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Studies on Nucleotidases in Plants

Isolation and Properties of the Monomeric Form of the Crystalline and Homogeneous Mung Bean Nucleotide Pyrophosphatase

Chandrasekharapuram V. BALAKRISHNAN, Chelakara S. VAIDYANATHAN, and Naropantul Appaji RAO

Department of Biochemistry and University Grants Commission Center of Advanced Study in Biochemistry, Indian Institute of Science, Bangalore

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Mung bean nucleotide pyrophosphatase isolated in a crystalline and homogeneous form as a dimer with a molecular weight of 65000 was converted by AMP into a tetramer. The tetramer was enzymatically active with altered kinetic properties. This conversion of the dimeric form by AMP to a tetrameric one was prevented by treating the dimer with *p*-hydroxymercuribenzoate. The molecular weight of the *p*-hydroxymercuribenzoate-treated enzyme was determined to be 32700 by a combination of Stokes' radius (2.4 nm) and sedimentation velocity ($s_{20,w}$ = 1.9 S), by thin-layer gel chromatography on superfine Sephadex G-200 and by sodium dodecylsulfate/polyacrylamide gel electrophoresis. The monomer obtained by treatment of the native enzyme with *p*-hydroxymercuribenzoate was isolated by passage of the dissociated enzyme through a column of Biogel P-200. The monomer was optimally active at 37 °C, whereas the dimer and tetramer were active at 49 °C. All the three enzyme forms were maximally active at pH 9.4. The K_m and V (measured as rate of FAD hydrolysis per mg protein) for FAD of the three enzyme forms were for the monomer, 0.5 mM and 7.0 μmol min^{-1}, for the dimer, 0.25 mM and 3.3 μmol min^{-1} and for the tetramer, 0.58 mM and 2.5 μmol min^{-1}, respectively. The time course of the reaction of the monomer was linear and comparable to the initial fast rate of the dimer. The monomer was not converted to a tetramer or a dimer on the addition of AMP; and it was irreversibly inhibited by urea and EDTA. ATP and ADP were noncompetitive inhibitors of the monomer.

Nucleotide pyrophosphatase was earlier isolated in a crystalline and homogeneous form from mung bean (*Phaseolus aureus*) seedlings [1]. The time course of the reaction catalyzed by this enzyme was biphasic [2]. Addition of AMP (50 μM) at the start of the reaction abolished the initial fast rate and caused it to proceed at the second slower rate [2]. It was demonstrated by us that AMP was bringing about a stable change in the quaternary structure of the enzyme and the altered enzyme was also active at the second slower rate [3,4]. Preincubation of the native enzyme with *p*-hydroxymercuribenzoate abolished the inhibition at low concentrations of AMP and the reaction proceeded for a longer time at the initial faster rate suggesting that this treatment was desensitizing the enzyme to AMP interaction [2].

Enzymes showing cooperative interaction with ligands have been desensitized by *p*-hydroxymercuribenzoate and this reagent has been shown to bring about dissociation of some polymeric enzymes [5–10]. It was of interest, therefore, to investigate the mode of action of *p*-hydroxymercuribenzoate on this enzyme. In this communication, we report the isolation and properties of the *p*-hydroxymercuribenzoate-dissociated mung bean nucleotide pyrophosphatase and a comparison of its properties with the native and the AMP-modified enzymes.

MATERIALS AND METHODS

Materials

Yeast alcohol dehydrogenase, bovine liver catalase, bovine serum albumin (monomer), chymotrypsinogen, pepsin, cytochrome *c*, FAD, FMN, ATP, ADP, AMP, dAMP, *p*-hydroxymercuribenzoate and sodium dodecylsulfate were obtained from Sigma Chemical Company (St Louis, Mo., U.S.A.). Superfine Sephadex

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Enzymes. Dinucleotide nucleotidohydrolase or nucleotide pyrophosphatase (EC 3.6.1.9); alcohol: NAD^+ oxidoreductase or alcohol dehydrogenase (EC 1.1.1.1); hydrogen-peroxide: hydrogen-peroxide oxidoreductase or catalase (EC 1.11.1.6); pepsin (EC 3.4.23.1).

G-200 was obtained from Pharmacia Fine Chemicals (Uppsala, Sweden). Biogel P-200 was obtained from Calbiochem (Los Angeles, Calif., U.S.A.). All other chemicals used were of the analytical reagent grade. The mung bean seeds were purchased from the local market. FAD, FMN, and ADP were purified on DEAE-cellulose columns [11].

Isolation of the Native, AMP-Modified, and p-*Hydroxymercuribenzoate-Treated Mung Bean Nucleotide Pyrophosphatase*

The mung bean nucleotide pyrophosphatase (native) purified as described earlier [1] was concentrated to approximately 5 mg/ml by placing the enzyme in a dialysis bag immersed in sucrose. The enzyme (3 mg) was treated with *p*-hydroxymercuribenzoate (5 mM) and kept aside at 0 °C for 20 min. The mixture was applied to a column of Biogel P-200 (1.25 × 50 cm) and the protein was eluted with water. Protein [12] and enzyme activity [13, 14] were estimated in the fractions (1.5 ml). The undissociated enzyme was eluted in the early fractions and the major fraction was the *p*-hydroxymercuribenzoate-dissociated enzyme which eluted as a second symmetrical peak. The fractions in the second peak were pooled and used as the *p*-hydroxymercuribenzoate-treated enzyme in these studies. The AMP-modified enzyme was isolated as described earlier [4].

Enzyme Assay

Hydrolysis of FAD was determined as follows. 1 ml of the assay mixture contained 40 mM sodium barbital/HCl buffer, pH 9.4, 2.0 mM FAD, and a suitable amount of the native enzyme (8 – 16 µg), or *p*-hydroxymercuribenzoate-dissociated enzyme (8 to 20 µg), or AMP-modified enzyme (8 – 20 µg). Reaction mixtures without the enzyme, without the substrate and with the reaction stopped at zero time, served as controls. The assay tubes were incubated at 37 °C for 1 min. The reaction was stopped by the addition of 2 ml of ethanol and centrifuged at 10000 × *g* for 10 min. Aliquot portions of the reaction mixture were subjected to circular paper chromatography using ethanol/acetic acid/water (4/1/5, v/v/v). After paper chromatography [13] the flavins were eluted into 5 ml of glass-distilled water and fluorescence of the eluates were measured in a Klett fluorimeter, with primary B_1 (480 nm) and secondary orange (560 nm) filters.

An additional method for assaying the activity was by direct fluorimetry. This assay is based on the observation that the hydrolysis of FAD to FMN results in a ten-fold increase in fluorescence [14]. Aliquot solutions (0.25 ml) of the reaction mixture were made up to 5 ml with 0.05 M sodium phosphate buffer, pH 7.0, and the amount of FMN was determined by measuring the increase in fluorescence [14].

The unit of activity (U) is defined as the amount of enzyme required to produce 1 µmol of FMN per min at pH 9.4 and at 37 °C. Specific activity is defined as U/mg protein.

Disc Gel Electrophoresis Using Sodium Dodecylsulfate

Polyacrylamide gel electrophoresis of the native, AMP-modified, and *p*-hydroxymercuribenzoate-dissociated enzymes in the presence of sodium dodecylsulfate was carried out according to the method of Weber and Osborn [15]. Enzyme samples and the standards (pepsin, chymotrypsinogen and monomeric bovine serum albumin) were denatured in 0.01 M sodium phosphate buffer, pH 7.0, containing 1% sodium dodecylsulfate and 1% 2-mercaptoethanol at 37 °C for 2 h. The proteins (80 – 120 µg each) were subjected to electrophoresis in 0.2 M sodium phosphate buffer, pH 7.0, containing 0.1% sodium dodecylsulfate. A 10% acrylamide gel was used and electrophoresis was carried out at 4 °C for 5 – 7 h. Bromophenol blue was used as the tracking dye. After electrophoresis, the protein bands were located by staining the gels with Coomassie brilliant blue in CCl_3COOH for 2 h. The gels were destained by repeated washing with methanol/acetic acid/water (1/2/20, v/v/v).

Thin Layer Gel Chromatography

Thin-layer gel chromatography on superfine Sephadex G-200 was carried out according to Radola [16, 17] using the following marker proteins: catalase, yeast alcohol dehydrogenase bovine serum albumin (monomer), chymotrypsinogen, pepsin [18], cytochrome *c* [16] and blue dextran. The native, AMP-modified and *p*-hydroxymercuribenzoate-dissociated enzymes were used along with the markers. Sodium chloride (0.1 M) was used as the solvent and the proteins were identified by staining with Coomassie brilliant blue.

Determination of Stokes' Radius

Catalase, yeast alcohol dehydrogenase, bovine serum albumin (monomer), chymotrypsinogen and cytochrome *c* (1 mg of each) dissolved in a total volume of 1 ml of water and loaded on to a column (1.25 × 50 cm) of Biogel P-200 [19]. Fractions (1.5 ml) were collected and assayed for activity in the case of catalase [20] and alcohol dehydrogenase [21]. In all the fractions, protein was estimated by measuring absorbance at 260 and 280 nm. The native enzyme (3 mg) treated with *p*-hydroxymercuribenzoate (5 mM)

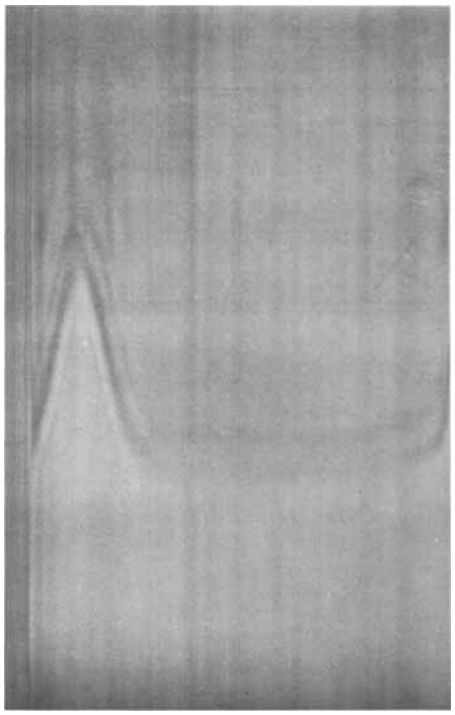

Fig. 1. *Sedimentation velocity pattern of mung bean nucleotide pyrophosphatase.* Protein concentration was 10 mg/ml. The photograph was taken at a bar angle of 45° at 64 min after maximum speed of 57800 rev./min was attained

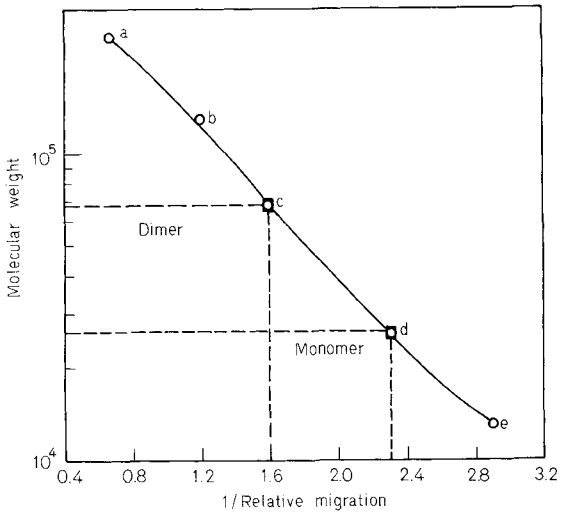

Fig. 2. *Thin-layer gel chromatography of marker proteins and of monomeric and dimeric forms of mung bean nucleotide pyrophosphatase.* Glass plates (20 × 20 cm) were coated with superfine Sephadex G-200 as described by Radola [16, 17]; (a) catalase (250000); (b) yeast alcohol dehydrogenase (125000); (c) monomeric bovine serum albumin (65000); (d) pepsin (35000); (e) cytochrome *c* (12000) and blue dextran were spotted along with the monomer, dimer and tetramer. The numbers in parentheses indicate molecular weight. The plates were developed with 0.1 M NaCl at a flow rate of 3 cm/h. The proteins were adsorbed on to a Whatman no. 3 filter paper, dried and then stained with Coomassie brilliant blue in CCl_3COOH. Excess dye was removed by repeated washing with methanol/acetic acid/water (1/2/20, v/v/v). The distance migrated by the protein with reference to catalase was measured. The inverse of the relative migration was plotted against the molecular weight of the protein on a log scale. The mobility of the monomer was identical to that of pepsin and that of the dimer to the monomeric form of bovine serum albumin. From the figure the molecular weight of the dimer and monomer was calculated to be 65000 and 35000 respectively

and incubated at 0 °C for 20 min was loaded on to the same column and fractions (1.5 ml) were collected. Protein [12] and enzyme activity [14] was estimated in each fraction. Stokes' radius was calculated according to the method of Ackers [22]. Stokes' radii of the native and AMP-modified enzymes were similarly determined [4].

Analytical Ultracentrifugation

The native, AMP-modified and the *p*-hydroxymercuribenzoate-dissociated enzymes (10 mg/ml) were subjected to analytical ultracentrifugation at 57800 rev./min in a Beckman model E centrifuge. From the sedimentation pattern, the $s_{20,w}$ values were calculated.

RESULTS

Molecular Weight of the p-Hydroxymercuribenzoate-Dissociated Mung Bean Nucleotide Pyrophosphatase

The *p*-hydroxymercuribenzoate-dissociated enzyme sedimented as single symmetrical peak on analytical ultracentrifugation (Fig. 1). The $s_{20,w}$ value of the *p*-hydroxymercuribenzoate-dissociated enzyme was calculated to be 1.9 S. $s_{20,w}$ values of the native and AMP-modified enzymes, similarly determined, were 2.4 and 4.1 S, respectively [4].

Stokes' radius of the *p*-hydroxymercuribenzoate-dissociated enzyme determined using a calibrated Biogel P-200 column was found to be 2.4 nm, and that of the native and AMP-modified enzymes were 3.5 and 4.6 nm, respectively [4].

The molecular weight of the *p*-hydroxymercuribenzoate-dissociated enzyme calculated according to the method of Siegel and Monty [24], combining the Stokes' radius and $s_{20,w}$ value, was found to be 32700. The molecular weight of the native and AMP-modified enzymes were similarly determined to be 65000 and 136000, respectively. Using catalase as the standard, inverse of the relative migration of reference proteins on thin-layer gel chromatography on Sephadex G-200 (Fig. 2) was plotted against the log of the molecular weight of the reference proteins. The molecular weight of the native as well as of the *p*-hydroxymercuribenzoate-dissociated enzyme were determined from the graph obtained using reference proteins (Fig. 2), to be 65000 and 35000, respectively.

The *p*-hydroxymercuribenzoate-treated enzyme on dodecylsulfate gel electrophoresis (Fig. 3 B) moved

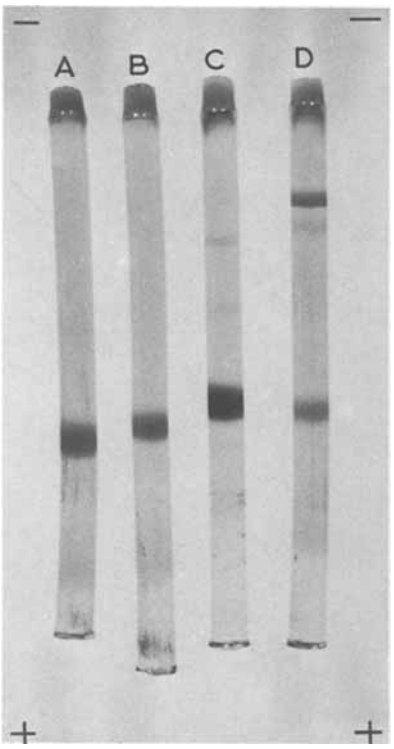

Fig. 3. *Disc gel electrophoresis in sodium dodecylsulfate of marker proteins and the monomer of mung bean nucleotide pyrophosphatase.* The marker proteins (pepsin, chymotrypsinogen and bovine serum albumin) and the monomer of the mung bean nucleotide pyrophosphatase were denatured in 0.01 M sodium phosphate buffer pH 7.0 containing 1% of 2-mercaptoethanol and sodium dodecylsulfate at 37 °C for 2 h. The proteins (80–120 μg) were subjected to electrophoresis in 0.2 M sodium phosphate buffer pH 7.0 containing 0.1% sodium dodecylsulfate [16]. A 10% gel was used and electrophoresis was caried out at 4 °C for 5–7 h. Bromophenol blue was used as the tracking dye. After electrophoresis, the protein bands were located by staining with Coomassie brilliant blue in CCl_3COOH for 2 h. Gels were destained by repeated washing with methanol/acetic acid/water (1/2/20, v/v/v). (A) Chymotrypsinogen; (B) monomer; (C) pepsin; (D) bovine serum albumin + chymotrypsinogen

as a single band with a mobility similar to that of pepsin and chymotripsinogen (Fig. 3A and C) suggesting that its minimum molecular weight was approximately 35000. This value is in agreement with molecular weight determined by other methods (see above). These observations indicate that treatment of the native enzyme with *p*-hydroxymercuribenzoate was dissociating it to a monomeric form. This observation was confirmed by subjecting a mixture of the native, AMP-modified and *p*-hydroxymercuribenzoate-treated enzyme to dodecylsulfate gel electrophoreses. A single protein band identical to that of *p*-hydroxymercuribenzoate-treated enzyme was noticed (not shown in the figure). The following conclusion can be drawn from these results. All the three enzyme forms breakdown on dodecylsulfate treatment to an apparently single subunit of molecular weight 33000 indicating that the enzyme is made up of identical subunits. The native enzyme is a dimer made up of similar subunits and addition of AMP converts it to a tetramer, whereas treatment with *p*-hydroxymercuribenzoate dissociates the native enzyme to a monomer. The three enzyme forms will be referred to hereafter as dimer (native enzyme), tetramer (AMP-modified enzyme) and monomer (*p*-hydroxymercuribenzoate-treated enzyme).

Time Course of the Dimeric, Tetrameric and Monomeric Forms of the Mung Bean Nucleotide Pyrophosphatase

The reaction mixture, scaled up to 3 ml, contained either the dimer (48 μg), tetramer (60 μg) or monomer (60 μg). Aliquot solutions (0.25 ml) of the reaction mixture were withdrawn at time intervals shown in Fig. 4. The amount of FMN formed was determined by direct fluorimetry [14] as well as after chromatography separation from FAD [13]. In all the cases, the products of the reaction were identified as FMN and AMP as described earlier [1] and the stoichiometry of the reaction catalyzed by the isolated monomer was similarly established [1], *i.e.*, hydrolysis of 1 mole of FAD resulted in the formation of 1 mole of FMN and AMP. The rate of reaction of the monomer (Fig. 4) was linear and comparable to the initial fast rate of the dimer. The reaction rate of the dimer was biphasic [2]. Time course of reaction of the tetramer was linear and similar to the second slower rate of the dimer (Fig. 4). Addition of AMP, ADP, ATP, IMP, dAMP, adenosine and FMN at the start of the reaction did not alter the time course of the monomer, whereas that of the dimer was altered to the second slower rate on the addition of AMP (not shown in Fig. 4, see [2, 4]).

Effect of pH and Temperature

The monomer was optimally active at pH 9.4 like the dimer and tetramer (see Table 2 below). The monomer functioned optimally at 37 °C (Fig. 5) whereas the dimer and tetramer were maximally active at 49 °C (Fig. 5).

Heat Stability

The monomer (2 mg), dimer (2 mg) and tetramer (2 mg) were separately incubated at 55 °C and aliquots of the solution were withdrawn at time intervals shown in Fig. 6, and chilled rapidly to 0 °C. From the figure it is evident that the monomer was denatured rapidly and complete loss of activity was observed in 15 min. On the other hand, the dimer and tetramer were relatively more stable. The times for 50%

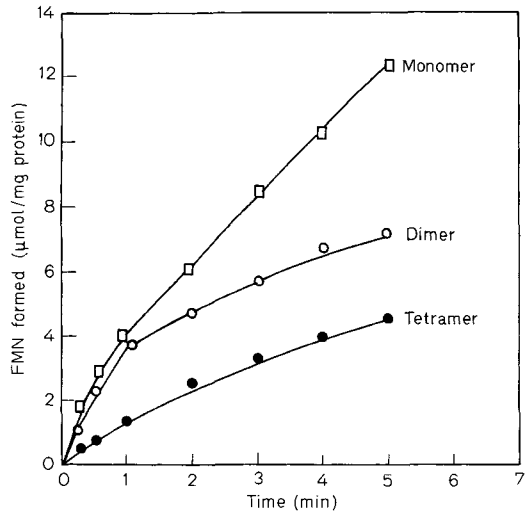

Fig. 4. *Progress of the reaction catalyzed by the monomeric, dimeric and tetrameric forms of mung bean nucleotide pyrophosphatase.* The reaction mixture (3 ml containing 48 μg of the monomer) was incubated at 37 °C and aliquots (0.25 ml) of the reaction mixture were withdrawn at time intervals indicated in the figure. Enzyme activity was estimated by determining the amount of FMN formed [13]. The activity of the dimer (48 μg per 3-ml reaction mixture) and of the tetramer (60 μg per 3-ml reaction mixture) was similarly assayed at time intervals indicated in the figure

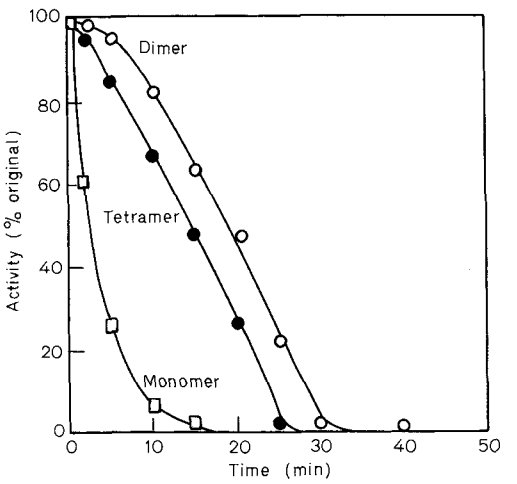

Fig. 6. *Heat stability of the mung bean nucleotide pyrophosphatase.* Monomer, dimer and tetramer (2 mg) were incubated at 55 °C and aliquot (0.25-ml) portions were withdrawn at time intervals in the figure. The enzyme activity was assayed at 37 °C

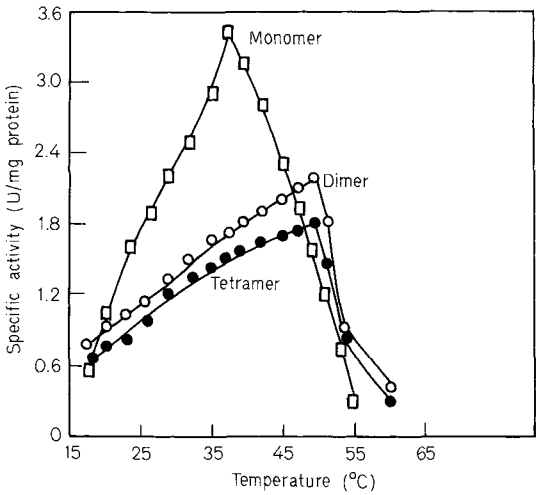

Fig. 5. *Effect of temperature on the activity of the monomeric, dimeric and tetrameric mung bean nucleotide pyrophosphatase.* 8 μg of the dimer or 12 μg of the tetramer or monomer were used per ml of assay mixture. Enzyme activity was estimated as described earlier at the temperatures indicated in the figure

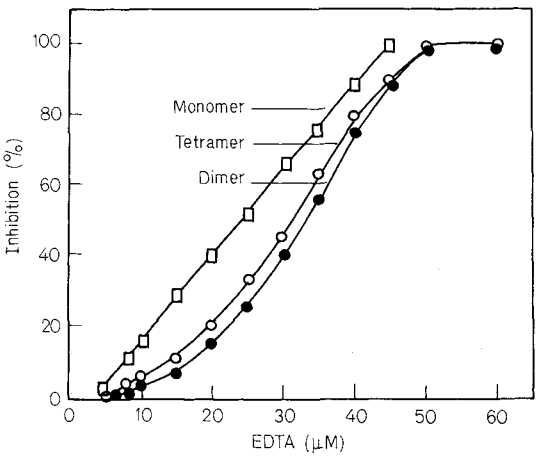

Fig. 7. *Effect of EDTA on the various forms of mung bean nucleotide pyrophosphatases.* The monomer, dimer and tetramer (20 μg/ml reaction mixture) were preincubated at 0 °C for 10 min with EDTA (concentrations indicated in the figure). The reaction was started by the addition of FAD and enzyme activity estimated as described earlier [13, 14]

inactivation at 55 °C of monomer, dimer, and tetramer were 3, 20 and 15 min respectively.

Effect of EDTA on Mung Bean Nucleotide Pyrophosphatase Monomer

A number of pyrophosphatases require metal ions for activity [25–30]. From Fig. 7, it is evident that concentrations of EDTA below 4 μM were without effect but complete inhibition of the monomer activity was observed at a concentration of 45 μM EDTA. On the other hand, activity of the dimer and tetramer was not significantly inhibited up to 10 μM. Further increase in the concentration of EDTA resulted in a sigmoid pattern of inhibition and complete inhibition was observed at 50 μM. The enzyme activity was

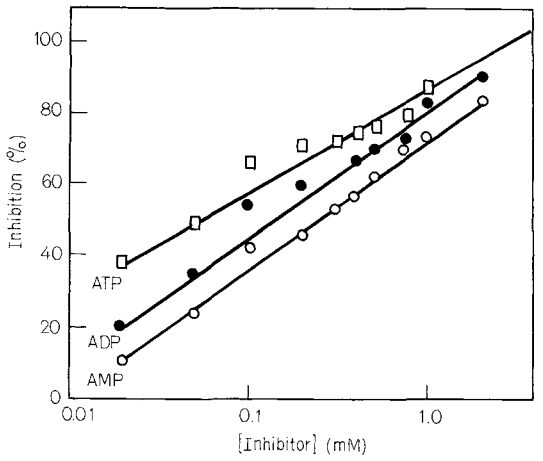

Fig. 8. *Effect of adenylates on the monomeric form of mung bean nucleotide pyrophosphatase.* The adenine nucleotides, AMP, ADP or ATP (concentrations shown in figure) were incubated with the monomeric form (20 μg) of the mung bean nucleotide pyrophosphatase at 0 °C for 15 min. This mixture was added to reaction mixtures (1.0 ml) and the reaction was started by the addition of FAD. The enzyme activity was assayed by estimating FMN formed [13] in 1 min at 37 °C. The activity in the absence of added adenine nucleotide was similarly estimated and the percentage inhibition is calculated using this value

assayed at different pH values in the range 6.0–9.8, in reaction mixtures containing EDTA (4, 8, 10, 20, 30, 40, 45 and 50 μM). No increase in activity was observed at any of the pH values suggesting that EDTA did not alter the pH optimum of the enzyme. The activity of the tetramer and monomer inhibited by EDTA could not be restored by adding excess of metal ions like Zn^{2+}, Mg^{2+}, Mn^{2+} or Co^{2+}, while the activity of the dimer inhibited by EDTA could be reversed specifically by Zn^{2+} and Co^{2+} [1].

Michaelis Constant for FAD

The K_m for FAD calculated for the monomer from Lineweaver-Burk plot was 0.50 mM. V for this enzyme form was 7.0 μmol FAD hydrolysed min^{-1} (mg protein)$^{-1}$.

Effect of Adenine Nucleotides on the Monomer

Adenine nucleotides are known to regulate the activity of a number of enzymes, and they participate in the biosynthesis of flavin coenzymes. AMP is one of the reaction products of the mung bean nucleotide pyrophosphatase and it alters the biphasic time course of reaction catalyzed by the dimer [3,4]. Adenine nucleotides inhibited the activity of the dimer and tetramer [2–4]. It was of interest to examine the effect of AMP, ADP and ATP on the monomer. It is evident from Fig. 8 that AMP, ADP and ATP inhibit the activity of the monomer linearly. This is in contrast to the sigmoidal pattern of inhibition obtained with the dimer and tetramer [2,4]. In Table 1 are presented the K_m and slopes of the Lineweaver-Burk plots of the monomeric enzyme at different fixed concentrations of AMP, ADP and ATP. All three nucleotides made no significant change to the K_m but altered the slope of the reciprocal plots, suggesting that they might be noncompetitive inhibitors of the nucleotide pyrophosphatase.

Table 1. *Effect of adenylates on the monomeric mung bean nucleotide pyrophosphatase*
The monomeric form of mung bean nucleotide pyrophosphatase was preincubated with AMP, ADP and ATP (concentrations indicated) for 15 min at 0 °C. FAD (0.05–2.0 mM) was used as the substrate. The reaction mixture (1.0 ml contained 8.0 μg of the enzyme) was incubated for 1 min at 37 °C and at pH 9.4. FMN formed was estimated [13,14]. K_m and slope were determined from Lineweaver-Burk plots

Adenylate	Concn	K_m	Slope
	mM		
AMP	0.1	0.40	0.10
	0.2	0.40	0.11
	0.3	0.40	0.12
	0.4	0.40	0.13
	0.5	0.40	0.15
	0.7	0.40	0.20
	1.0	0.40	0.23
	2.0	0.40	0.33
	5.0	0.40	0.53
ADP	0.1	0.25	0.08
	0.2	0.25	0.09
	0.4	0.25	0.10
	0.5	0.25	0.11
	0.7	0.25	0.12
	1.0	0.25	0.23
	2.0	0.25	0.35
	5.0	0.25	0.56
ATP	0.1		0.10
	0.2		0.11
	0.3		0.12
	0.4		0.13
	0.5		0.14
	0.7		0.16
	1.0		0.24
	2.0		0.30
	5.0		0.40
None		0.50	0.07

Effect of Urea

It was earlier reported [3,4] that the dimeric mung bean nucleotide pyrophosphatase denatured with urea could be reactivated by removal of urea. The monomer, dimer and tetramer were treated with urea (concentrations indicated in Fig. 9) and assayed for activity in reaction mixtures containing the same

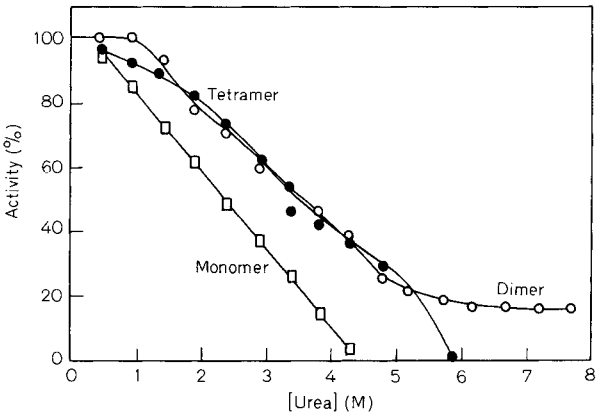

Fig. 9. *Effect of urea on the three forms of mung bean nucleotide pyrophosphatases.* The monomer, dimer and tetramer (8 μg each) were incubated with urea (0–8 M) for 10 min at 0 °C. The enzyme activity was determined in reaction mixtures containing the same concentration of urea. A duplicate set was assayed in reaction mixtures without urea. The results are given as a percentage of the activity without urea. The dilution of urea in the reaction mixtures was 50-fold

Table 2. *Summary of the properties of monomeric, dimeric and tetrameric forms of the mung bean nucleotide pyrophosphatase*

Property	Value for		
	monomer	dimer	tetramer
Molecular weight	32700	65000	136000
Minimum molecular weight[a]	33000	33000	33000
pH optimum	9.4	9.4	9.4
Temperature optimum	37 °C	49 °C	49 °C
K_m for FAD	0.50 mM	0.25 mM	0.58 mM
V	7.0[b]	3.3[b]	2.5[b]
Time course	linear	biphasic	linear
Inhibition by AMP:			
10 μM	0	25%	0
1 mM	100%	100%	100%
EDTA inhibition	+	+	+
Reversal by Zn^{2+}	no	yes	yes
Urea denaturation	+	+	+
Activity after removal of urea	0	100%	0

[a] From dodecylsulfate electrophoresis.
[b] Units are μmol FAD hydrolyzed min^{-1} (mg protein)$^{-1}$.

concentration of urea. It is evident from Fig. 9 that urea denatured the monomer as it did the dimer and tetramer. Complete inhibition of monomer activity was observed at 4.5 M urea whereas 6 M urea was required for the inhibition of the tetramer. The dimer was maximally inhibited (80%) at 6 M urea and further increase in urea concentration had no additional effect on the activity. Removal of urea by dialysis or by passage through a Sephadex G-25 column did not result in the restoration of activity of the urea-denatured monomer or tetramer. The shape of the denaturation curve of the monomer was different from that of the dimer or tetramer (Fig. 8).

DISCUSSION

One of the common mechanisms of regulation of enzyme activity is by an alteration of the quaternary structure of enzymes. A number of effectors are known to cause either dissociation or association of polymeric enzyme [5, 30–41]. A significant observation made in our study is that the monomeric, dimeric and tetrameric forms of mung bean nucleotide pyrophosphatase could be isolated and all of these forms were active with altered physicochemical and kinetic properties [4, 5] (and this paper).

A comparison of the properties of the three enzyme forms is given in Table 2. These results permit us to conclude that the mung bean nucleotide pyrophosphatase isolated as described earlier [1] occurs as a dimer made up of similar subunits. The dimer could be converted by AMP into an active tetramer and could be dissociated by *p*-hydroxymercuribenzoate to an active monomer. It has not yet been possible for us to convert the monomer to an active dimer or tetramer, or to dissociate the tetramer to an active dimer or monomer. Attempts are now in progress to achieve this.

An explanation for the difference in the temperature optima of the three enzyme forms (Fig. 5) may be that the monomer is less stable at higher temperatures than the dimer or tetramer. This inference is supported by the observation that the monomer is more rapidly inactivated by proteolytic enzymes compared to the dimer, and the helical content of the dimer decreases from 45% to 24% on dissociation to the monomer (unpublished observations). A comparison of V for the three forms (Table 2) showed the monomer to be the most active and the tetramer the least active; the K_m values of the three forms were similar. These results suggest that the monomer is the most active but the least stable of the three enzyme forms.

The denaturation (Fig. 9) of the monomer by urea as well as the inactivation by EDTA (Fig. 8) was different from that of the dimer and tetramer suggesting that the process of denaturation was different [3] for the three forms. However, it is too premature to clearly identify the reasons for this difference.

Our earlier observations [2] showed that AMP had two sites of interaction and also that low concentrations of AMP were converting the dimer to tetramer [4]. The tetramer was not affected by low concentration of AMP (50 μM). Higher concentrations of AMP, ADP, and ATP inhibited the activity of all the three enzyme forms. Low concentrations of AMP had no effect on the activity of the monomer. The sigmoid nature of the inhibition curve suggests that

AMP may be acting as an allosteric effector for the dimer and tetramer whereas it may be acting as a catalytic inhibitor for the monomer.

These results suggest that the activity of mung bean nucleotide pyrophosphatase is regulated through association/dissociation phenomenon.

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C. V. Balakrishnan, C. S. Vaidyanathan, and N. Appaji Rao*, Department of Biochemistry, Indian Institute of Science, Bangalore, India, 560012

* To whom all correspondence should be addressed