J. Biosci., Vol. 2, Number 3, September 1980, pp. 211-225 © Printed in India.

Comparison of the conformation and stability of the native dimeric, monomelic, tetrameric and the desensitized forms of the nucleotide pyrophosphatase from mung bean *(Phaseolus aureus)* seedlings

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MS received 26 May 1980; revised 18 August 1980

Abstract. A homogenous and crystalline form of nucleotide pyrophosphatase (EC 3.6.1.9) from *Phaseolus aureus* (mung bean) seedlings was used for the study of the regulation of enzyme activity by adenine nucleotides. The native dimeric form of the enzyme had a helical content of about 65% which was reduced to almost zero values by the addition of AMP. In addition to this change in the helical content, AMP converted the native dimer to a tetramer. Desensitization of AMP regulation, without an alteration of the molecular weight, was achieved either by reversible denaturation with 6 M urea or by passage through a column of Blue Sepharose but additionof *p*-hydroxymercuribenzoate desensitized the enzyme by dissociating the native dimer to a monomer. The changes in the quaternary structure and conformation of the enzyme consequent to AMP interaction or desensitization were monitored by measuring the helical content, EDTA inactivation and Zn^{2+} reactivation, stability towards heat denaturation, profiles of urea denaturation and susceptibility towards proteolytic digestion. Based on these results and our earlier work on this enzyme, we propose a model for the regulation of the mung bean nucleotide pyrophosphatase by association-dissociation and conformational changes. The model emphasizes that multiple mechanisms are operative in the desensitization of regulatory proteins.

Keywords. *Phaseolus aureus*; nucleotide pyrophosphatase; regulation; desensitization; conformational changes; circular dichroism; optical rotatory dispersion.

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Abbreviations used: Ultraviolet, uv; circular dichroism, CD; optical rotatory dispersion, ORD; sodium dodecyl sulphate, SDS.

Introduction

There have been several examples of allosteric enzymes that are susceptible to desensitization of their regulatory responses by certain reagents (Carney *et al*. 1978; Clark and Yielding, 1971; Gerhart and Pardee, 1962; Graves and Wang, 1972; Mangiarotti and Pontremoli, 1963; Wang and Tu, 1969; Weitzman, 1966). The study of the desensitized enzymes, which behave with markedly altered kinetic and physical properties, has been useful in understanding the details of the aliosteric regulation of the enzyme activity. Nucleotide pyrophosphatase (dinucleotide nucleotide-hydrolase, EC 3.6.1.9) catalyzes the hydrolysis of dinucleotides at the pyrophosphate bond to yield the corresponding mononucleotides and AMP. It was earlier reported from this laboratory that the enzyme from mung bean seedlings was a dimer (M_r 65,000) and was converted to a tetramer on the addition of AMP. The native dimer was dissociated into a monomer by *p*-hydroxymercuribenzoate. The monomer, dimer and tetramer were all enzymatically active. The dimer was desensitized by treatment with *p*-hydroxymercuribenzoate or by reversible denaturation with urea (Ravindranath and Appaji Rao, 1969; Balakrishnan *et al*., 1975,1977). We have recently reported a method for the isolation of a dimeric form of the enzyme which was desensitized to interactions with AMP. The desensitized enzyme had enhanced catalytic activity and increased temperature optimum (Reddy *et al*., 1979). It was, therefore, of interest to examine the conformation and stability of the desensitized enzyme and compare it with the native dimeric and tetrameric forms of the enzyme. The results of such a study are presented in this paper along with a model to explain the regulation of mung bean nucleotide pyrophosphatase by AMP.

Materials and methods

Materials

FAD, AMP, *p*-hydroxymercuribenzoate, α -chymotrypsin EDTA, urea, sodium dodecyl sulphate (SDS) and bovine serum albumin were from Sigma Chemical Company, St. Louis, Missouri, USA. Trypsin (diphenyl carbamyl chloride treated) was from Serva Feinbiochemica, Heidelberg, Germany. Urea was recrystallized twice before use. Mung bean seeds were purchased from the local market.

Methods

The native enzyme, the monomer, the tetramer and the desensitized enzymes were prepared as reported earlier (Ravindranath and Appaji Rao, 1968; Balakrishnan *et al*. 1974, 1975, 1977; Reddy *et al*., 1979). The monomeric, dimeric and tetrameric forms of the enzyme were assayed using 0.5 mM FAD and the desensitized enzyme using 1 mM FAD as the substrate (Ravindranath and Appaji Rao, 1968; Reddy *et al*., 1979). Protein was estimated by the method of Lowry *et al*. (1951) using crystalline bovine serum albumin as the standard.

Circular dichroism (CD) and optical rotatory dispersion (ORD) spectrawere recorded using a Jasco J-20 automatic recording spectropolarimeter at an appropriate chart speed and time constant to obtain maximal resolution of the spectrum. Calibration of the CD and ORD scales were done using *d*-10-camphor sulphonic acid and

sucrose, respectively (Adler *et al*., 1973). All measurements were made at 25°C. Quartz cells with a light path of 5 mm were used. The CD and ORD spectra are reported in terms of mean residue ellipticities [θ] and mean residue rotation [*m*] respectively in deg. cm^2. $dmol^{-1}$, taking a value of 115 for the mean residue weight of the enzyme.

The helical content was calculated from the CD spectra by the method of Greenfield and Fasman (1969) employing the following equation:

$$\% \text{ Helix} = \frac{[\theta]_{208} - 4000}{33000 - 4000} \times 100$$

The helical content was also determined from the ellipticity value at 222 nm and from the ORD data using the following equations (Chen and Yang, 1971):

$$[\theta]_{222} = -30{,}300\, f_H - 2340$$
$$[m]_{223} = -12{,}700\, f_H - 2520$$

Where f_H is the fractional helical content. The reference values of $[\theta]_{208}$ and $[m]_{233}$ for fully helical and random coil forms of poly L-lysine were used (Green field *et al*., 1967).

Results

Effect of AMP

The far-ultraviolet (U.V.) CD spectrum (figure 1) of the native dimer showed two troughs at 208 and 222 nm with mean residue ellipticities of –22,200 and –21,300 deg. cm^2. $dmol^{-1}$, respectively. The CD spectrum of the tetramer or of the native

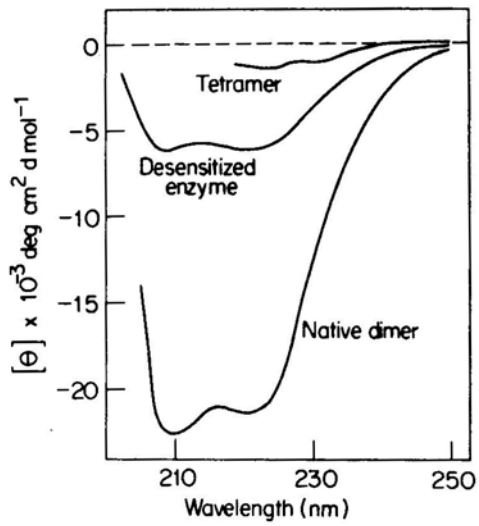

Figure 1. Far-ultraviolet CD spectra of the native dimeric, tetrameric, and the desensitized forms of the mung bean nucleotide pyrophosphatase. The spectrawere recorded at 25°C in 1ml of 0.1M sodium phosphate buffer, pH 7.4 containing 67.5 μg of the native enzyme, 46 μg of the tetramer of 54 μg of the desensitized enzyme. The spectrum of the desensitized enzyme plus AMP was identical to that of the desensitized enzyme alone.

dimer in the presence of AMP showed no characteristic features suggesting that AMP was either masking the spectrum or the tetramer had very low helical content (figure 1). The presence of bound *p*-hydroxymercuribenzoate in the monomeric form of the enzyme interfered with the recording of the CD spectrum (not shown in the figure). The desensitized dimer obtained by passage through Blue Sepharose showed two troughs at 210 and 222 nm with mean residue ellipticity value of –6,000 deg. cm^2 $dmol^{-1}$ at both the wavelengths (figure 1). Addition of AMP had no effect on the CD spectrum of the desensitized enzyme. The helical content of the four enzyme forms, *viz*., native dimer, monomer, tetramer and the desensitized forms of the mung bean nucleotide pyrophosphatase, calculated from mean residue ellipticity values at 208 and 222 nm are shown in table 1.

Table 1. Helical content of the native dimer, tetramer, monomer and the desensitized dimer forms of mung bean nucleotide pyrophosphatase.

Enzyme form	Helical content (%)		
	a	*b*	*c*
Native dimer	63	63	67
Tetramer	Nil	Nil	Nil
Monomer	4	9	8
Desensitized dimer	6	12	11

[a] from CD data using the method of Greenfield and Fasman (1969)

[b] from CD data using the method of Chen and Yang (1971)

[c] from ORD data using the method of Chen and Yang (1971).

The ORD spectrum of native dimer (figure not given) showed a trough at 233 nm with a mean residue rotation of –11,000 deg. cm^2 . $dmol^{-1}$ which .decreased to –2,200 deg. cm^2. $dmol^{-1}$ on treatment of the native enzyme with 50 μM AMP (figure 2) and to —3,500 deg. cm^2, $dmol^{-1}$ on treatment with 0.5 mM *p*-hydroxymercuribenzoate. The spectra of the tetramer and the monomer were identical with those of the native dimer in the presence of AMP or *p*-hydroxymercuribenzoate, respectively. The desensitized enzyme obtained by passage through a Blue Sepharose column (Reddy *et al*., 1979) showed a trough at 231.5 nm and a cross-over point at 221 nm (figure 2). The mean residue rotation of the desensitized enzyme was was –4,100 deg. cm^2, $dmol^{-1}$ at 231.5 nm and, unlike in the case of the native dimer, there was no change in the rotation at 231.5 nm or in the overall shape of the ORD spectrum on the addition of AMP to the desensitized enzyme. The helical contents of the various enzyme forms calculated by the method of Chen and Yang (1971) is given in table 1. It can be seen from the table that the helical content of the native dimer calculated from mean residue ellipticity values at 208 or 222 nm or from the mean residue rotation at 233 nm is in good agreement validating the equaions used in these calculations. The helical content decreased markedly when the native dimer was either desensitized to AMP interactions or when AMP was bound to it

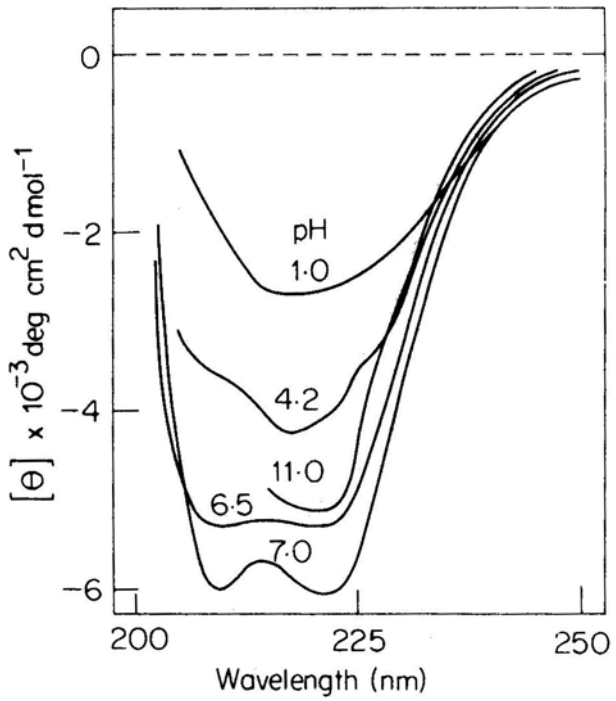

Figure 2. Efect of pH on the CD spectra of the desensitized mung bean nucleotide pyrophosphatase. Enzyme concentration used was 90 μg. pH changes were effected by the addition of either dilute HCl or NaOH.

Effect of pH on the conformation and the activity of the desensitized enzymes

Figure 2 depicts the CD spectra of the desensitized enzyme as a function of pH in the range of 1-11. At pH 11.0, the CD spectrum below 215 nm could not be recorded because of the high absorbance due to the OH^- ions (Visser and Blout, 1971). It can be seen that as the pH is changed from neutrality in either direction, the helical content decreases. The decrease in the acid side was more pronounced. The spectra of the desensitized enzyme at pH 4.2, 6.5, 7.0 and 11.0 returned to the spectrum at pH 7.0 suggesting that the conformational change in this pH range was reversible. However, decreasing the pH to below 4.2 caused irreversible denaturation as indicated by the observation that the original spectrum was not obtained on readjusting the pH to 7.0. The helical content at different pH values and the activity of the enzyme at that pH and after adjusting to pH 9.2, which is the optimal pH for the enzyme activity are shown in table 2. It can be seen that the recovery of activity correlates well with the recovery of the helical content.

Effect of urea and sodium dodecyl sulphate on the helical content and activity of the desensitized enzyme

It can be seen from figure 3 that the addition of urea causes a decrease in the helical content of the desensitized enzyme. From the inset, it can be seen that the loss of helicity correlates with the loss of enzyme activity, complete loss occurring when the helical content decreases to about 2%.

Table 2. Effect of pH on the helical content and activity as well as stability of the desensitized enzyme.

pH	Helical content (%)	Activity	
		Before[a]	After adjusting to pH 9.2[b]
7.5	12	27	100
6.5	9	0	90
5.6	8	0	86
4.2	6	0	80
1.0	1	0	0
9.0	12	100	100
11.0	7	0	100

[a] The enzyme activity was assayed at the pH indicated
[b] The enzyme was adjusted to pH 9.2 and assayed for activity.

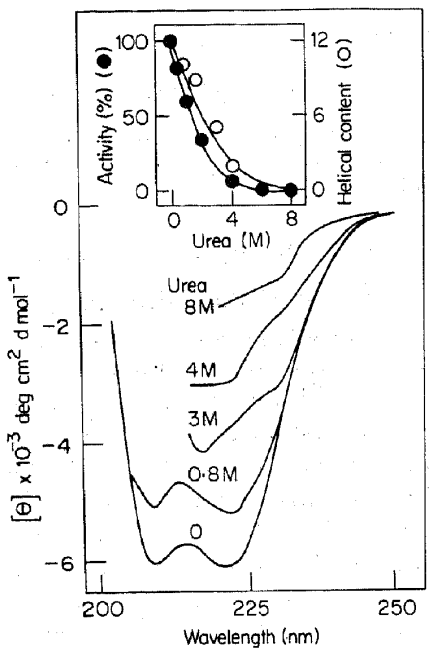

Figure 3. Changes in the CD spectra of the desensitized nucleotide pyrophosphatase on the addition of urea. To the desensitized enzyme (55 μg) at pH 7.4, calculated amounts of urea were added to obtain 0.8, 3.0, 4.0 and 8.0 M and the spectra were recorded. The pH change on the addition of urea was marginal and a value of 7.2 was reached at 8 M urea. Volume changes consequent to the addition of urea were measured and corrected for.
Inset represents the correlation between change in the helical content (calculated according to the method of Chen and Yang (1971) and the catalytic activity at the different concentrations of urea (0-8M). The activity in the absence of urea was normalised to 100 and the activity at the various concentrations of urea were expressed as per cent of this activity.

The effect of sodium dodecyl sulphate (0.11, 0.32 and 0.53%) on the conformation of the enzyme as monitored by the changes in the CD spectrum of the desensitized enzyme, showed a significant change in the wavelength region (205-225 nm). The absence of characteristic features in the CD spectra of the desensitized enzyme made it difficult to assign specific conformational changes, except to say that sodium dodecyl sulphate causes a change in the conformation of the protein.

Stability towards denaturants

Alterations in the structure of proteins have been monitored by comparing the profiles of the loss of catalytic activity on subjecting the enzyme to denaturation by heat and urea (Wang and Tu, 1969; Clark and Yielding, 1971; Weitzman, 1966) inactivation by EDTA (Ravindranath and Appaji Rao, 1968; Balakrishnan, *et al*., 1974) and proteolytic digestion (Carney, *et al*., 1978; Murakami and Murachi, 1978; Schultz and Colowick, 1969; Yamato and Murachi, 1979).

It can be seen from the figure 4 that while the Blue Sepharose-desensitized enzyme was stable at 50° C for 30 min, the native dimer, monomer and tetramer forms of the enzyme progressively lost the activity with time of preincubation at 50°C. The monomer was most susceptible to inactivation losing complete activity in 15 min., whereas 25 and 30 min were needed to completely inactivate the tetramer and the native dimer, respectively.

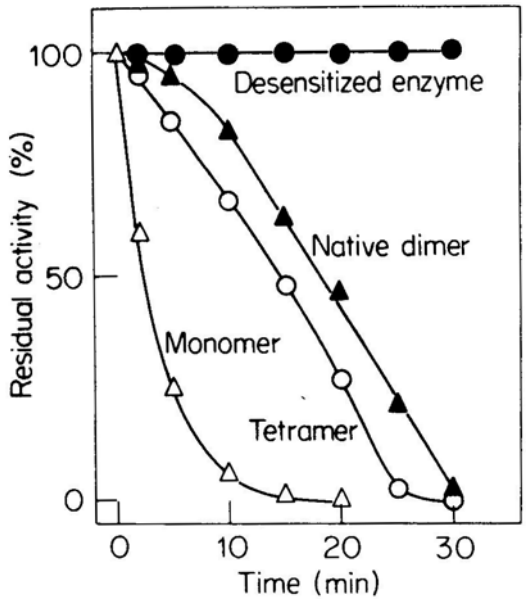

Figure 4. Effect of heating on the activity of the native dimer (▲) tetramer (O), monomer (Δ) and the desensitized (●) forms of the mung bean nucleotide pyrophosphatase. The native dimmer (12 μg), tetramer (20μg), monomer (20 μg), and the desensitized enzyme (30 μg) were heated at 50°C. At time intervals indicated in the figure, aliquots were withdrawn and rapidly cooled to 0°C. Activity of the various forms of the enzyme kept at 0°C for 30 min and assayed at 37°C was normalized to 100 and the residual activity is expressed as per cent of this value.

The native dimeric form of the nucleotide pyrophosphatase was maximally inhibited at 6 M urea and further increase upto 8 M caused no additional inhibition (figure 5). The monomer, tetramer and the desensitized dimer were also inactivated by increasing concentrations of urea and complete loss of activity occurred at 4.5, 6 and 4 M respectively. The native dimer and the desensitized enzyme could be renatured by removal of urea whereas the monomer and the tetramer were irreversibly inactivated.

The mung bean nucleotide pyrophosphatase like several other pyrophosphatases was shown to be a zinc containing protein (Pattabhiraman *et al*., 1964; Cabib *et al*., 1965; Sonnino *et al*., 1966; Corder and Lowry, 1969; Brown and Reichard, 1969; Ravindranath and Appaji Rao, 1968). The inhibition by increasing concentrations of EDTA of the activity of the native dimeric, monomeric, tetrameric and the desensitized forms of the enzyme are shown in figure 6. The desensitized enzyme was the most sensitive to EDTA inhibition as indicated by the concentration of EDTA required for 50% inhibition (3.5 μM) compared to 35, 32, and 25 μM required for the tetramer, native dimer and the monomer respectively.

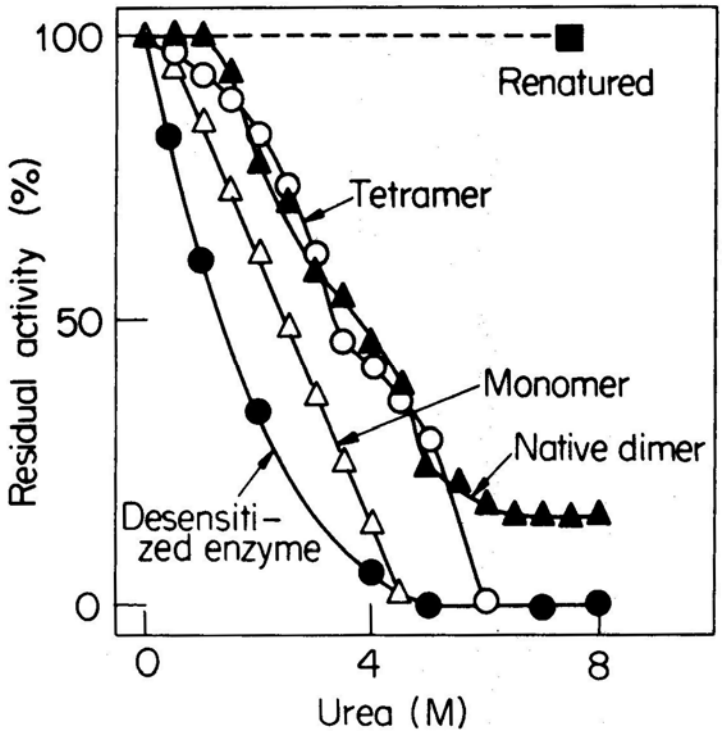

Figure 5. Loss of catalyticactivity of native dimeric, tetrameric, monomeric and the desensitized forms of the mung bean nucleotide pyrophosphatase on treatment with urea. The four enzyme forms were assayed at different concentrations of urea (indicated in the figure) in the reaction mixture. Activity in the absence of urea was normalized to 100. The activity of the renatured enzyme obtained upon removal of urea from the native dimeric or the desensitized enzyme is shown (■) the figure. This enzyme form obtained from the native dimer was desensitized to AMP regulation.

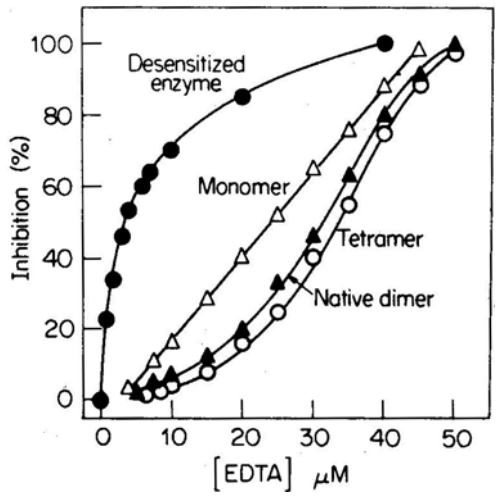

Figure 6. Effect of EDTA on the catalytic activity of the native dimer, tetramer, monomer and the desensitized forms of the mung bean nucleotide pyrophosphatase. The enzymes were assayed in reaction mixtures containing the concentrations of EDTA indicated in the figure. Acivity in the absence of EDTA was normalized to 100.

The EDTA inhibition could be reversed by adding Zn^{2+} . The reactivation profiles of the native dimer, tetramer and the desensitized enzyme are shown in figure 7. However, the inhibition of the monomer could not be reversed by the addition of Zn^{2+} or any other metal ion. Very low concentrations of Zn^{2+} were sufficient to reactivate the desensitized dimer suggesting the active site was more accessible than in the case of the native dimer (table 3).

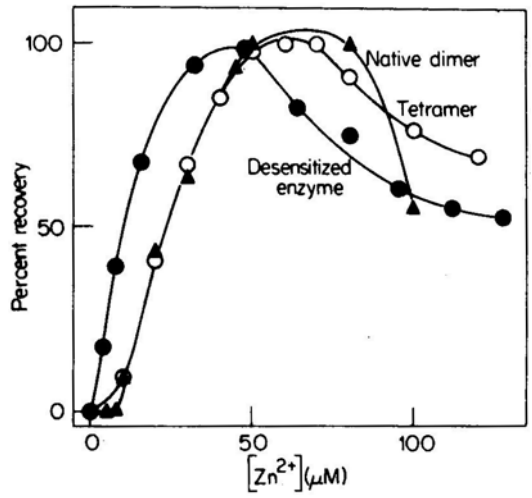

Figure 7. Reactivation of the apoenzyme by Zn^{2+}. The native dimeric, tetrameric and the desensitized forms (20 μg each) of the enzyme were incubated with EDTA (l mM) and excess EDTA removed by passage through a Sephadex G-25 column. The apoenzymes thus obtained were reactivated by assaying for activity in reaction mixtures containing concentrations of Zn^{2+} in the range of 0-150 μM . Activity of the enzyme before the addition of EDTA was normalized to 100 and the apoenzymes had no detectable activity.

Table 3. Physico-chemical and kinetic properties of the native dimeric, monomeric, urea-denatured and renatured, tetrameric and the Blue Sepharose-treated forms of mung bean nucleotide pyrophosphatase.

Property	Monomer	Native dimer	Urea denatured and renatured	Blue Sepharose	Tetramer
Molecular weight	35,000	65,000	65,000	65,000	130,000
Electrophoretic mobility	0.82	0.32	ND	0.63	0.79
Time course	Linear	Biphasic	Linear	Linear	Linear
pH optimum	9.4	9.2-9.4	9.2-9.4	9.3	9.4
Temperature optimum	37°C	49°C	49°C	67°C	49°C
K_m for FAD (mM)	0.50	0.33	1.8	0.33	0.58
V_{max} for FAD	7.0	3.3	8.0	24	2.5
AMP regulation (high affinity site)	−	+	−	−	-
AMP inhibition (low affinity site)	+	+	+	+	+
K_i values for AMP (mM)	0.7	0.1 (competitive)	ND	0.1 (competitive)	0.64
K_i for ADP (mM)	ND	2.2	ND	0.5-1.0	ND
K_i for ATP (mM)	ND	0.26 (non-competitive)	ND	0.88 (Partial non-competitive)	ND
EDTA inhibition (conc. required for 50% inhibition) (μM)	25	32	ND	3.5	35
Zn^{2+} reactivation (conc. required for 50% reactivation) (μM)	ND	21	ND	9	21
Urea denaturation (conc. required for 50% inhibition) (M)	2.5	3.7	ND	1.3	3.4
Ability to be reversibly denatured by urea	−	+	+	+	−
Helical content (%)	9	63	ND	12	Nil
Heat inactivation (time required for 50% inactivation at 50°C) (min)	3	18	ND	Not inactivated	14
Susceptibility to proteolysis (time required for 50% inactivation)					
(a) Trypsin (min)	10	35	ND	Not inactivated	15
(b) Chymotrypsin (min)	15	34	ND	Not inactivated	20

+ Present; – Absent; ND Not determined

It can be seen from figures 8 and 9, that the desensitized enzyme was refractory to proteolytic digestion, whereas the activities of the other forms of the enzyme were rapidly lost. The absence of the release of detectable amounts of trichloro acetic acid-soluble, ninhydrin-positive material at the end of the incubation of the

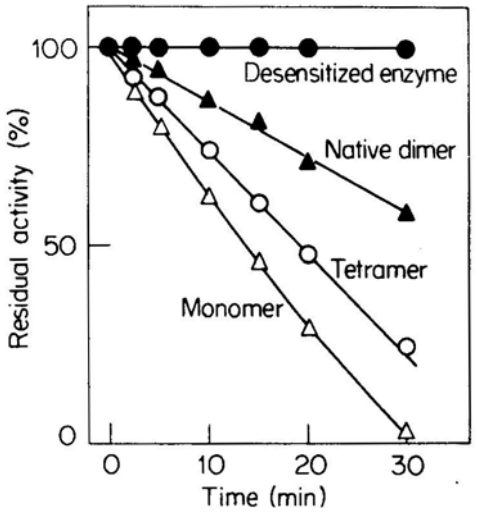

Figure 8. Effect of chymotrypsin on the catalytic activity of the native dimer, tetramer, monomer and the desensitized forms of the mung bean nucleotide pyrophosphatase. The native dimer (250 μg), tetramer (250 μg), monomer (250 μg), or the desensitized enzyme (100 μg) were incubated with 5 μg of chymotrypsin in Tris-HCl buffer pH 8.0 at 37°C in a total volume of 1 ml and aliquots were withdrawn at time interval s indicated and assayed for catalytic activity.

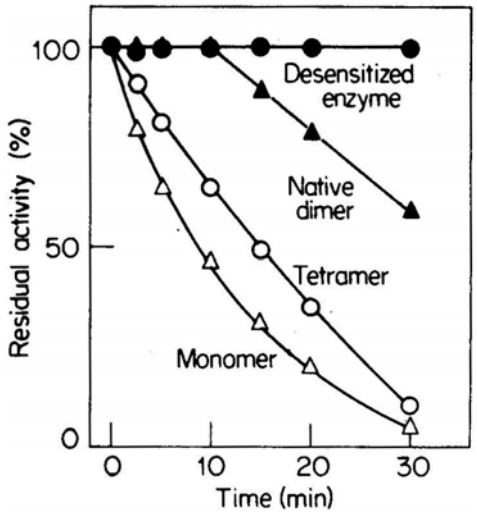

Figure 9. Effect of trypsin on the catalytic activity of the various forms of the enzyme. The native dimer, tetramer, monomer (250 μg each) or the desensitized enzyme (100 μg) were incubated with 5 μg of tripsin in Tris-HCl buffer, pH 8.0 at 37°C in a total volume of 1.0 ml. Aliquots portions of the reaction mixture were withdrawn at time intervals indicated in the figure and assayed for nucleotide pyrophosphatase activity.

desensitized enzyme with trypsin or chymotrypsin suggested that proteolysis was not occurring. The possibility that the desensitized enzyme was functioning as a protease inhibitor was ruled out by assaying for proteolytic activity of trypsin and chymotrypsin using casein as substrate in the presence of the desensitized enzyme. The observation that an enzyme can become protease-resistant as a consequence of a change in its structure has been documented in the case of fumarase (Yamoto and Murachi, 1979).

Discussion

Although conformational changes in proteins on binding of ligands are well documented, comparatively less information is available on the effect of desensitization of regulatory enzymes on their conformation. The availability of native and desensitized forms of mung bean nucleotide pyrophosphatase enabled us to probe into these effects.

The addition of AMP brought a marked change in the CD spectrum of the native enzyme and also a change in its kinetic properties (decrease in V_{max} from 3.3 to 2.5, increase in K_m from 0.25 mM to 0.58 mM, loss of inhibition at low concentraions of AMP and the loss of ability to be reversibly denatured by urea). The Blue Sepharose desensitized enzyme showed difference in its kinetic properties and the CD spectrum when compared with the native enzyme (V_{max} increased from 3.3 to 24, K_m remained unaltered, temperature optimum increased from 49° to 67° C and inhibition by low concentrations of AMP was lost). The desensitization of the high-affinity binding site for AMP was confirmed by the absence of any change in the CD spectrum of the desnsitized enzyme on the addition of AMP. In the case of rabbit muscle phosphorylase, small differences in the conformation were observed when phosphorylase *b* was converted to phosphorylase *a* (Graves and Wang, 1972). Changes in the catalytic activity in the absence of regulators, decrease in solubility and stability to cold denaturation were also observed (Graves and Wang, 1972). The conformational changes in the desensitized enzyme brought about by changes in the pH suggested that there was good correlation between the helical content and activity.

The stability of the desensitized enzyme compared to that of the native enzyme showed that using certain parameters, the stability was enhanced but with others it decreased. The desensitized enzyme was more stable than the native enzyme towards heat similar to the observation with citrate synthase (Weitzman, 1966), phosphorylase *b* (Wang and Tu, 1969) and glutamate dehydrogenase (Clark and Yielding, 1971). The stability to proteolytic digestion markedly changed on desensiization, reminiscent of the observation with phosphorylase *b* (Carney *et al*., 1978).

While the sensitivity to urea denaturation was decreased in the case of citrate synthase (Weitzman, 1966) and phosphorylase *b* (Wang and Tu, 1969), the sensitivity was increased in the case of nucleotide pyrophosphatase.

Based on the results presented in this paper and from our earlier work on the enzyme (Ravindranath and Appaji Rao, 1968; 1969; Balakrishnan *et al*., 1974,1975, 1977; Reddy *et al*., 1979), we postulate the following model to explain the regulation of mung bean nucleotide pyrophosphatase by AMP (figure 10).

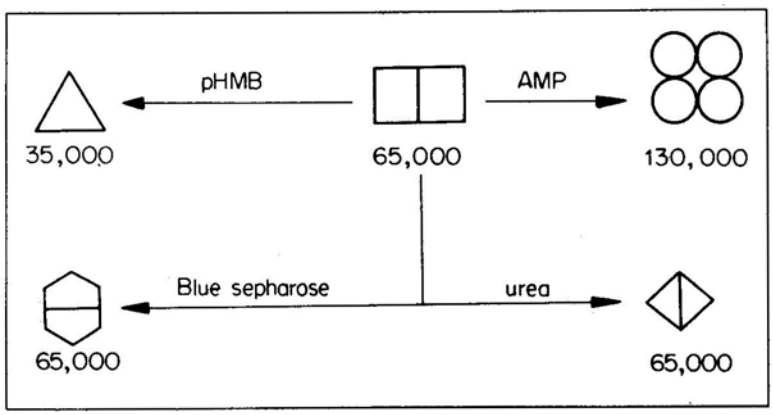

Figure 10. The model describing the mechanism of the regulation of the activity of the mung bean nucleotide pyrophosphatase.

The enzyme as isolated from the germinated seedlings was a dimmer, ⊡ (Ravindranath and Appaji Rao, 1968, 1969), which became associated to form a tetramer on the addition of AMP, ⊠ (Balakrishnan *et al*., 1975) and was dissociated into a monomer by the addition of *p*-hydroxymercuribenzonate, Δ (Balkrishnan *et al*., 1977). Urea denaturation and renaturation by the removal of urea, resulted in the conversion of the native dimer to another dimeric form with an altered conformation, ◈ (Balakrishnan *et al*., 1974).The enzyme purified by using Blue sepharose column, resulted in the isolation of yet another dimeric form, ⊖ (Reddy *et al*., 1979). All the enzyme forms were catalytically active but the monomer,tetramer, urea-denatured-renatured dimer and the Blue Sepharose-dimer were insensitive to the allosteric regulation by AMP, unlike the native dimer. The physicochemical and kinetic properties of these various forms of mung bean nucleotide pyrophosphaase are listed in table 3 and lends support to the model (figure 10) proposed for AMP regulation.

The native dimer catalyzed the hydrolysis of FAD with an initial fast rate followed by a second slower rate. This observation suggested that the enzyme was being converted to another stable form during the course of the reaction. Addition of AMP (6 μM) before the start of the reaction abolished the initial fast rate and the reaction now followed the second slower rate (Ravindranath and Appaji Rao, 1969). Concentrations of AMP below 1 μM were without effect and in between 6 and 40 μM inhibited the enzyme activity to a constant value of 20%. Increasing the concentration of AMP above 40 μM inhibited the enzyme activity linearly and complete inhibition was observed at about 1 mM AMP. These results suggested the possibility of two classes of sites for AMP binding on the enzyme – a high affinity site responsible for the regulation by AMP and a low-affinity site for product inhibition. The formation of a stable enzyme form in the presence of AMP was indicated by the change observed in the electrophoretic mobility (0.33 for the native dimer and 0.79 for the tetramer). It was later shown that AMP converted the native dimer (M_r = 65,000) into a tetramer (M_r = 130,000)whose pH andtemperature

optima were unaltered (Balakrishnan *et al*., 1977). The K_m value of FAD was not altered but the V_{max} decreased from 3.3 to 2.5, for the native dimer and tetramer, respectively. Urea (6 M) irreversibly denatured the tetramer but could reversibly denature the native dimer. The changes in the structure were reflected by the decrease in the helical content, the time required for 50% inactivation by heat and the susceptibility to proteolytic digestion by trypsin and chymotrypsin, when the native dimer was converted to a tetramer by AMP (figures 1, 4, 8, 9 and tables 1 and 3).

Dissociation of the native dimer was brought about by treatment with *p*-hydroxymercuribenzoate and the resulting monomer had a M_r of 35,000 and functioned with the initial fast rate of FAD hydrolysis observed for the native dimer. The observation that low concentrations of AMP could neither inhibit the rate nor convert the monomer to a dimer or tetramer confirmed the hypothesis that the enzyme was desensitized to allosteric interactions of AMP on dissociation. The electrophoretic mobility of the monomer was 0.82 as compared to 0.32 for the native dimer (table 3). Although the pH optima for both the enzymes were identical, they differed in their temperature optima (37°C and 49°C, for the monomer and native dimer, respectively), suggesting that dissociation had made the enzyme more unstable. This conclusion was reaffirmed by the observations that there was a decrease in the helical content; time required for 50% inactivation by heat at 50°C and susceptibility to proteolytic digestion by trypsin and chymotrypsin of the enzyme on dissociation (table 3). The K_m values for both the forms were same but they differed in their $V_{\max}$ 3.3 (dimer) to 7.0 (monomer). Our attempts at interconverting the monomeric, native dimeric and tetrameric forms of the enzyme were unsuccessful.

An altered dimeric form was obtained when the native one was subjected to urea (6 M) denaturation and renaturation by the removal of urea. The altered form had M_r of 65,000, catalyzed the FAD hydrolysis with a linear time-course and low concentrations of AMP could not convert this form to a tetramer. There was a concomitant increase in the K_m and $V_{\max}$ values of the altered enzyme. Sodium dodecyl sulphate gel electrophoreses of the native dimer, tetramer, monomer and the urea-denatured-renatured form elicited a single band (M_r 35,000) suggesting that all the enzyme forms hitherto mentioned were made up of identical subunits.

Yet another method of desensitizing the native dimer is by its interaction with Cibacron Blue F3GA. This interaction had no effect on the molecular weight and pH optimum, but increased the $V_{\max}$ from 3.2 to 24 and the temperature optimum 49°C to 67°C. A similar increase of $V_{\max}$ on desensitization of regulatory enzymes is well documented with other allosteric enzymes (Gerhart and Pardee, 1962; Mangiarotti and Pontremoli, 1963). The altered conformation of this desensitized dimer was indicated by its refractiveness to inactivation by heating at 50° C for 30 min. and proteolytic digestion by trypsin and chymotrypsin (figures 4, 8 , 9 and table 3). In contrast to these observations, there was a decrease in the concentrations required for 50% inhibition by urea and EDTA (3.6 M and 32 μM for the native dimer and 1.3 M and 3.5 μM for the desensitized enzyme, respectively).

The model (figure 10) highlights the regulation of mung bean nucleotide pyrophosphatase by AMP. Although the monomer, desensitized dimer and tetramer have lost

the ability to interact with low concentrations of AMP, the site for product inhibition is still retained by all of them indicating that they function by similar catalytic mechanism.

The results emphasize that multiple mechanisms are operative in desensitization of regulatory proteins.

References

Adler, A. J., Greenfield, N. J. and Fasman, G. D. (1973) *Methods Enzymol*., **27,** 675.
Balakrishnan, C. V., Ravindranath, S. D. and Appaji Rao, N. (1974) *Arch. Biochem. Biophys*., **164,** 156.
Balakrishnan, C. V., Ravindranath, S. D. and Appaji Rao, N. (1915) *Arch. Biochem. Biophys*., **168,** 163.
Balakrishnan, C. V., Vaidyanathan, C. S. and Appaji Rao, N. (1977) *Eur. J. Biochem*., **78,** 95.
Brown, N. C. and Reichard, P. (1969) *J. Mol. Biol*., **46,** 39.
Cabib, E., Carminatti, H. and Woyskovsky, N. M. (1965) *J. Biol. Chem*., **240,** 2114.
Carney, I. T., Beynon, R. J., Kay, J. and Birket, N. (1978) *Biochem. J*., **175,** 105.
Chan, W. W. C., and Enns, C. A. (1978) *Can. J. Biochem*., **56,** 654.
Chen, Y. H., and Yang, J. T. (1971) *Biochem. Biophys. Res. Commun*., **44,** 1285.
Clark, C. E., and Yielding, K. L. (1971) *Arch. Biochem. Biophys*., **143,** 158.
Corder, C. N., and Lowry, O. H. (1969) *Biochim. Biophys. Acta*, **191,** 579.
Gerhart, J. C. and Pardee, A. B. (1962) *J. Biol Chem*., **237,** 891.
Graves, D. J. and Wang, J. H. (1972) *The enzymes*, ed. P. D. Boyer, **7,** 435.
Greenfield, N., Davidson, B. and Fasman, G. D. (1967) *Biochemistry*, **6,** 1630.
Greenfield, N. and Fasman, G. D. (1969) *Biochemistry*, **8,** 4108.
Lowry, O. H. Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem*., **193,** 265.
Mangiarotti, G. and Pontremoli, S. (1963) *Biochem. Biophys. Res. Commun*., **12,** 305.
Mihalyi, E. (1972) *Application of proteolytic enzymes to protein structure studies* (Cleveland, Ohio: Chemical Rubber Company Press)
Murakami, T. and Murachi, T. (1978) *J. Biochem*. (Tokyo), **84,** 83.
Pattabhiraman,T.N., SekharaVarma,T.N. and Bachhawat, B. K. (1964) *Biochim. Biophys.Acta*, **83,** 74.
Ravindranath, S. D. and Appaji Rao, N. (1968) *Indian J.Biochem*., **5,** 137.
Ravindranath, S. D. and Appaji Rao, N. (1969) *Arch. Biochem. Biophys*., **131,** 54.
Reddy, A. R.V., Ananathanarayanan, V. S. and Appaji Rao, N. (1979) *Arch. Biochem. Biophys*., **198,** 89.
Schultz, I. T. and Colowick, S. P. (1969) *J. Biol. Chem*., **244,** 2306.
Sonnino, S., Carminatti, H. and Cabib, E. (1966) *Arch. Biochem. Biophys*., **116,** 26.
Visser, L. and Blout, E. R. (1971) *Biochemistry*, **10,** 743.
Wang, J. H. C. and Tu, J. I. (1969) *Biochemistry*, **8,** 4403.
Weitzman, P. D. J. (1966) *Biochem. J*., **101,** 44C.
Yamato, S. and Murachi, T. (1979) *Eur. J. Biochem*., **93,** 189.