p-NITROPHENYL PHOSPHATE AS SUBSTRATE FOR RABBIT LIVER FRUCTOSE DIPHOSPHATASE*

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Summary: Purified rabbit liver fructose diphosphatase has been found to catalyze the hydrolysis of p-nitrophenyl phosphate, PNPP. It has been established that the hydrolysis of p-nitrophenyl phosphate is due to fructose diphosphatase through studies of the chromatographic properties of the enzyme, its temperature sensitivity, dependence on divalent cations and its inhibition by fructose diphosphate. The K_m for PNPP is 6×10^{-3}M at pH 9.2, 5×10^{-4}M at pH 7.5. This substrate should facilitate studies of the kinetics and mechanism of action of fructose diphosphatase and the comparison of this enzyme with other alkaline phosphatases.

FDPase** isolated from rabbit liver is known to hydrolyze both fructose diphosphate and sedoheptulose diphosphate (1), and it is also known that this enzyme can hydrolyze phosphoenol pyruvate at pH 7.5 (2). Earlier, Mendicino reported (3) that kidney FDPase can hydrolyze PNPP very weakly and the activity is less than 0.4% of that when FDP is used as the substrate under identical conditions. However, Scala reported (4) that navy bean leaf FDPase can hydrolyze PNPP ten times better than FDP. Since he used only partially purified enzyme, the result could be caused by contamination by alkaline phosphatase or other hydrolytic enzymes. In this communication, we report that purified rabbit liver FDPase catalyzed the hydrolysis of PNPP with significantly high activity. At pH 9.2, PNPP gives an activity which is 4% of that when FDP is used as substrate.

Rabbit liver FDPase was purified according to the procedures of

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** Abbreviations used are: FDPase, fructose diphosphatase (EC 3.1.3.11); FDP, fructose-1,6-diphosphate; PNPP, p-nitrophenyl phosphate; PNP, p-nitrophenol.

Pontremoli (5) and Pogell (6) with some modifications. Frozen rabbit liver, obtained from Pel-freez, was homogenized, fractionated with ammonium sulfate, chromatographed on carboxymethyl cellulose and crystallized (2). The purified enzyme has a specific activity comparable with that reported by Pontremoli (7).

PNPP (obtained from Sigma) was used as substrate and the rate of formation of PNP, measured by its absorbance at 410 mμ, agreed with that as determined by phosphate release (8). The activity of the enzyme is either stated as Δ O.D./min or μmoles/min product formation. In the case of FDPase activity, the coupled enzyme assay method was used (5). For determining PNPPase activity, the molar extinction coefficient $\epsilon = 17.1 \times 10^3$ at pH 9.2 for PNP was used. The enzyme required either Mn^{++} or Mg^{++} for activity with the former as the preferred one.

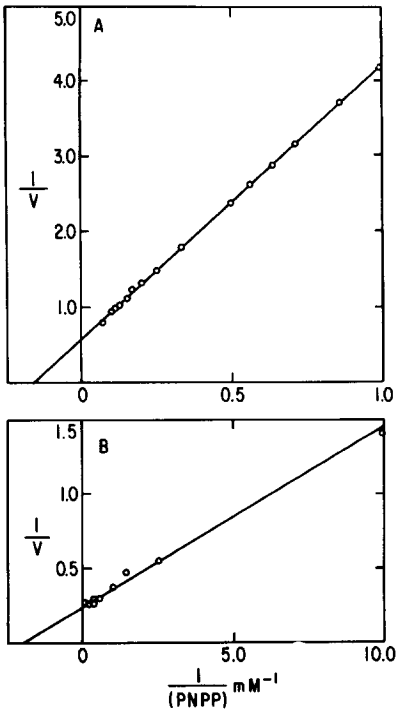

Fig. 1 Reciprocal plot of the initial rate of hydrolysis of PNPP as a function of PNPP concentration. The Mn^{++} concentration was 0.5 mM. A. Glycine buffer (0.4 M), pH 9.4, was used, and B. Tris buffer (0.01 M), pH 7.5, was used.

The K_m value for Mn^{++} in the PNPPase assay is 3.3×10^{-2}M and since it is impractical to use the optimal concentration of Mn^{++} in the assay, we used a final concentration of 0.5 mM to avoid any precipitate formation. This is also the concentration of Mn^{++} used for the FDPase assays.

By using the arbitrarily chosen Mn^{++} concentration, we obtained the K_m values for PNPP. At pH 9.2 it is 6×10^{-3}M, and at pH 7.5 it is 5×10^{-4}M (Fig. 1). The value at low pH is smaller than that at the high pH, in agreement with the FDPase assay. The pH optimum for PNPPase activity is around pH 8.5 as shown by the pH profile (Fig. 2). The sharp drop of activity beyond pH 8.7 probably is caused by some unknown complex formation which removes some Mn^{++} from the solution. The size of the shoulder near pH 7.5 depends on the "age" of the enzyme preparation. In some freshly prepared samples of enzyme, the PNPPase activity was higher at pH 7.5 than at pH 9.2. Since most K_m determina-

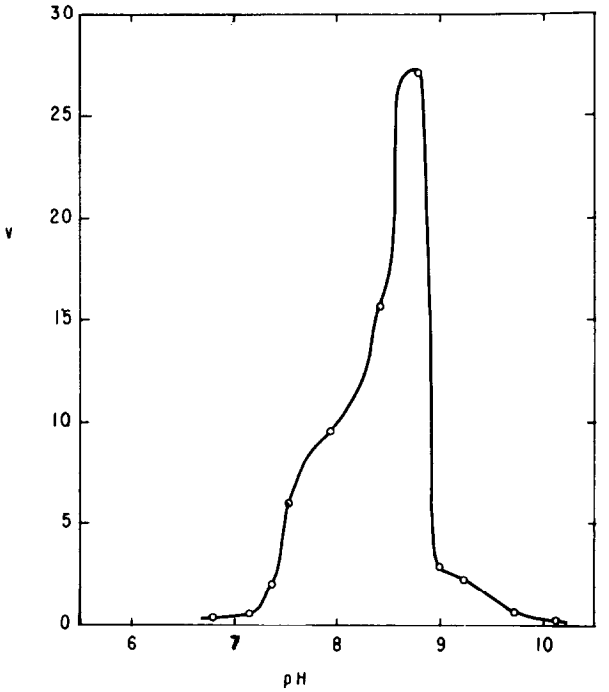

Fig. 2 pH Profile of PNPPase activity. Below pH 7.5, phosphate buffer (0.01 M) was used. Between pH 7.5 and 9.0, Tris buffer (0.01 M) was used. Above pH 9.0, glycine buffer (0.4 M) was used. The PNPP concentration in each case was 4 mM.

tions on FDP are done at pH 7.5 and 9.3, for comparison, we used the same pH values in our studies with PNPP.

In order to show that the PNPPase activity is due to FDPase, we examined

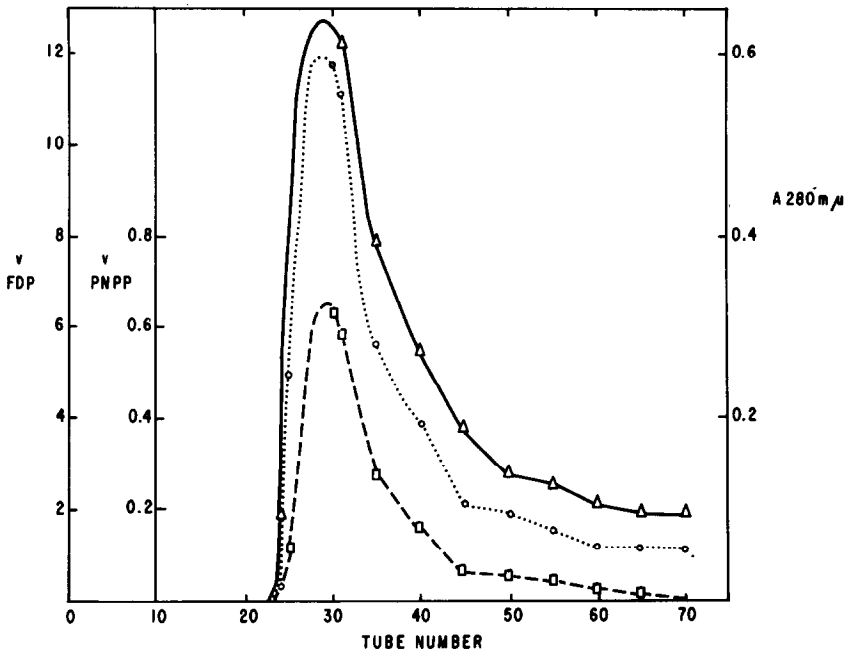

Fig. 3 Carboxymethyl cellulose column elution pattern. The column size is 25 mm x 50 cm, and each tube collected 10 ml. Tube 1 was the beginning of the substrate elution (5 mM malonate, 1.5 mM FDP buffer, pH 6.0). The triangle curve (Δ——Δ) is the absorbance at 280 mμ, the circle curve (O----O) is the initial rate of FDPase activity and the square curve (□– – –□) is the PNPPase initial rate.

the elution pattern of the carboxymethyl cellulose column (Fig. 3). In this column, FDPase is specifically eluted with FDP (6) and it is clear that the peaks for protein, FDP hydrolysis activity and PNPP hydrolysis activity coincide with each other. As further confirmation, the purified enzyme was heated at 50-51°C and the decrease in FDPase and PNPPase activity was determined (Fig. 4). The almost identical slopes between the FDP and PNPP activity at two different pH readings further confirm that PNPP hydrolysis is catalyzed by FDPase.

FDP is a good inhibitor of PNPP hydrolysis. At pH 9.2, FDP inhibits PNPP competitively and has a K_I of 2.2×10^{-4}M (Fig. 5A). At pH 7.5, FDP also

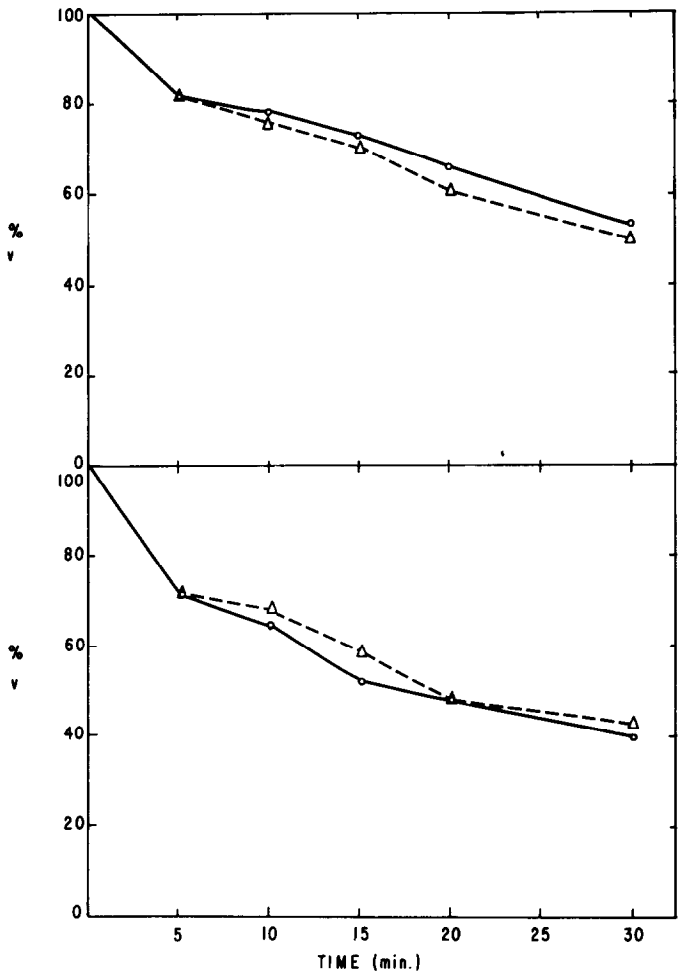

Fig. 4 Heat denaturation curves of FDPase and PNPPase activity. The enzyme was incubated in 5 mM malonate buffer, pH 6.0, at 50-51°C. The curves in the top half were assayed at pH 9.2 (0.4 M glycine buffer) and the curves in the bottom half were assayed at pH 7.5 (0.01 M Tris buffer). For the FDPase assay, 0.1 mM FDP was used (O——O) and 4 mM PNPP was used for the PNPPase assay (Δ- - -Δ). For both assays, 0.5 mM Mn^{++} was used.

inhibits the hydrolysis of PNPP but in this case the inhibition is non-competitive (Fig. 5B). The K_I for FDP at pH 7.5 is 2.5 x 10^{-4}M.

PNPP is a well established substrate for alkaline phosphatase assay, and it is now clear that rabbit liver FDPase can hydrolyze this compound. Preliminary experiments show that PNPP can also be used as a substrate for rabbit muscle FDPase. PNPP should be particularly useful for studies of the mechanism of action

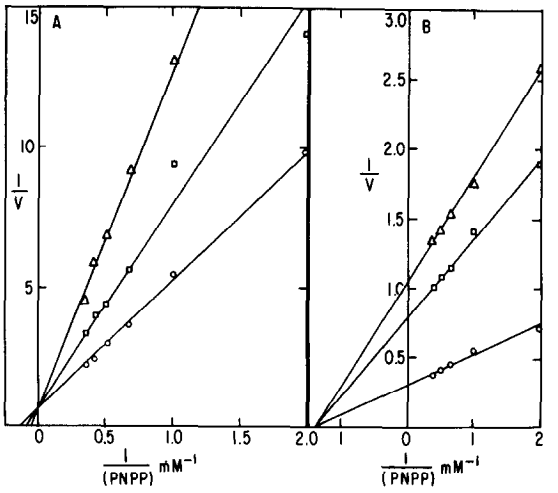

Fig. 5 The inhibition of PNPPase activity by FDP. A. Glycine buffer, 0.4 M, pH 9.2 was used and the Mn^{++} concentration was 0.5 mM. The three lines were obtained at FDP concentrations of 0, 0.1 and 0.2 mM (bottom to top curves). B. Tris buffer, 0.01 M, pH 7.5 was used and the Mn^{++} concentration was 0.5 mM. The three lines were obtained at FDP concentrations of 0, 0.01 and 0.02 mM (bottom to top curves).

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of FDPase since its hydrolysis can be followed spectrophotometrically without the necessity of using a coupled enzyme system or a discontinuous phosphate determination.

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