NAPHTHYL PHOSPHATES AS SUBSTRATES FOR FRUCTOSE 1,6-DIPHOSPHATASE

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1. Introduction

Mammalian liver and kidney fructose 1,6-diphosphatase (EC 3.1.3.11) (FDPase) is considered to be a highly specific enzyme for the hydrolysis of the 1-phosphate from fructose 1,6-diphosphate and sedoheptulose 1,7-diphosphate [1]. However, under appropriate conditions FDPase also hydroloyzes at reduced rates several other phosphate esters: phosphoenol pyruvate [2], β-glycerol phosphate [3], *p*-nitrophenyl phosphate [4], and fructose-1-phosphate [5]. To a certain extent, the enzyme could be regarded as a non-specific phosphatase with highly increased affinities and catalytic activities for two substrates: fructose 1,6-diphosphate and sedoheptulose 1,7-diphosphate. This view led us to study the reaction of FDPase with naphthyl phosphates, since the hydrolysis of these well established substrates for most non-specific phosphatases [6–8] could be used for detection of FDPase on polyacrylamide gels.

2. Methods

Pig kidney FDPase with maximal activity at neutral pH was prepared as described [9]. The purified enzyme gave a single protein band on polyacrylamide disc gel electrophoresis. Its specific activity, measured by the rate of formation of inorganic phosphate from fructose 1,6-diphosphate [9], was of 30 units per mg of protein. FDPase concentration was determined by its absorbance at 280 nm using a value of 0.755 for the absorbance per mg per ml [10].

The hydrolysis of either α- or β-naphthyl phosphate (Sigma) was measured by the initial rate of formation of α- or β-naphthol at 30°C, measured by absorbance at 340 nm on a Gilford 2400 spectrophotometer with the absorbance recording system set at maximum sensitivity so that full scale deflection of the 10-inch recording chart corresponded to an absorbance of 0.1. Molar extinction coefficients at pH 9.2 of 2700 for α-naphthol, and of 1150 for β-naphthol, were used. Appropriate corrections were made when assays were carried out at other pH values. Unless otherwise stated, the assay mixture of 1 ml contained 50 mM Tris–HCl (pH 9.2), 50 mM $MgSO_4$ 0.1 mM EDTA, either 5 mM α-naphthyl-P or 5 mM β-naphthyl-P, and 100–200 μg per ml of FDPase. Activity of *E. coli* alkaline phosphatase (Sigma, Type III) was measured by hydrolysis of β-naphthyl-P, as described above.

Polyacrylamide disc gel electrophoresis was performed in 7.5% gels according to Davis [11] with 0.05 M Tris–glycine buffer, pH 8.6. Prior to the application of the sample, persulfate was removed by electrophoresis [12]. The contaminated buffer was removed and fresh buffer employed for electrophoresis of the sample at a current of 2 mA per tube for 90 min. The protein was stained with Coomassie blue. For the location of activity, non-stained gels were placed into a slightly larger tube holding 4 ml of freshly prepared solution of a staining reagent containing 50 mM Tris–HCl (pH 9.2), 50 mM $MgSO_4$, 0.1 mM EDTA, 10 mM β-naphthyl-P, and 1 mg per ml of Fast Blue BB (Sigma). After 15–20 min incubation in the dark at room temperature the gels were washed in 50% (v/v) ethanol and scanned at 520 nm on a 2410-S Gilford linear transport attachment.

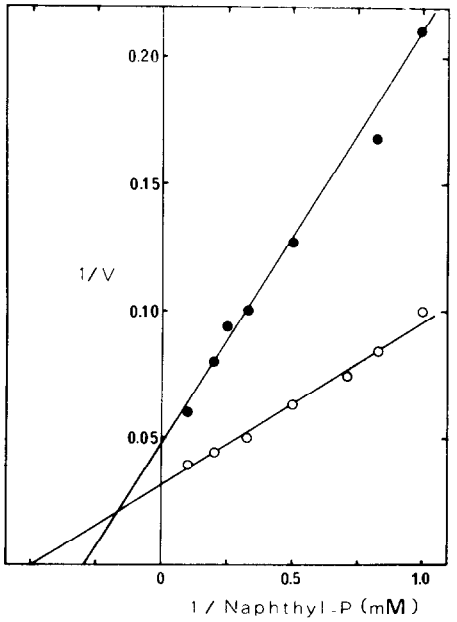

Fig. 1. Double reciprocal plots for the hydrolysis of naphthyl phosphates by FDPase. Except for the variable concentration of substrate, the assays were performed as described under Methods. •, α-naphthyl-P; ○, β-naphthyl-P. V = nmoles of product formed per min per mg of protein.

3. Results and discussion

Although purified pig kidney FDPase exhibits maximal activity at neutral pH with fructose 1,6-diphosphate [9], the hydrolysis of napthyl phosphates be FDPase is characterized by optimum activity at pH 9.2–9.5. Kinetic studies with naphthyl phosphates as substrates for FDPase demonstrate that the enzyme displays Michaelis–Menten kinetics. Lineweaver–Burk plots are shown in fig. 1. The extrapolated Michaelis constants (K_M) are 3.4 mM for α-naphthyl-P, and 2 mM for β-naphthyl-P. V_{max} was 1.4-fold higher with β-naphthyl-P than with α-naphthyl-P. The observed V_{max} value for β-naphthyl-P is 1% of that observed with fructose 1,6-diphosphate, under similar experimental conditions (pH, Mg^{2+} concentration, no K^+ added). At pH 9.2 and 5 mM substrate, K_M for Mg^{2+} was found to be 8.3 mM with α-naphthyl-P and 6.6 mM with β-naphthyl-P.

Potassium ions, which are activators of the reaction of FDPase with fructose 1,6-diphosphate [9, 13], were not used in the studies of the reaction with naphthyl phosphates since activity decreased approximately 68% by the addition of 150 mM KCl. In this respect, it seems that potassium ions only increase activity of FDPase with its natural substrate fructose 1,6-diphosphate, while there is an inhibitory effect of potassium on the reaction of FDPase with other substrates: Fructose-1-P (5), naphthyl phosphates (this report), *p*-nitrophenyl-P and β-glycerol-P [14].

As expected for two compounds which are substrates for the same enzyme, fructose 1,6-diphosphate inhibits the hydrolysis of naphthyl phosphates (table 1). The characteristic allosteric AMP inhibition of FDPase [9, 15, 16] is also observed when naphthyl phosphates are the substrates of the reaction (fig. 2). From the corresponding Hill plots (not shown), K_i and n values were calculated [15]. With α-naphthyl-P the K_i value for AMP is 0.26 mM and n equals 2.9, whlle with β-naphthyl-P the K_i value for AMP is 0.38 mM and n equals 2.8. The above results strongly suggest that FDPase is responsible for the hydrolysis of both fructose 1,6-diphosphate and naphthyl phosphates. Further confirmation was obtained by experiments on heat inactivation. As shown in table 2, the relative rates of hydrolysis of fructose 1,6-diphosphate and β-naphthyl-P did not change during the course of heat inactivation.

Since α- or β-naphthyl-P, in conjunction with a stabilized diazonium salt, have been successfully used for location of alkaline phosphatase activity on polyacrylamide gels [17], the reaction of FDPase with naphthyl phosphates was visualized as a tool for rapid location of this enzyme after disc gel electrophoresis. Up to now, stains for FDPase activity have been only

Table 1
Inhibition of the hydrolysis of naphthyl phosphates by fructose 1,6-diphosphate

Substrate	Other additions	Naphthyl-P hydrolysis (nmoles/min)
α-Naphthyl-P	None	2.50
α-Naphthyl-P	0.02 mM Fru-1, 6-P_2	2.03
α-Naphthyl-P	0.20 mM Fru-1, 6-P_2	1.09
β-Naphthyl-P	None	2.86
β-Naphthyl-P	0.10 mM Fru-1, 6-P_2	2.60
β-Naphthyl-P	0.40 mM Fru-1, 6-P_2	1.88

Experimental procedures were as indicated in Methods.

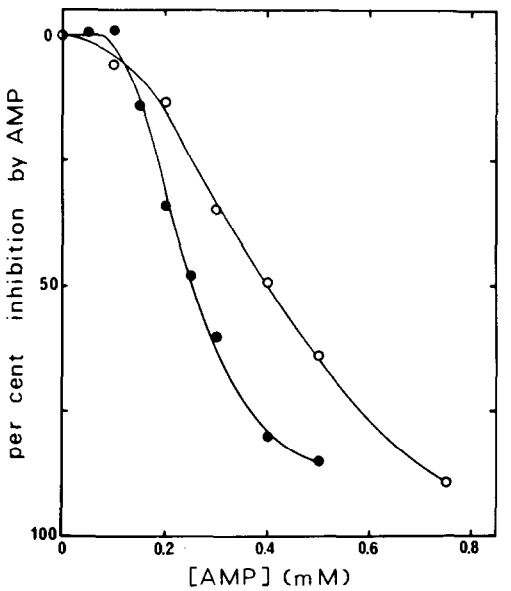

Fig. 2. Inhibition by AMP of FDPase activity with naphthyl phosphates as substrates. The assays were performed as described under Methods, except that AMP was added as indicated. •, α-naphthyl-P; ○, β-naphthyl-P.

occasionally used [18], since in this case the method involving the usual reduction of a tetrazolium dye by NADPH, formed by a coupled system requiring two other enzymes (P-glucose isomerase and glucose-6-P dehydrogenase), is expensive and gives poorly defined

Table 2
Effect of heating on FDPase activities

Temperature (°C)	% Initial Activity with: β-naphthyl-P	Fructose 1,6-diphosphate
–	100	100
55°	98	99
60°	86	92
65°	79	82
70°	62	57
72°	34	32
75°	0	4

FDPase at 0.5 mg per ml was heated for 10 min at different temperatures, as indicated. After cooling in ice and removing the precipitate, the solutions were tested for enzyme activity with β-naphthyl-P (as described in Methods) and with fructose 1,6-diphosphate at pH 9.3 as described [9], except that K_2SO_4 was not added to the reaction mixture.

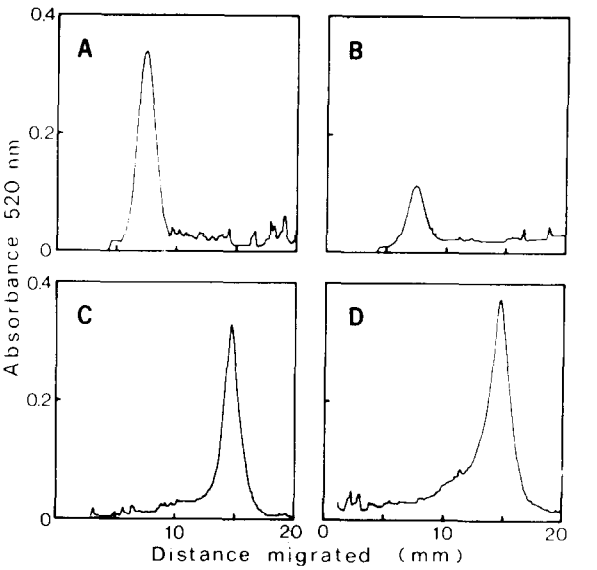

Fig. 3. Densitometric scans of polyacrylamide gels, stained for β-naphthyl phosphatase activity: (A) 0.0017 Units of FDPase; (B) 0.0017 Units of FDPase, but staining reagent contained 2 mM AMP; (C) 0.0010 Units of *E. coli* alkaline phosphatase; (D) 0.0010 Units of *E. coli* alkaline phosphatase, stained in the presence of 2 mM AMP. For experimental details see Methods. The direction of migration is left to right, and only the first 20 mm of the densitometric scans are illustrated. In each case, the position of the band observed with the activity stain corresponded to the position of the band obtained in a duplicate get stained the protein.

bands. The studies of the reaction of FDPase with naphthyl phosphates reported herein, provided the experimental conditions for designing a rapid procedure capable of producing sharp enzyme activity bands on acrylamide disc gels. Fig. 3, a composite diagram of densitometric scanned gels, illustrates the potentiallity of the method using the activity stain with β-naphthyl phosphate and Fast Blue BB. The densitometric scan of a sample containing 60 μg of purified pig kidney FDPase (0.0017 Units of β-naphthyl phosphatase activity) is shown in fig. 3A. As expected, the addition of 2 mM AMP to the staining reagent resulted in significant inhibition of the activity stain for FDPase (fig. 3B). On the contrary, no inhibitory effect was observed when the activity stain plus AMP was used for *E. coli* alkaline phosphatase (figs. 3C and 3D). These results show that β-naphthyl phosphate can be used as a substrate for the rapid detection of

FDPase on acrylamide gels, and that the inhibition of FDPase by AMP allows a clear distinction between FDPase and alkaline phosphatase.

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