Ultrasensitive glycogen synthesis in *Cyanobacteria*

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Abstract ***Cyanobacter*** **ADPglucose pyrophosphorylase exhibits a ultrasensitive response in activity towards its allosteric effector 3-phosphoglycerate, elicited by orthophosphate and polyethyleneglycol-induced molecular crowding. The ultrasensitive response was observed either when the enzyme operates in the zero or first order region for its physiological substrates. The ultrasensitivity exhibited maximal amplification factors of 15–19-fold with respect to 1% of the maximal system velocity. Only a 2.4–3.8-fold increase in 3PGA concentration was necessary to augment the flux from 10% to 90% through AGPase as compared with 200-fold required for the control. The results are discussed in terms of finely tuned regulatory mechanisms of polysaccharide synthesis in oxygenic photosynthetic organisms.**

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Key words: ADPglucose pyrophosphorylase; Glycogen and starch biosynthesis; Enzyme regulation; Molecular crowding; *Anabaena* PCC 7120

1. Introduction

Metabolic pathways for the synthesis of glycogen in bacteria and of starch in plants exhibit two main features in common: (i) both occur through a pathway using ADPglucose (ADPGlc) as the glucosyl donor and (ii) the regulatory step in both biosynthetic routes takes place at the level of the sugar nucleotide production, a reaction catalyzed by ADPGlc pyrophosphorylase (AGPase: EC 2.7.7.27): glucose-1P + ATP ⇔ ADPglucose + PPi (for reviews see [1–4]).

AGPases from different sources are allosterically regulated by effectors derived from the dominant carbon assimilation route in the respective organism [1–4]. Thus, the enzyme from different bacteria is inhibited by ADP or Pi and activated by Fru1, 6bisP or Fru6P and pyruvate, depending upon the operative route for glucose degradation existing in the respective microorganism [1–3]. The enzyme from higher plants, green algae and *Cyanobacteria* is allosterically regulated by 3P-glycerate (3PGA, activator) and Pi (inhibitor), two key metabolites in the photosynthetic process [1–4]. It has been clearly demonstrated that the fine regulation of the enzyme by these metabolites directly determines the levels of starch accumulated in plant tissues [4,5].

Studies on the characterization of enzymes playing key roles in bacterial and plant metabolism were mostly performed with pure enzymes in aqueous media. A few reports deal with the behavior of enzymes in environments which could be closer to in vivo conditions, either physiological or stressful ones [6–9]. Concerning AGPase, extensive work has been performed to determine its kinetic and regulatory properties, from which the physiological relevance of the enzyme was pointed out [1–4]. However, because all of this work was carried out in aqueous buffered media, the understanding of the actual properties of the enzyme under in vivo conditions is limited. Previous studies showed that well characterized allosterically regulated enzymes could have a higher significance when analyzed under conditions resembling the intracellular environment [8,10–13]. In the present work we carried out studies on the changes of the regulatory properties of AGPase from *Cyanobacteria*, induced by molecular crowding. We found that the interplay between the allosteric effectors (3PGA and Pi) and polyethyleneglycol (PEG)-induced molecular crowding triggers a ultrasensitive phenomenon in AGPase. The enzyme ultrasensitivity and its classical allosteric modulation were finely up- or down-modulated by molecular crowding. As far as we are aware, this is the first time that this phenomenon is shown to exist in an enzyme from photosynthetic organisms.

2. Materials and methods

2.1. Chemicals

[^{14}C]Glc1P and [^{32}P]PPi were purchased from DuPont NEN. Glc1P, 3PGA and alkaline phosphatase were from Sigma Chemical (St. Louis, MO, USA). ATP, ADPGlc and PEG 8000 were purchased from ICN (Argentina). All other reagents used were of the highest quality available.

2.2. Bacterial strains and media

Bacterial strains used in this study are a generous gift of Prof. Jack Preiss (Dept. Biochemistry, MSU, USA). *Escherichia coli* mutant strain AC70R1-504, which has no AGPase activity, was used for the expression of the *Anabaena* AGPase gene, as previously described [14]. AC70R1-504 cells were grown in enriched medium containing 1.1% K_2HPO_4.

2.3. 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 as previously described [14,15].

2.4. Enzyme assay

AGPase activity was determined in the ADPGlc synthesis direction at two saturation conditions for the substrates ATP and Glc1P. At saturating levels of both substrates, the reaction mixture contained (unless otherwise specified) 20 μmol of MOPS-KOH pH 7.5, 1.25 μmol of $MgCl_2$, 0.3 U of inorganic pyrophosphatase, 0.5 μmol of ATP and 0.1 μmol of [^{14}C]Glc1P (specific activity 9.9×10^6 cpm/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]ADPGlc was assayed as previously described [16]. Assays performed at subsaturating levels of one or both of the substrates were essentially identical, except for the amount of Glc1P and ATP in the mixture that was changed to 0.01 μmol and 0.1 μmol, respectively.

2.5. Kinetic studies

3PGA activation and Pi inhibition kinetics of AGPase were exhaus-

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Abbreviations: ADPGlc, ADPglucose; AGPase, ADPglucose pyrophosphorylase; PEG, polyethyleneglycol; 3PGA, 3-phosphoglycerate

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tively determined. AGPase activity was assayed at different concentrations of allosteric effectors, in the presence of subsaturating and saturating levels of substrates (see above) and different concentrations of PEG or its absence. The experimental data were fitted through the generalized Hill equation by a non-linear least square regression kinetics computer program [17]. This program was used to calculate kinetic parameters: V_{max} and Hill coefficients (n_H), as well as $3PGA_{0.01}$, $3PGA_{0.1}$, $3PGA_{0.5}$ and $3PGA_{0.9}$, corresponding to the effector concentrations giving 1%, 10%, 50% and 90% maximal activation, respectively. All kinetic parameters are means of at least three determinations and are reproducible within at least ±10%. To evaluate the effect of the non-essential activator 3PGA, net velocities (v_N) were determined by subtracting the velocity in the absence of the activator from each of the velocities determined at different 3PGA concentrations [18]. Therefore, V_{Nmax} corresponds to the net maximal velocity of the enzyme. Amplification factors, *As*, were obtained using the equation described by Koshland et al. [19]: $As = ((v_{Nf} - v_{Ni})/v_{Ni})/((3PGA_f - 3PGA_i)/3PGA_i)$ where v_{Nf} is the net velocity in ADPGlc 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% or 10% of the maximal activity.

3. Results

We undertook a detailed study of the rate exhibited by AGPase when analyzed as a function of 3PGA and Pi effector levels at a zero or first order concentration of the enzyme substrates (Glc1P and ATP) under PEG-induced molecular crowding conditions.

3.1. AGPase ultrasensitivity in the zero order region for its substrates

AGPase assayed in the ADPGlc synthesis direction (the physiological reaction) at saturating levels of substrates, exhibited different saturation curves for the allosteric activator 3PGA, depending upon the absence or the presence of Pi and PEG in the medium (Fig. 1). The stimulus-response curves for 3PGA, plotted as v_N/V_{Nmax} versus $3PGA/3PGA_{0.5}$ [19], exhibited a sigmoidal behavior that appeared when either Pi (0.05 or 1 mM) was present or a combination of both, Pi and PEG-induced molecular crowding (Fig. 1A,B, Table 1). In the absence of Pi and molecular crowding, the enzyme showed hyperbolic sensitivity (Fig. 1A,B), in agreement with previous reports [15,20]. The sigmoidal behavior was only slight at Pi concentrations of 0.05 mM (Fig. 1A) but increased markedly when accompanied by 9% PEG (Fig. 1B). The highest cooperativity, n_H, was achieved with a combination of 1 mM Pi and 9% PEG (Fig. 1B, Table 1). However, Pi concentrations of 1 mM (Fig. 1A, Table 1) or different PEG concentrations (Table 1) were able by themselves to sensitively amplify the AGPase response. In Table 1, it can be clearly seen that the sigmoidal response of the enzyme rate as a function of the effector concentration stimulus, increases with PEG-induced molecular crowding.

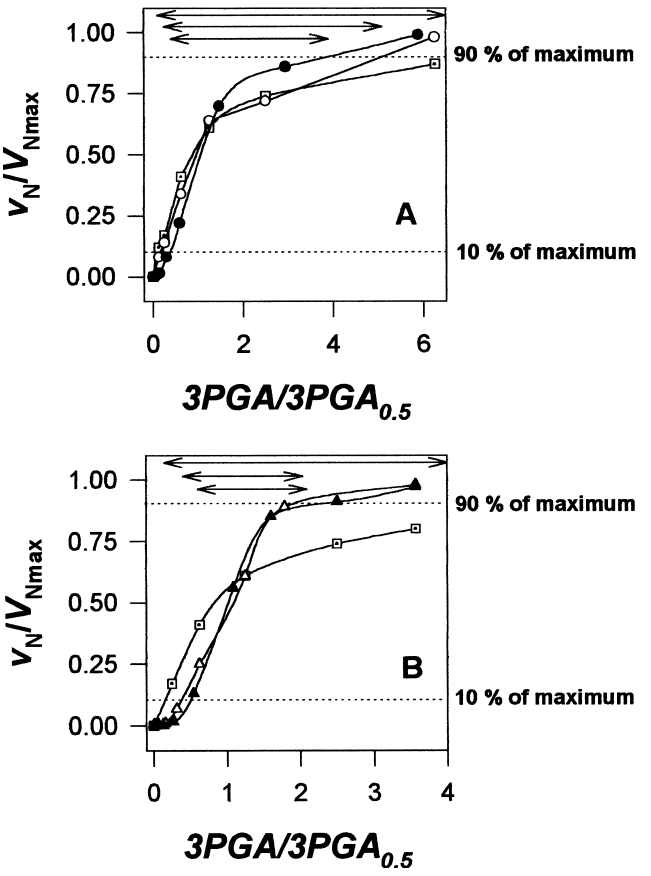

Fig. 1. Stimulus-response behaviour of AGPase as a function of the allosteric effector 3PGA. Ratios of net velocities over the net maximal velocity were determined at different ratios of 3PGA over the effector concentration, giving 50% of the maximal activation for assays of ADPGlc synthesis activity in media containing 0.05 mM (A) or 1 mM Pi (B) without further additions (○, △) or plus 9% (w/w) PEG (●, ▲). In A and B, controls runned in the absence of Pi and PEG are illustrated by a dotted square. Arrows(↔) indicate the amplitude in the 3PGA ratio producing an increase in the net velocity ratio from 10% to 90%.

In order to ascertain that the increase in n_H corresponded to ultrasensitivity, the amplification factor *As* ([19], see Section 2) was determined from the increase in velocity versus the 3PGA concentration, in the presence or the absence of Pi and PEG. An *As* greater than one implies that the percentage change in the response is greater than the percentage change in the stimulus. However, the sensitivity amplification factor is not au-

Table 1
Ultrasensivity behavior of AGPase in the presence of allosteric effectors and PEG-induced molecular crowding

Condition Pi (mM)	PEG (% w/w)	$3PGA_{0.01}$ (μM)	$3PGA_{0.1}$ (μM)	$3PGA_{0.9}$ (μM)	$3PGA_{0.9}/3PGA_{0.01}$	$3PGA_{0.9}/3PGA_{0.1}$	n_H	*As* 0.01[a]	0.1[a]
0	0	0.13	2.5	500	3846	200	1.0	< 0.01	< 0.01
0.05	0	0.7	5.3	229	327	43.0	1.3	1.4	1.4
0.05	9	18.0	59.0	500	27.8	8.5	2.0	6.3	1.9
1	0	160	470	1800	11.3	3.8	2.3	14.9	3.2
1	9	350	745	1800	5.14	2.4	3.6	19.3	4.4
0	3	0.9	7.7	353	371	45.0	1.2	1.2	1.0
0	9	12.3	45.0	480	39.0	10.7	2.1	11.1	2.7
0	15	26.4	72.0	451	17.0	6.2	2.5	15.3	3.5

[a]With respect to 1% (0.01) or 10% (0.1) of AGPase activity.

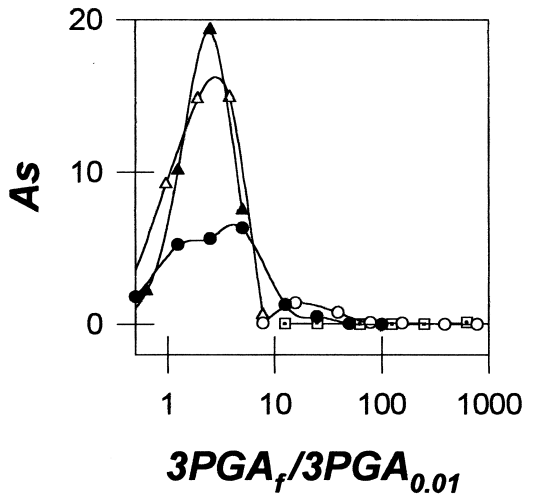

Fig. 2. The maximum sensivity amplification factor exhibited AGPase as a function of the ratio (logarithm) of the 3PGA concentration over the effector concentration corresponding to 1% of the background enzyme activity. Assays were carried out in buffered aqueous media without further additions (dotted squares), in the presence of 0.05 mM Pi alone (○) or plus 9% (w/w) PEG (●), or in the presence of 1 mM Pi alone (△) or plus 9% (w/w) PEG (▲).

tomatically larger than one if a system is ultrasensitive, it will depend on the range over which the stimulus-response ratio is observed [19]. Fig. 2 depicts a semilogarithmic plot of *As* as a function of the concentrations ratio of 3PGA with respect to 1% of the enzyme its maximal velocity. It is clearly shown that the maximal *As* (19.3-fold) is obtained in the presence of both 1 mM Pi and 9% PEG. Significant amplifications (15- or 11-fold) were determined in the presence of 1 mM Pi or 9% PEG, respectively (Fig. 2, Table 1). Interestingly, the highest amplification factors (towards the left of the plot in Fig. 2) were obtained close to one of the $3PGA_{0.9}/3PGA_{0.01}$ ratio which means that only small stimuli (i.e. small increases over the background level of 1%) were able to trigger a significant amplification of the response (i.e. the percentage change in AGPase rate). These *As* values, obtained for AGPase under different conditions, fit very well with maximum sensitivity amplification factors estimated from a theoretical model of a cooperative protein with a given n_H ([19,21,22], see also Section 4). Furthermore, the results obtained also show that molecular crowding may up- or down-modulate the enzyme depending upon the presence or the absence of Pi, respectively (Fig. 2, Table 1).

Quantitatively, ultrasensitivity allows for a more restricted change in the 3PGA concentration over which to increase from 10% to 90% activity of AGPase. As illustrated in Table 1, the amplification factor increases with the Hill coefficient. For instance, in the presence of 1 mM Pi with or without 9% PEG, only a 2.4-fold or 3.8-fold change in 3PGA, respectively, was needed to increase the activity from 10% to 90%, whereas 200-fold was necessary in the absence of PEG. The latter values drastically increased when the enzyme response had to scale up from 1% of background activity to 90% (Table 1).

3.2. AGPase ultrasensitivity in the first order domain

We asked whether the ultrasensitive phenomenon observed in the zero order region for the substrates, could still remain in the first order one. The results are shown in Table 2, where we can see that in the presence of 9% PEG, a sigmoidal comportment appears in the first order domain (i.e. around $S_{0.5}$) for both Glc1P and ATP, or zero order for one or the other substrate alternatively. In the absence of molecular crowding only hyperbolic behavior of the enzyme was observed.

When compared with the control for these experiments (9% PEG, zero order for ATP and Glc1P : see Table 1, line 7), it is shown that *As* values are even improved except for the situation in which Glc1P is saturating and ATP subsaturates the enzyme (Table 2). Under those conditions the *As* decreased almost 2-fold with respect to the case in which ATP saturates and Glc1P subsaturates the enzyme. Even for the latter most unfavorable case, the amount of 3PGA necessary to increase from 10% to 90% of the enzyme flux was still lower for the first order domain than the zero order condition (Table 1). The improvement observed in the *As* for the first order domain was obtained with only a modest increase in cooperativity as measured by n_H with respect to the zero order behavior.

4. Discussion

The main contribution of the present work is to show an ultrasensitivity phenomenon in AGPase from *Cyanobacteria*. The positive cooperativity exhibited by the enzyme resulted from the interplay between both effectors, 3PGA and Pi, constituting the basic biochemical mechanism for achieving ultrasensitivity by AGPase. Furthermore, another important finding of this work is that PEG-induced molecular crowding provides an additional level of regulation of amplification

Table 2
Ultrasensivity of AGPase induced by PEG in the zero or first order region respect to the substrates (Glc1P and ATP)

Condition Glc1P	ATP	$3PGA_{0.01}$ (μM)	$3PGA_{0.1}$ (μM)	$3PGA_{0.9}$ (μM)	$3PGA_{0.9}/3PGA_{0.01}$	$3PGA_{0.9}/3PGA_{0.1}$	n_H	*As* 0.01[a]	0.1[a]
No PEG									
Low*	Low	1.6	10.1	262	164	25.9	1.1	0.5	0.3
Low	High*	1.1	6.9	200	200	29.0	1.1	0.6	0.5
High	Low	1.7	8.9	162	95.3	18.2	1.2	0.9	0.7
+PEG (9% w/w)									
Low	Low	0	98	490	12.3	5.0	2.6	13.5	2.0
Low	High	28	64	303	10.8	4.7	2.7	16.6	3.7
High	Low	24	70	517	21.5	7.3	2.2	7.6	2.2

[a]With respect to 1% (0.01) or 10% (0.1) of AGPase activity.
*Low and high concentrations of the substrates correspond to subsaturating or saturating conditions respectively. Concentrations in the assay medium were 0.05 mM or 0.5 mM for Glc1P and 0.5 mM or 2.5 mM for ATP.

either per se or in synergy with Pi. The amplified response shown by the enzyme towards the activator 3PGA is in agreement with the ultrasensitivity phenomenon described by Koshland, Goldbeter and coworkers [19,21,22]. Previous reports on the regulation of AGPase from oxygenic photosynthetic organisms highlighted the interaction of the regulatory effects of 3PGA and Pi [1–5,15,20]. However, these studies never demonstrated the fine interplay of the two allosteric effectors to establish a coordinated regulation of the enzyme as is established herein.

Ultrasensitivity has been defined as a response in a biological system which is more sensitive than that to be expected from the classical hyperbola of Michaelis-Menten kinetics (Fig. 1) [19,21,22]. One main advantage of ultrasensitivity is that as a device in biochemical networks, it allows to increase several-fold the flux through a metabolic step in a narrow range of change in substrate or effector concentration. The latter was indeed observed for AGPase (Fig. 1, Table 1). It must be stressed that quantitatively, the experimentally observed *As* values for AGPase are very similar to the maximal *As* values expected for an allosteric enzyme exhibiting sensitivity amplification [21]. In fact, the *As* values for AGPase were 14.9 ($n_H = 2.3$) and 19.3 ($n_H = 3.6$) or 3.2 ($n_H = 2.3$) and 4.4 ($n_H = 3.6$) with respect to 1% or 10% of the maximal activity, respectively (see Table 1). For the sake of comparison, an allosteric enzyme performing ultrasensitivity with an n_H of 2 or 4 shows, theoretically, *As* values of 5.5 and 23.9 or 2.1 and 5.5 with respect to 1% or 10% of the maximal activity, respectively [19,21]. Indeed, the *As* values for an allosteric enzyme without sensitivity amplification are significantly lower, i.e. for n_H of 2 or 4, are 1 or 4, respectively, with respect to 10% of the maximal activity [19,21].

In turn, sensitivity amplification deals with the percentage change in a response compared to the percentage change in the stimulus. According to these concepts (considering that the enzyme from *Cyanobacteria* has similar regulatory properties than the protein from higher plants: see [2,20]), the sensitivity amplification phenomenon exhibited by AGPase may represent a significant regulation of plant cell physiology. In fact, the enzyme would be sensing Pi levels in the chloroplast, that, when high enough, would imply the ultrasensitive response of the enzyme activity toward only slight changes of 3PGA concentrations over the background level. Let us recall that sensitivity amplification operates when going from a background non zero level to another finite value [22]. The conditions for significant sensitivity amplification are not only that the overall system exhibits ultrasensitivity, but also that the range over which the effector 3PGA, or the substrates that are changing, are close to the optimal. The positive effector 3PGA concentration in the chloroplast operates within the region in which the optimal amplification factors shown in Fig. 2 are observed [23,24].

Interesting enough, the ultrasensitive phenomenon was observed either in the first or zero order region for enzyme substrates Glc1P and ATP (Tables 1 and 2). However, the ultrasensitivity phenomenon described in the present work does not correspond to zero order ultrasensitivity since the latter arises in steady state systems [21]. Besides, the original theoretical work of Koshland and coworkers dealt with substrate-induced ultrasensitivity whilst in our work it is allosteric effector- (3PGA, Pi) and/or molecular crowding-provoked (Fig. 1, Tables 1 and 2).

In the absence of Pi, the ultrasensitive phenomenon was only possible in the presence of PEG-induced molecular crowding (Tables 1 and 2). Thus, our results point out the possibility that AGPase could be sensing the molecular crowding status of the chloroplast stroma likely induced by starch itself as well as other proteins known to be present at a high concentration [7]. Indeed, the ultrasensitivity is also observed in the presence of a protein as a crowding agent (Gómez Casati, Aon and Iglesias, manuscript in preparation). The polysaccharide accumulation through molecular crowding could induce lower amplifications along with a Pi decrease.

Synthetically, the ultrasensitive phenomenon described is quite likely that could apply not only to *Cyanobacteria* glycogen synthesis but to starch synthesis in higher plants as well. In this way, the present results allow to suggest that the known relevance of the 3PGA/Pi ratio, that is supposed to regulate starch synthesis within the chloroplast [1–4], may establish a feasible mechanism for the fine modulation of the process under different physiological conditions. This ultrasensitive biochemical device, although very simple (operating only by allosteric regulation), reveals itself as very sensitive to small changes in the levels of the metabolites exerting regulation.

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