Kinetic and structural analysis of the ultrasensitive behaviour of cyanobacterial ADP-glucose pyrophosphorylase

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The kinetic and (supra)molecular properties of the ultrasensitive behaviour of ADP-glucose pyrophosphorylase (AGPase) from *Anabaena* PCC 7120 (a cyanobacterium) were exhaustively studied. The response of the enzyme toward the allosteric activator 3-phosphoglycerate (3PGA) occurs with ultrasensitivity as a consequence of the cross-talk with the inhibitor P_i. Molecular 'crowding' renders AGPase more sensitive to the interplay between the allosteric regulators and, consequently, enhances the ultrasensitive response. In crowded media, and when orthophosphate is present, the activation kinetics of the enzyme with 3PGA proceed with increased co-operativity and reduced affinity toward the activator. Under conditions of ultrasensitivity, the enzyme's maximal activation takes place in a narrow range of 3PGA concentrations. Moreover, saturation kinetics of the enzyme with respect to its substrates, glucose 1-phosphate and ATP, were different at low or high 3PGA levels in crowded media. Only under the latter conditions did AGPase exhibit discrimination between low or high levels of the activator, which increased the affinity toward the substrates and the maximal activity reached by the enzyme. Studies of fluorescence emission of tryptophan residues, fourth-derivative spectroscopy and size-exclusion chromatography indicated that the ultrasensitive behaviour is correlated with intramolecular conformational changes induced in the tertiary structure of the homotetrameric enzyme. The results suggest a physiological relevance of the ultrasensitive response of AGPase *in vivo*, since the enzyme could be subtly sensing changes in the levels of allosteric regulators and substrates, and thus determining the flux of metabolites toward synthesis of storage polysaccharides.

Key words: *Anabaena* PCC 7120, enzyme structure and regulation, glycogen and starch biosynthesis, molecular crowding, ultrasensitivity.

INTRODUCTION

ADP-glucose pyrophosphorylase (ATP:α-D-glucose-1-phosphate adenylyltransferase; EC 2.7.7.27; AGPase) catalyses the regulatory step in the biosynthesis of glycogen in bacteria and starch in higher plants [1,2]. This enzyme mediates the production of ADP-glucose and PP_i from glucose 1-phosphate and ATP, and the product, ADP-glucose, serves as the activated glucosyl donor in α-1,4-glucan synthesis [1]. Studies based on a wide range of sources have shown that AGPase is generally modulated by allosteric effectors, which are key intermediates of the major pathway of carbon assimilation in the respective organism [2].

AGPase from cyanobacteria constitutes a good experimental system to study the relationships between structure and function of the protein, because it has properties intermediate between those of bacteria and higher plants [3]. The enzymes from cyanobacteria and higher plants have similar specificities for 3-phosphoglycerate (3PGA; allosteric activator) and P_i (inhibitor). However, the enzyme from cyanobacteria is homotetrameric in structure, as are those of other bacteria [3].

The structural and kinetic properties of AGPase from different organisms have been previously studied [1,2], though these studies were performed with low enzyme concentrations in aqueous media. It has been reported [4–6] that enzymes such as AGPase operate *in vivo* at higher concentrations than those used *in vitro*. Furthermore, intracellular conditions resemble those of molecularly crowded environments [5,7–9]. Recently [10,11] we have shown that the regulatory properties of AGPase from the cyanobacterium *Anabaena* PCC 7120 are highly affected by molecular-crowding conditions mimicking those occurring in the cell. Major changes were observed in a media 'crowded' by the presence of poly(ethylene glycol) (PEG). One of the aims of the present study was to analyse the kinetic and structural properties of AGPase comparatively in aqueous and crowded media. We sought a better understanding of the molecular basis for the differences in the regulatory behaviour of the enzyme elicited by PEG.

Media containing large amounts of macromolecules have been described as 'crowded' [7,12,13]. The addition of neutral hydrophilic solutes (i.e. PEG) mimicks molecular crowding and promotes specific protein–protein interactions [7,13]. In crowded media, protein aggregation is promoted through its exclusion from the solvent and the consequent local increase in protein concentration [7,13]. The kinetic and structural properties of oligomeric proteins may be affected by both homologous and heterologous protein–protein interactions [8,14]. Previous experimental evidence exists which shows that the presence of PEG increases the activity of a variety of enzymes [12,15].

Our own previous data show that PEG increases AGPase activity approx. 2-fold in the presence of 3PGA, whereas a 5-fold inhibition of the enzyme activity was observed in the absence of the activator [10]. Very recently [11] we have reported that the interplay between the allosteric effectors (3PGA and P_i) of AGPase, and PEG-elicited molecular crowding, trigger an ultrasensitive phenomenon in the cyanobacterial AGPase. The enzyme exhibiting ultrasensitivity responds very sensitively to small changes in its allosteric effectors in crowded media. An ultrasensitive behaviour indicates higher responsive properties of

Abbreviations used: AGPase, ADP-glucose pyrophosphorylase; 3PGA, 3-phosphoglycerate; PEG, poly(ethylene glycol); DTT, dithiothreitol.

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an enzyme than the one expected from the classical hyperbola of Michaelis–Menten kinetics [11,16–18]. This amplification mechanism enables the enzyme to: (i) discriminate small changes in an effector above a certain background, and (ii) to increase several-fold the flux in a narrow range of change in substrate or effector concentrations. It was demonstrated that allosteric enzymes exhibiting ultrasensitive behaviour give higher amplification responses than those proteins showing allosterism without sensitivity amplification [11,16–18]. Remarkably, analysis of ultrasensitive systems has emerged as a key tool for the development of the quantitative era in the study of metabolic processes [19].

In the present study we analysed the kinetic and (supra)-molecular properties of the ultrasensitive behaviour of AGPase from *Anabaena* PCC 7120. The effects of PEG-induced molecular crowding on the ultrasensitive behaviour exhibited by the enzyme were also investigated. The physiological relevance of the results obtained are discussed in terms of the *in vivo* regulation of bacterial glycogen and plant starch synthesis.

EXPERIMENTAL

Reagents

[^{14}C]Glucose 1-phosphate and [^{32}P]PP$_i$ were purchased from DuPont–NEN. Unlabelled glucose 1-phosphate, ATP, ADP-glucose, 3PGA, PP$_i$, inorganic pyrophosphatase, alkaline phosphatase, PEG 8000, BSA and rabbit liver glycogen were from Sigma. All other reagents were of analytical grade.

Bacterial strains and growth media

Bacterial strains used in the present study were generously provided by Professor Jack Preiss (Department of Biochemistry, Michigan State University, East Lansing, MI, U.S.A.). *Escherichia coli* AC70R1-504, which has no AGPase activity, was used for the expression of the *Anabaena* AGPase gene, as previously described [20]. *E. coli* AC70R1-504 cells were grown in enriched medium containing 1.1 % K_2HPO_4.

Enzyme purification

Recombinant enzyme resulting from the expression of the gene encoding AGPase from *Anabaena* PCC 7120 in *E. coli* AC70R1-504 was purified to electrophoretic homogeneity essentially as previously described in [20,21].

Enzyme assay

AGPase activity was determined in the ADP-glucose synthesis direction at different saturation conditions of the substrates ATP and glucose 1-phosphate, in the absence or in the presence of the indicated concentrations of the allosteric effectors (3PGA and P$_i$) and different concentrations of PEG or its absence. Unless otherwise specified, the standard reaction mixture contained 20 μmol of Mops/KOH, pH 7.5, 1.25 μmol of $MgCl_2$, 0.3 unit of inorganic pyrophosphatase, 0.5 μmol of ATP and 0.1 μmol of glucose 1-phosphate (sp. radioactivity 9.9×10^6 c.p.m./μmol) in a final volume of 0.2 ml. Assays were initiated by the addition of enzyme, incubated for 10 min at 37 °C and stopped by heating in a boiling-water bath for 45 s. [^{14}C]ADP-glucose formed was assayed as described previously [22]. One unit of AGPase activity is defined as the amount of enzyme required to produce 1 μmol of ADP-glucose/min at 37 °C. Before assaying, the enzyme was desalted in a Sephadex G-75 column, equilibrated with a buffer containing 20 mM Mops/KOH and 2 mM 2-mercaptoethanol.

Table 1 Kinetic parameters and amplification factor (A_s) for the activation of AGPase by 3PGA in the presence or absence of P$_i$ and in crowded or non-crowded media

The sensitivity amplification factors at 1 % ($A_{s,0.01}$) were obtained as described in [11]. Briefly, the following equation was used in order to calculate A_s:

$$A_{s,0.01} = [(v_{Nf} - v_{Ni})/v_{Ni}]/[(3PGA_f - 3PGA_i)/3PGA_i]$$

where v_{Nf} is the net velocity (determined by subtracting the velocity in the absence of the activator from each of the velocities determined at different 3PGA concentrations; see [11]) in ADP-glucose synthesis at a concentration corresponding to 3PGA$_f$; and v_{Ni} is the rate at a fixed amount of 3PGA$_i$ which corresponds to 1 % of maximal activity. If A_s is greater than 1, the percentage change in the response is greater than the percentage change in the stimulus [16]. For a Michaelian-type enzyme, the A_s values are always less than unity for any finite value of effector. In contrast, for cooperative enzymes, A_s depends on the initial level of stimulus and, for a given value of h, it becomes higher, since the initial stimulus is lower [16,17].

Condition PEG (% w/w)	P$_i$ (mM)	Activation by 3PGA ($A_{0.5}$) (mM)	h	V_{max} (%)	$A_{s,0.01}$
0	0	0.035	1.0	100*	< 0.01
	0.05	0.05	1.3	88	1.4
	0.10	0.07	1.5	88	3.0
	0.25	0.20	1.8	87	4.9
	1.0	1.05	2.3	74	14.9
3	0	0.05	1.2	178	1.2
9	0	0.14	2.0	184	11.1
	0.05	0.18	2.3	98	6.3
	0.10	0.25	2.8	110	15.7
	0.25	0.38	3.0	108	16.4
	1.0	1.50	3.6	112	19.3
15	0	0.19	2.5	130	15.3

* 100% activity corresponds to 36 units per mg of protein.

Kinetic studies

The experimental data were fitted through the generalized Hill equation by a non-linear least-squares regression kinetics computer program [23], and thus used to calculate kinetic parameters V_{max}, $s_{0.5}$, $A_{0.5}$ and $I_{0.5}$ (the last three being the concentrations of substrate, activator and inhibitor giving 50 % of maximal velocity, activation and inhibition respectively) and $3PGA_i$ (see Table 1). All kinetic parameters are the means of at least three determinations and are reproducible to within at least ± 10 %.

Protein quantification

Total protein was determined by the modified method of Lowry described in [24], using BSA as standard.

Gel-filtration chromatography

Size-exclusion chromatograpy was performed on both a Pharmacia Superose B-12 column and a Sephacryl S300 (1.5 cm × 32 cm) column, plugged to a FPLC® system (Pharmacia) at room temperature. Columns were equillibrated with 50 mM Mops/KOH (pH 7.5)/1 mM dithiothreitol (DTT) and 10 mM KCl in the absence or in the presence of 9 % PEG 8000. The flow rate was 0.4 ml/min and the sample volume was 200 μl. The elution of AGPase was monitored at 280 nm. Columns were calibrated with the following standard proteins (all purchased from Sigma Chemical Co.): thyroglobulin (669 kDa), apoferritin (443 kDa), β-amylase (200 kDa), alcohol dehydrogenase (150 kDa) and BSA (66 kDa). The void volume was determined with Blue Dextran.

Fourth-derivative spectroscopy of AGPase

Fourth-derivative spectroscopy was performed with a Hitachi U-2000 or a Spectronic Genesys 2PC UV–visible spectrophotometer, using a slitwidth of 2 nm and a scan rate of 10 nm/min as previously described [25]. Before measurement, samples were filtered through a 0.22-μm-pore-size filter membrane (Millipore). Triplicates from each sample were analysed at 37 °C. Fourth-derivative spectra were numerically calculated with the built-in program provided with the spectrophotometer.

Fluorescence studies

Fluorescence emission spectra were obtained with a spectrofluorimeter Bio-Tek Kontron SFM 25 (Kontron Instruments, Zürich, Switzerland). The intrinsic fluorescence of tryptophan was measured using an excitation wavelength of 295 nm (slitwidth 5 nm). The emission spectrum was analysed within the 305–400 nm range. Before assay, samples were filtered through a 0.22-μm-pore-size filter membrane (Millipore). Each spectrum was recorded in triplicate at 37 °C.

RESULTS

Ultrasensitive response and allosteric regulation of AGPase in crowded medium

It was previously shown that positive co-operativity was the basic biochemical mechanism through which AGPase achieved ultrasensitivity [11]. Supposedly the positive co-operativity arises through the fine interplay between both allosteric effectors of AGPase, 3PGA and P_i. Moreover, PEG-induced molecular crowding provides a level of further regulation by amplifying the enzyme's response [11]. Although the highest amplification factor attained by the enzyme was achieved with a combination of 1 mM P_i and 9 % PEG, the latter was able by itself to trigger the AGPase ultrasensitive response (Figure 1; Table 1). In the zero-order regime of AGPase for its substrates, 1 mM P_i and 9 % PEG (either together or separately) induced amplification factors ranging from 11- to 19-fold (Table 1). Amplification factors were even improved in the first-order domain of AGPase operation [11].

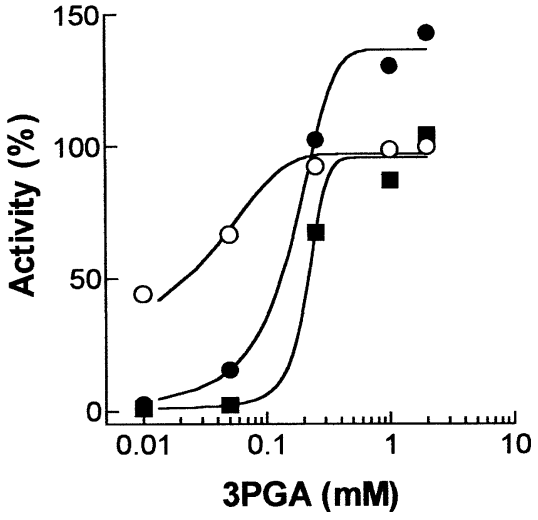

Figure 1 Effect of PEG-induced molecular crowding on the activation of AGPase by 3PGA

Assays were carried out using about 25 ng of pure enzyme in aqueous medium (○), or in the presence of 9% (●) or 15% (■) (w/w) PEG 8000.

Since AGPase could be sensing the molecular-crowding status of the intracellular environment, we decided to investigate more thoroughly the regulatory properties of the enzyme in the presence of PEG. AGPase activity was affected by PEG 8000 in a concentration-dependent manner [10]. When assayed in the presence of 3PGA, maximal activation of the enzyme was found at 5–10 % (w/v) PEG; and, contrarily, in the absence of 3PGA, PEG inhibited the enzymic activity approx. 5-fold. The combined effects of 3PGA and PEG on the kinetic behaviour of AGPase are shown in Figure 1. A remarkable result is that the enzyme activity changes from a hyperbolic to a sigmoidal type of response as a function of 3PGA concentration, in the presence of PEG (Figure 1; note the logarithmic scale for 3PGA concentrations). The sigmoidal response of AGPase was apparent in the presence of 9 % or 15 % (w/w) PEG (Figure 1 and Table 1), although not at 3 % (Table 1). In fact, the Hill coefficient, h, increased 2-fold, along with an 80 % increase in V_{max} in the presence of 9 % PEG with respect to its absence (Table 1). At lower concentrations of PEG or in its absence, AGPase did not exhibit the sigmoidal response with respect to 3PGA, yet a significant increase in V_{max} was already noticeable at 3 % PEG (Table 1). As a matter of fact, amplification factors with values of 11 or 15 were obtained in media containing 9 or 15 % PEG respectively ([11] and the present Table 1). Only an 11- or 6-fold increase of 3PGA in the presence of 9 and 15 % PEG, respectively, as compared with the 200-fold needed to increase AGPase activity from 10 to 90 % in the absence of PEG, were observed (see Table 1 in [11]).

Dependence of AGPase activation by 3PGA upon the presence of P_i and PEG-induced crowding

The regulatory properties of AGPase were also altered by the presence of its allosteric inhibitor, P_i [11]. Previous data showed that the sensitivity of AGPase toward its allosteric activator, 3PGA, is hyperbolic in the absence of P_i and PEG [10]. A significant amplification of AGPase (15-fold) was determined in the presence of 1 mM P_i with respect to 1 % of the maximal velocity of the system (Table 1). Since this ultrasensitive behaviour of AGPase was the consequence of the fine interplay between its allosteric effectors (3PGA and P_i) and the environmental crowding [11], we carried out an exhaustive analysis of the effect of P_i on the kinetic parameters for enzyme activation ($A_{0.5}$ for 3PGA, h), and vice versa (the effect of 3PGA on $I_{0.5}$ for P_i inhibition), as a function of crowding. Results are detailed in Figures 2 and 3.

As can be seen in Figure 2, the kinetics for activation of AGPase by 3PGA were affected as a function of P_i concentration. Changes were evident in both, the activation constant ($A_{0.5}$; Figure 2A) and co-operativity (h; Figure 2B) for 3PGA activation. Both parameters reach higher values in crowded than in aqueous media (Figure 2). Significantly higher co-operativity values were attained by AGPase in crowded media in the presence of P_i. A dependence of the changes occurring in h upon P_i concentration is observed; increasing rapidly at low P_i concentrations and steadily, although with a lower slope, at higher concentrations of the inhibitor (Figure 2B). An increase of h in both crowded and aqueous media was observed at low P_i concentrations (see the inset to Figure 2B). Moreover, significant differences are observed in the activation constant for 3PGA

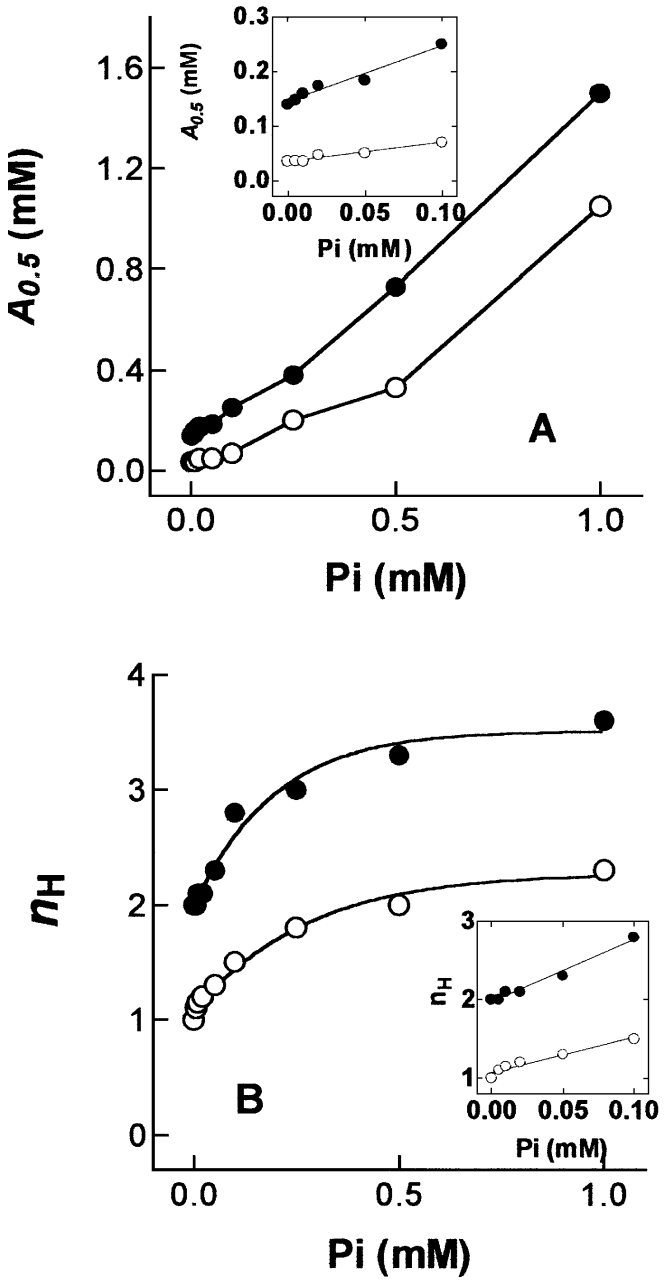

Figure 2 Effect of different P_i concentrations on the activation of AGPase by 3PGA

Activation constants (**A**) and the respective Hill coefficients (*h*, but called 'n_H' on the Figure; **B**) for 3PGA activation were determined at the specified concentrations of P_i in aqueous media (○) or crowded media containing 9% (w/w) PEG (●). Insets show the data obtained at low concentrations of P_i (lower than 0.1 mM) on an expanded scale.

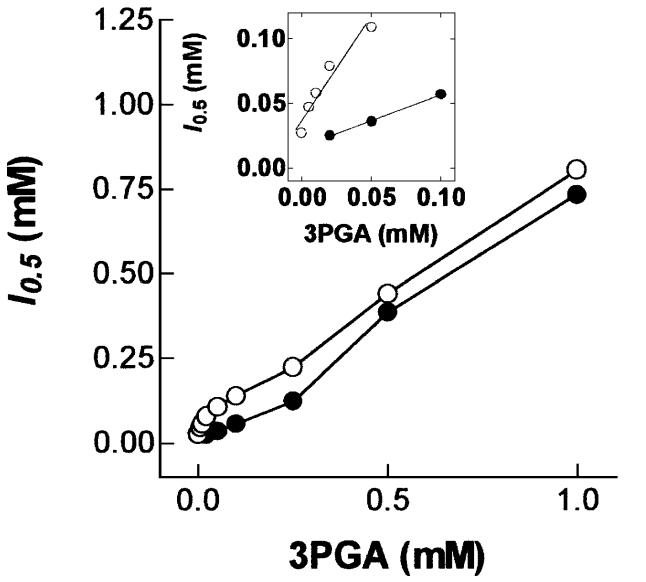

Figure 3 Inhibitory effect of P_i on AGPase at different concentrations of 3PGA

Inhibition kinetics were performed under aqueous conditions (○) or in crowded media (●). The inset shows a more detailed plot of the data obtained at concentrations of 3PGA lower than 0.1 mM.

Table 2 Kinetic parameters for glucose-1-P of AGPase assayed at different ATP concentrations, at two levels of the allosteric activator 3PGA, and in aqueous or crowded media

Conditions			Kinetic parameters for glucose-1-phosphate		
[PEG 8000] (%, w/w)	[ATP] (mM)	[3PGA]	$s_{0.5}$ (mM)	*h*	V_{max} (%)*
0	0.5	Low†	0.032	1.0	64
		High	0.025	1.0	89
	2.5	Low	0.029	1.1	89
		High	0.035	1.2	100
9	0.5	Low	0.08	1.3	5
		High	0.03	1.1	124
	2.5	Low	0.11	0.8	20
		High	0.034	1.2	184

* A V_{max} of 100% corresponds to that obtained in aqueous medium and saturating concentrations of substrates (as described in the Experimental section) and 3PGA (2.5 mM), a rate of 35 μmol of ADP-glucose produced per min. All assays were carried out by using between 10 and 50 ng of purified AGPase in the respective medium.

† Levels of 3PGA in the different media, specified as low or high, correspond to values of 0.05 or 2.5 mM respectively.

either at low (inset to Figure 2A) or high (Figure 2A) concentrations of P_i. At low concentrations, the increment of the $A_{0.5}$ for 3PGA was higher (a larger slope; inset to Figure 2A) in crowded than in non-crowded media.

By inspecting Figure 3, it can be seen that 3PGA in turn diminishes, in a concentration-dependent manner, the inhibition of AGPase by P_i. Clearly, at relatively low concentrations of the allosteric activator (see inset of Figure 3), the inhibition constant for P_i is markedly more affected in the absence of PEG than in its presence. These changes in inhibition constants for P_i on activator levels become independent on crowding conditions at 3PGA concentrations higher than 0.4 mM (Figure 3).

AGPase activity as a function of substrate concentration in aqueous and crowded media

AGPase kinetics in crowded and non-crowded media were analysed in order to study the extent to which the PEG-induced

Table 3 Kinetic parameters for ATP of AGPase assayed at different glucose-1-P concentrations, at two levels of 3PGA, and in aqueous or crowded media

Conditions			Kinetic parameters for ATP		
[PEG] (%, w/w)	[Glucose 1-phosphate] (mM)	[3PGA]	$s_{0.5}$ (mM)	h	V_{max} (%)*
0	0.05	Low†	0.60	1.8	47
		High	0.55	1.0	64
	0.5	Low	0.76	1.3	90
		High	0.40	1.2	100
9	0.05	Low	3.96	1.5	12
		High	0.26	1.3	67
	0.5	Low	1.80	1.3	20
		High	0.38	0.9	185

* A V_{max} of 100% and the amount of enzyme utilized in each assay are defined in Table 2.
† Concentrations of 3PGA for low or high levels are as in Table 2.

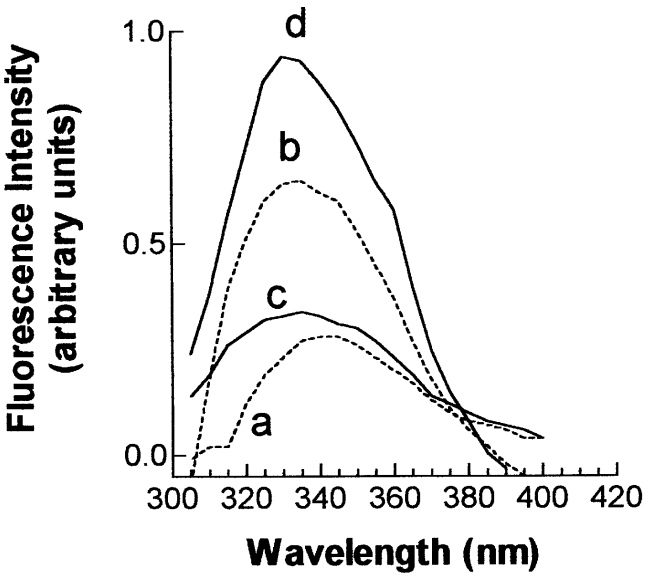

Figure 4 Intrinsic tryptophan emission spectra of AGPase under different conditions

The enzyme was incubated at 37 °C in a solution containing 50 mM Mops/KOH (pH 7.5) plus 1 mM DTT without further additions (curve a); in the presence of 1 mM P_i and 2.5 mM 3PGA (curve b); and with the addition of PEG (9%, w/w) alone (curve c) or plus 1 mM P_i and 2.5 mM 3PGA (curve d). Spectra were recorded as specified in the Experimental section.

crowding affects the kinetic parameters of the enzyme with respect to its substrates (glucose 1-phosphate and ATP). Table 2 shows the kinetic parameters for glucose 1-phosphate determined at low (0.05 mM) or high (2.5 mM) concentrations of 3PGA in media containing saturating levels (2.5 mM) of ATP and in the absence or in the presence of PEG. Interestingly, a comparison between the kinetic data in Table 2, obtained in non-crowded and crowded media, indicates that, under the latter conditions the enzyme was able to: (i) discriminate between high and low levels of 3PGA; and (ii) reach significantly higher maximal activities (at high 3PGA levels) when compared with the results obtained in non-crowded environments. More specifically, AGPase only exhibited low activity values in the presence of micromolar concentrations of 3PGA under crowding conditions, while there was almost no difference between high or low 3PGA in the absence of crowding (Table 2). Similar results on saturation kinetics for glucose 1-phosphate were obtained at low (0.5 mM) concentrations of ATP (Table 2).

This discriminating ability of AGPase toward its allosteric activator in crowded media is relevant, since the latter condition is expected to resemble more the intracellular environment [5,6]. The relevance is confirmed by the fact that it adds to a finer response of the enzyme to allosteric regulation. A similar result was achieved at increasing concentrations of ATP under conditions in which glucose 1-phosphate saturates the enzyme (Table 3). Again, in aqueous media, AGPase exhibited similar saturation kinetics for ATP at different levels of the allosteric activator, whereas the enzyme was able to clearly discriminate between high and low concentrations of 3PGA when assayed under crowding conditions (Table 3). At subsaturating concentrations of glucose 1-phosphate, which simulate physiological situations, closely similar data were obtained (Table 3).

It can be clearly seen that, in the presence of PEG, with a high ATP concentration (2.5 mM) and micromolar (low) concentrations of 3PGA, the enzyme exhibits about 5-fold lower V_{max} values with respect to media in the absence of PEG (i.e. 20% of its V_{max}; Table 2). The drastic decrease in the V_{max} of the AGPase under crowding conditions and low 3PGA was accompanied by a significant increase in the $s_{0.5}$ of glucose 1-phosphate (Table 2). Remarkably, the kinetic parameters of the enzyme were slightly affected by changes in the levels of 3PGA in aqueous media (Table 2). Similar results were obtained when assays were performed at low (0.5 mM) concentrations of ATP (Table 2). Thus, the ability of AGPase to distinguish low or high 3PGA concentrations under crowding conditions may be explained by the very low activities exhibited by the enzyme along with a significant decrease in its affinity toward glucose 1-phosphate (Table 2). Also remarkable was the 84% increase in V_{max} in crowded with respect to non-crowded media displayed by the enzyme in the presence of high 3PGA levels. The increase in V_{max} was also concomitant with the decrease in $s_{0.5}$ toward glucose 1-phosphate (Table 2).

Qualitatively, the results obtained with various ATP concentrations at saturating levels of glucose 1-phosphate were similar to those observed with various glucose 1-phosphate concentrations. Under crowding conditions, high glucose 1-phosphate concentrations (0.5 mM) and low concentrations of the allosteric activator, AGPase was about 5-fold less active, with a significantly lower affinity for ATP (Table 3). Conversely, at high 3PGA concentration AGPase was 85% more active in the presence of PEG with similar affinity for ATP as when assayed in non-crowded media (Table 3). Synthetically, the enzyme was notably more active in the presence of PEG and high 3PGA concentrations when compared with similar conditions in non-crowded media. Noteworthy is the notable shift in the affinity of the enzyme for ATP between low and high concentrations of 3PGA in crowded media, as could be judged through the 5–15-fold lower $s_{0.5}$ determined (Table 3).

Structural basis of AGPase ultrasensitivity

It is well known that conformational and kinetic behavioural changes of enzymes are contemporary phenomena [26,27]. The subtle cross-talk exhibited by AGPase allosteric effectors, along with the interplay accomplished by the enzyme's substrates

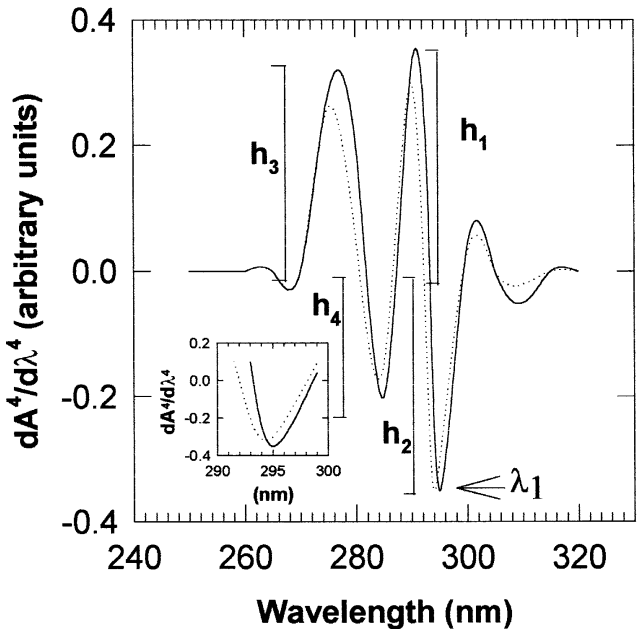

Figure 5 Fourth-derivative absorption spectra of AGPase in aqueous (dotted line) and crowded (continuous line) media

The enzyme (approx. 0.35 mg/ml) was incubated in 50 mM Mops-KOH (pH 7.5) plus 1 mM DTT in the absence (dotted line) or in the presence (continuous line) of 9% (w/w) PEG 8000 and the respective spectra were recorded at 37 °C. Characteristic λ_1 wavelength and valley-to-peak distances h_1, h_2, h_3, and h_4 are shown. The inset magnifies the spectra between 290 and 300 nm.

Table 4 Data corresponding to fourth derivative spectra of AGPase from cyanobacteria analysed under different conditions

Condition	R*	λ_1 (nm)†
Non-crowded medium		
AGPase	1.45	295.1
+2.5 mM 3PGA	1.45	295.2
+2.5 mM 3PGA and 1 mM P_i	1.40	295.4
+1 mM P_i	1.40	295.5
Crowded medium		
AGPase	1.35	295.4
+2.5 mM 3PGA	1.45	295.2
+2.5 mM 3PGA and 1 mM P_i	1.50	295.4
+1 mM P_i	1.40	295.3

* R is defined [30] as the relation of valley-to-peaks (see Figure 5): $R = (h_1 + h_2)/(h_3 + h_4)$

† λ_1 is the longest wavelength minimum. Protein concentration utilized is indicated in Figure 5.

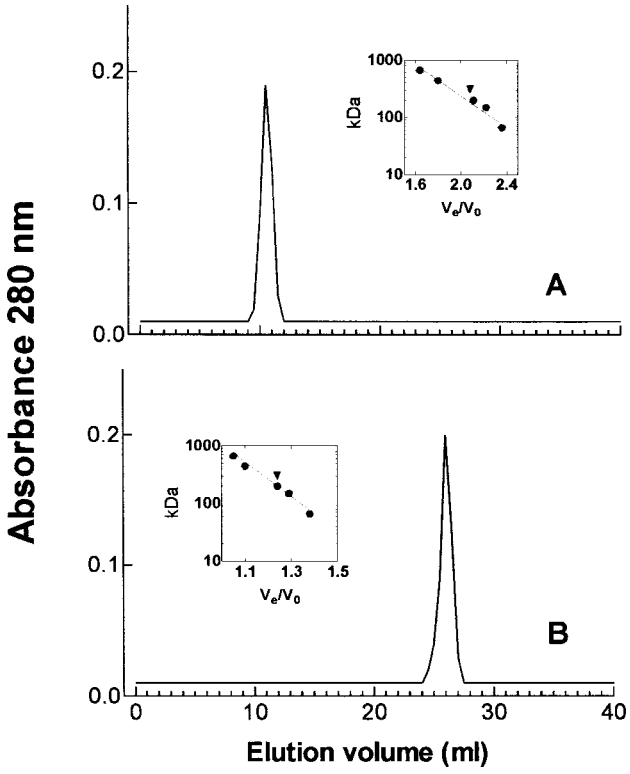

Figure 6 Gel-filtration chromatograms of AGPase using a Superose B-12 column in aqueous medium (A) or a Sephacryl S-300 column in crowded conditions (B)

Chromatography was performed as detailed in the Experimental section. Insets show the calibration curve for the respective column obtained with standard proteins under aqueous or crowded conditions, and arrows indicate the V_e/V_0 value obtained for AGPase in each case.

under crowding conditions, suggest a highly flexible and fine modulation of the enzyme activity. We decided to investigate more thoroughly the ultrasensitive phenomenon exhibited by AGPase from a conformational point of view. In order to address this problem, we employed three experimental strategies: (i) fourth-derivative spectroscopy in order to detect intramolecular changes; (ii) size-exclusion chromatography to determine whether changes in the quaternary structure of the enzyme are involved; and (iii) fluorescence-emission spectra, since the principal information on chromophore interaction with the environment is provided by spectral shifts.

As applied to AGPase, fluorescence spectra and fourth-derivative spectroscopy provided similar results with respect to the status of chromophores from tryptophan residues in aqueous or crowded medium (see Figures 4 and 5; Table 4). PEG-induced crowding increases the non-polarity of the environment of tryptophan residues, as can be judged through the short-wavelength shift and increase of fluorescence intensity. The UV shift of the maximal wavelength of emission of AGPase in the presence of PEG with respect to its absence (Figure 4) indicates that tryptophan residues relocate in a non-polar environment [28,29]. The maximal wavelength of fluorescence emission exhibited by AGPase in aqueous (λ_{max} 340 nm) and PEG (9%)-crowded (λ_{max} 330 nm) media, belongs to class II (i.e., surface tryptophan residues surrounded by bonded water dipoles with low mobility) and class I (i.e., tryptophan residues in a non-polar environment) respectively, according to Burstein's model [28,29]. The 12-nm-short-wavelength shift and drastic increase of fluorescence intensity of the AGPase spectrum, when the enzyme is in the presence of its effectors, 3PGA and P_i (Figure 4) is remarkable. PEG-induced crowding produced even more pronounced changes by increasing fluorescence intensity and shifting the spectrum even further away into the UV region (Figure 4).

Although fourth-derivative spectroscopy is unable to separate the contributions of tryptophan and tyrosine, the peaks given

by tryptophan dominate the spectrum whenever the ratio of tryptophan to tyrosine is at least 1:4 [25]. This is the case for AGPase from *Anabaena* PCC 7120; the four tryptophan and 16 tyrosine residues per subunit of homotetrameric enzyme [20] allowed us to apply fourth-derivative spectroscopy to obtain data mainly about changes occurring in the tryptophan environment and thus studying conformational changes undergone by the protein.

PEG induced changes in the fourth-derivative spectra of tryptophan residues from the cyanobacterial AGPase, as can be judged from the shift toward longer wavelengths of λ_1 from the long-wavelength peak ($\lambda_{1,aq.}$ 295.1 nm $\rightarrow$ $\lambda_{1,cr.}$ 295.4 nm (where $_{aq.}$ is aqueous and $_{cr.}$ is crowded; see Figure 5 and Table 4). Although small, the 0.3 nm shift was reproducible and consistent. As a matter of fact, the addition of both allosteric effectors in aqueous media caused a similar quantitative shift in λ_1 as the one registered by adding PEG (Table 4). In all cases, only changes in λ_1, but not in the geometrical parameter *R*, were observed (Table 4), thus indicating that the shape of the different spectra were similar [25,30]. These results would be indicative of tryptophan in a non-polar environment; that is, λ_1 values near 292 nm indicate an aqueous environment, whereas λ_1 values of 293 nm or higher result from a non-polar environment [30].

That AGPase did not change its oligomerization status in the presence of PEG was shown by size-exclusion chromatography (Figure 6). Indeed, in the absence of PEG, the enzyme behaved as a 200 kDa tetramer, as could be judged by gel-filtration chromatography on a Superose 12 column (Figure 6A), and retained the same quaternary structure, despite the presence of its substrates or allosteric effectors (results not shown). In agreement with previous reports [14], we observed that the Superose 12 column was unable to separate the different standard proteins when chromatographed in the presence of PEG. To solve this inconvenience, we used a Sephacryl S-300 column for running in the presence of the inert polymer (Figure 6B). Under the latter conditions we found that AGPase stayed in its tetrameric form in crowded media (Figure 6B), independently of the presence or the absence of substrates or effectors (results not shown).

DISCUSSION

The kinetic and allosteric regulation of AGPase has always been an important topic because this enzyme catalyses a key step in the biosynthetic pathway of glycogen in bacteria or starch in plants [1–3]. The role played by different levels of enzyme activity in determining the amount of storage polysaccharide accumulated in the cell has been clearly established [31]. It was pointed out previously [32] that, in plants, the interplay between the allosteric regulators of AGPase exerts a fine physiological modulation over its activity. The accuracy of this metabolic scenario was recently reinforced by observations showing that, under conditions mimicking the intracellular environment, the enzyme exhibits an ultrasensitive response to allosteric regulation [11].

A main finding of the present work is that the ultrasensitive response achieved by AGPase toward its allosteric activator 3PGA in molecularly crowded media (Figure 1) results from the cross-talk characteristics with the allosteric inhibitor, P_i. Crowding renders AGPase more sensitive to the cross-talk between 3PGA and P_i. Key evidence for the latter is given by the 2-fold higher positive co-operativity exhibited by the enzyme in crowded as opposed to aqueous media as a function of the increase in P_i concentration (Figure 2B). The narrow 3PGA concentration range over which AGPase becomes ultrasensitive (Figure 1) when the P_i concentration increases arises from the interplay of opposing actions exerted by the higher concentrations of the allosteric activator that are needed: (i) to achieve half-maximal velocity (i.e., higher $A_{0.5}$ for 3PGA) (Figure 2A and Table 1); and (ii) to reduce the inhibitory power of P_i (given by the $I_{0.5}$ for P_i) (Figure 3). Indeed, the cross-talk between the allosteric effectors of AGPase is finely modulated under PEG-elicited molecular-crowding conditions, whereas this was not the case in aqueous media (Figure 1).

Taken together, the results shown in Figures 2 and 3 indicate that: (i) the inhibitory action of P_i interferes with the activating role exerted by 3PGA; (ii) half-maximal AGPase activities were obtained with higher concentrations of 3PGA in crowded as opposed to aqueous conditions, as P_i concentration increases in the medium; (iii) in turn, to overcome the inhibitory power of P_i, relatively higher 3PGA concentrations were needed in crowded media; (iv) the co-operativity of the enzyme almost doubled under crowding conditions. Thus the allosteric effectors of AGPase exhibit subtle cross-talk, either in the presence or the absence of PEG. The cross-talk differences in both media depicted in Figures 2 and 3 allow one to explain the ultrasensitive behaviour of the enzyme in molecularly crowded media. In fact, in crowded media, AGPase is distinctly sensitive to P_i; i.e., a 2-fold higher positive co-operativity is induced by the allosteric inhibitor. This sensitively higher co-operative behaviour of AGPase in crowded media allows the enzyme to overcome the greater 3PGA concentrations needed either to achieve half-maximal velocity or to decrease the inhibitory action of P_i.

The increased AGPase sensitivity toward the cross-talk of its allosteric effectors under molecular crowding is also exemplified by the discriminating ability of the enzyme toward high or low 3PGA concentrations when assayed at different concentrations of the enzyme's substrates (Tables 2 and 3). Qualitatively, the same behaviour was obtained with increasing concentrations of glucose 1-phosphate (Table 2) or ATP (Table 3) while keeping (or not) the remaining substrate at saturation. Interestingly, only under crowding conditions was the enzyme able to discriminate between low and high levels of 3PGA with respect to the activity reached at a certain level of each of the substrates (Tables 2 and 3). Thus, at low 3PGA, very small changes in the activity were observed at various levels of glucose 1-phosphate or ATP, whereas significant differences were found at high concentrations of the allosteric activator. These results may have a physiological relevance, since the utilization of the enzyme's substrates (mainly ATP) for storage-polysaccharide synthesis is maximized only at appropriate levels of 3PGA, a metabolite signalling active photosynthesis [2,31,32]. Remarkably, in crowded media and at relatively low concentrations of glucose 1-phosphate (conditions expected to occur *in vivo*), the affinity of the enzyme for ATP is highly affected by 3PGA levels. The latter suggests a very fine regulatory mechanism of the use of substrate under physiological conditions.

The results obtained suggested to us that the subtle cross-talk exerted by 3PGA and P_i on AGPase activity could, in turn, be correlated with conformational changes. Evidence for the involvement of conformational changes in the ultrasensitive behaviour of AGPase, came from: (i) fluorescence emission of tryptophan residues (Figure 4); (ii) fourth-derivative spectroscopy (Figure 5; Table 4); and (iii) size-exclusion- chromatography analysis (Figure 6). Taken together, the results obtained by fluorescence spectra (Figure 4) and fourth-derivative spectroscopy (Figure 5; Table 4) are in agreement with a shift of chromophores from tryptophan residues of AGPase toward non-polar environments with unaltered oligomerization of the enzyme (Figure 6). In aqueous media, the wavelength shift was

induced by the enzyme's allosteric effectors, whereas PEG itself or with the additional presence of the effectors, gave similar results, although with more pronounced effects (see Figures 4 and 5; Table 4). It must be stressed that the conformational changes registered by AGPase in aqueous or crowded media are not the same. This is so despite the fact that the short-wavelength shifts in fluorescence spectra (Figure 4) or the increase in the longest wavelength minimum (Figure 5, Table 4) were similar, both qualitatively or quantitatively. However, the data in Table 4 clearly show that, in the presence of PEG, which corresponds to an inhibited AGPase, the Trp residues do exhibit similar λ_1 when compared with the enzyme in the presence of 3PGA and P_i (see Table 4) that in turn gives a 150-fold increase in activity [10,11].

The kinetic and structural analysis of AGPase under crowding conditions is undertaken to approach the functioning of the enzyme intracellularly. The understanding of the kinetic, regulatory and structural properties of enzymes within cells is limited by our lack of knowledge of the interactions involved between homologous and heterologous proteins, substrate and regulatory metabolite concentrations, as well as other cellular macromolecules [9,33–35]. Studies of the kinetic properties of cyanobacterial AGPase have been mainly performed in aqueous media. Under the latter conditions, saturation kinetics of the enzyme with 3PGA gives a Michaelian-type of curve (Figure 1). However, under conditions supposed to resemble the intracellular molecular crowding (PEG-induced), the enzyme becomes exquisitely ultrasensitive to 3PGA concentrations over background (Figure 1) [11]. The advantage of ultrasensitivity has been mainly rationalized through its role in amplifying several-fold the flux through a metabolic step in biochemical networks, within a narrow range of change in substrate [17] or effector [11] concentration. Indeed, the ultrasensitivity phenomenon exhibited by AGPase was only possible in the presence of PEG-induced crowding, when P_i is absent. The results presented in this and previous work [10,11] suggest that AGPase could be sensing the molecular-crowding status of the chloroplast stroma probably induced by starch itself, as well as other proteins known to be present at a high concentration [6]. At present it is unclear what are the actual conditions of the intracellular medium in bacteria (like cyanobacteria) or in the chloroplast stroma (where AGPase localizes) of higher plants, although a molecular-crowding condition at least exists [5,9]. Recent evidence revealed important differences in molecular crowding between different subcellular compartments, suggesting considerable diffusional heterogeneity for small metabolites within the different intracellular organelles [36].

It has been stated that the spatio-temporal organization of cellular metabolism, energetics and gene expression is highly dependent on cytoplasmic organization, particularly cytoskeleton and intracellular molecular crowding [9,35]. This organization may provide a crucial link between the stress response/perception/transduction to environmental challenge by plant cells. The results presented in this and previous work allude to the fine-tuning ability of molecular crowding on AGPase activity and the subtle cross-talk exerted by its allosteric effectors as operative mechanisms for the fine physiological regulation of storage-polysaccharide synthesis.

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