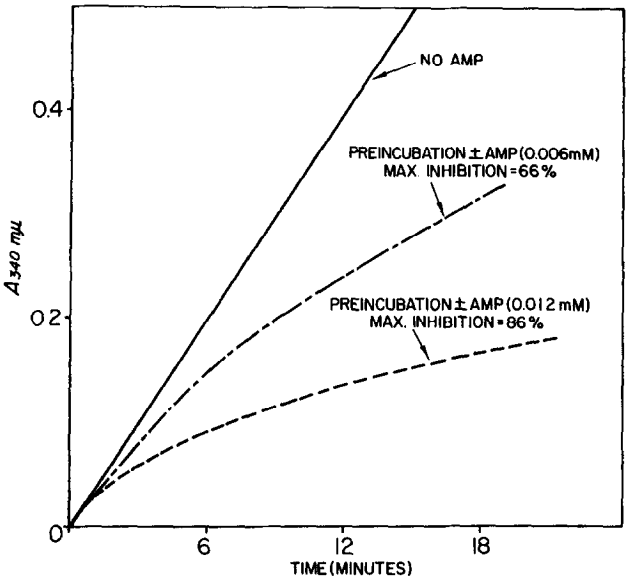

Figure 1. Kinetics of AMP inhibition of FDPase at 3°. The enzyme was assayed spectrophotometrically by following TPNH formation at 340 mμ. Partially purified enzyme, 50 mM Tris-HCl (pH 6.7)[1], 10 mM $MgSO_4$, 0.1 mM EDTA, 0.015 mM TPN^+, phosphohexoisomerase (7.8 U/ml), and glucose-6-P dehydrogenase (1.4 U/ml) were preincubated for 10 min with or without AMP addition. The reaction was started by the addition of 0.1 mM FDP. In the controls for preincubations without AMP, an equal amount of AMP was added to the final reaction system immediately after FDP.

[1]Measured at room temperature. At 3°, pH=ca.7.3.

AMP for periods from 0-40 min and starting the reaction by addition of Mg^{++}. These results suggest that a conformational change in protein structure occurs in the presence of inhibitor, with an obligatory requirement for both substrate and Mg^{++}. It should be emphasized that this final conformational alteration, as measured by maximal AMP inhibition, occurs very rapidly at higher temperatures (1).

Direct Binding Studies - Definite evidence for the substrate-dependent binding of AMP to FDPase was obtained by gel filtration through a Sephadex G-25 column according to the method developed by Hummel and Dreyer (2). When

the protein was filtered through the column preequilibrated with buffer containing Mg^{++}, EDTA, and ^{14}C-AMP in the absence of FDP, there was no appreciable increase in radioactivity associated with the protein peak, whereas in the presence of FDP, there was a marked rise in AMP bound to protein (Fig. 2A). In the absence of Mg^{++}, similar results were obtained with en-

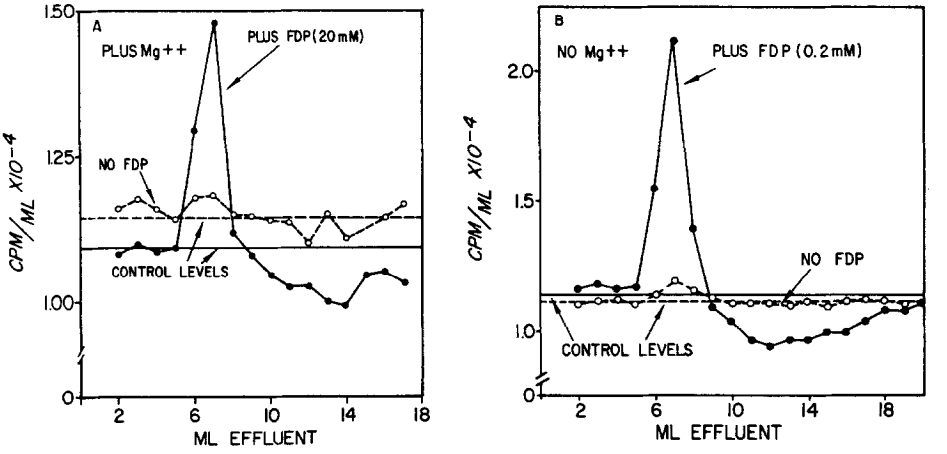

Figure 2. Chromatographic elution profile of AMP binding by FDPase in the presence and absence of FDP. Preparation of enzyme: FDPase was purified by substrate elution followed by gel filtration through Sephadex G-200 (3) and had a specific activity of 15 μmoles product formed per min per mg protein at 22° and pH 9.3, assayed according to Pontremoli (4). Quantities of FDPase were calculated from enzyme activity measurement assuming a specific activity of 22.5 for homogeneous enzyme (4).

A. A 1 cm diameter column containing 15 ml Sephadex G-25 gel was equilibrated with 50 mM Tris-HCl (pH 6.7) containing 10 mM $MgSO_4$, 0.1 mM EDTA, 5.26 μM ^{14}C-AMP, ±20 mM FDP. 0.327 mg FDPase (stock solution lyophilized and redissolved in appropriate eluant solution) was applied to the top of the column and eluted with the same solution. 1 ml fractions were collected at a flow rate of 1 ml per min. Radioactivity of each fraction was determined on a 0.2 ml aliquot in a Nuclear-Chicago Mark I scintillation counter employing a scintillation solution containing 1.5 ml NCS solubilizing reagent (Nuclear-Chicago) and 10 ml of a 1:25 dilution in toluene of Spectrafluor (Nuclear-Chicago). FDPase appeared in the same peak as radioactivity. All the binding studies were done at 0-4°.

B. Same as (A) omitting Mg^{++} and using 0.2 mM FDP and 0.283 mg FDPase prepared as follows: 20 mM $MgSO_4$ was added to the concentrated Sephadex G-200 effluent in malonate buffer (pH 6.0). After standing 15 min at room temperature and 2 hr at 0°, the preparation was dialyzed overnight at 0-4° against 5 mM sodium malonate (pH 6.0).

TABLE I

AMP binding to FDPase by gel filtration under different conditions.

Procedure same as described in Fig. 2. $MgSO_4$, Na_4FDP, or K_2glucose-1-P were added only where indicated. Amount of protein, 0.28-0.33 mg; ^{14}C-AMP, 5.3-5.5 μM; temperature, 0-4°. A. With purified enzyme before treatment with Mg^{++} and dialysis. B. With enzyme after treatment with Mg^{++} and dialysis (see Fig. 2 legend).

A. Additions	AMP bound mμmoles per 0.25 mg protein	B. Additions	AMP bound mμmoles per 0.25 mg protein
Mg^{++}	0.48	None	0.66
Mg^{++}, 20 mM FDP	2.64	20 mM FDP	6.32
None	3.07	0.2 mM FDP	7.02
0.2 mM FDP	6.23	10 mM glucose-1-P	1.18

hanced binding in the presence of FDP (Fig. 2B, Table I). Neither Mg^{++} nor EDTA was essential for binding, and Mg^{++} decreased the amount of AMP bound both in the presence and absence of EDTA. No enhancement of AMP binding was found in the presence of 10 mM glucose 1-phosphate (Table I).

Further confirmation of the FDP-dependent binding of AMP to enzyme was obtained by sucrose density gradient centrifugation. As is shown in Fig. 3a, a large peak of AMP was found associated with the enzyme when FDP was included in the gradient. In contrast, control experiments, either without enzyme or substrate, showed no AMP in this region. The slight shift of the AMP peak from that of the enzyme and the small trail behind it (Fig. 3b) were probably due to a constant dissociation of the FDP-protein-

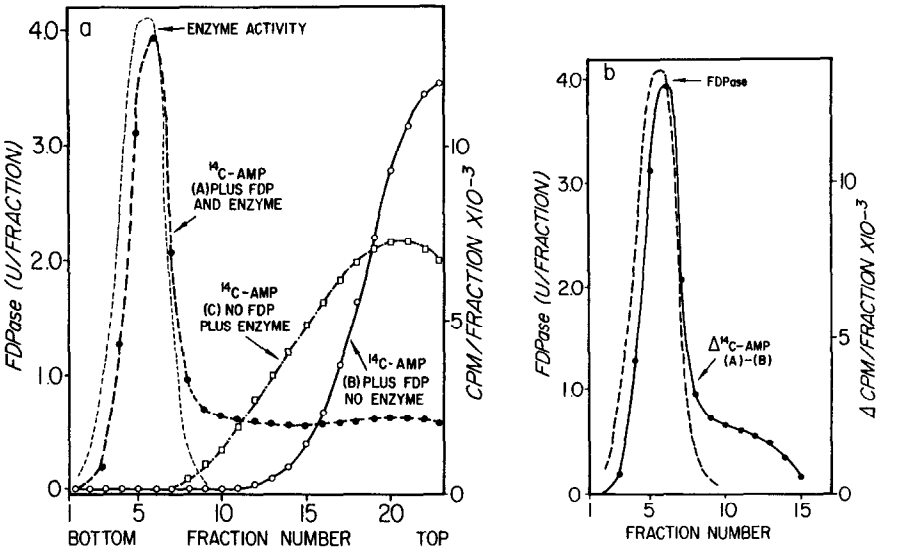

Figure 3. Binding of AMP by FDPase during sucrose density gradient centrifugation. a. Ultracentrifugation studies in sucrose gradients were carried out by the method of Martin and Ames (5). A. 0.2 mM FDP, 9.47 μM ^{14}C-AMP (specific radioactivity=1.6x10^{6} cpm per μmole AMP), 0.64 mg FDPase , 50 mM Tris-HCl (pH 6.7), and 0.1 mM EDTA in a volume of 0.2 ml were layered on 4.6 ml of a 5-20% sucrose gradient containing 50 mM Tris-HCl (pH 6.7), 0.1 mM EDTA, and 0.2 mM FDP with the use of band-forming cups (6). B. Complete system as in (A) minus enzyme. C. Complete system as in (A) minus FDP. Centrifugation was performed in a Spinco model L2 preparative ultracentrifuge with a SW-39L rotor run at 39,000 rpm for 20 hr at 3°. Fractions of 15 drops were collected. The FDPase activity and radioactivity of each fraction were determined by the same procedures described in Fig. 2. The location of FDPase activity was identical in gradients (A) and (C).
b. Plot of excess radioactivity associated with protein fractions in (A) over the control levels in (B).

AMP complex as it moved down through the gradient into regions containing no AMP. The AMP inhibition of FDPase is known to be a readily reversible reaction (1). In the absence of FDP, a small but definite binding was observed with a resultant broadening of the AMP band. This apparent binding was somewhat larger than that found with gel filtration in the absence of substrate. Similar results were observed with a D_2O gradient in phosphate

buffer and a sucrose gradient in D_2O. In the presence of Mg^{++} and no added substrate, there was no evidence for binding of AMP in a sucrose gradient.

No indication of any change in the molecular weight of FDPase in the presence of FDP and AMP was observed in any of the gradients studied.

Discussion - The present results are somewhat analogous to the 40-fold decrease in the dissociation constant for guanosine triphosphate binding to glutamate dehydrogenase in the presence of DPNH (7). It is still not known whether the increased binding of AMP in the presence of FDP is due solely to a decreased dissociation constant or whether there may also be an altered "n" value. The small binding of AMP in the absence of substrate suggests that the former is the primary change involved.

It is apparent from our current studies that FDPase and FDP form an intermediate enzyme-substrate complex in the absence of added divalent cation, which is required for substrate hydrolysis. The formation of this complex with resultant change both in conformation and charge is presumably the reason for the specific purification of FDPase obtained upon substrate elution from substituted cellulose columns (8). Enzyme-substrate complex formation in the absence of divalent cation is also suggested by the reported protection of enzyme activity by FDP during chemical acetylation of FDPase by acetylimidazole (9). This enzyme-substrate complex may be quite stable, since enzyme purified by substrate-elution followed by Sephadex gel filtration (3) could still bind significant amounts of AMP in the absence of both Mg^{++} and FDP. These values were reduced to very low levels either in the presence of Mg^{++} or by treatment of purified enzyme with Mg^{++} and subsequent dialysis (Table I). It also appears from the current studies that the respective dissociation constants for ligands in the ternary complex, nFDP-FDPase-mAMP, must be very low.

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