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ORIGINAL ARTICLE

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Ultrasensitive behavior in the synthesis of storage polysaccharides in cyanobacteria

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Abstract The glycogen synthetic pathway operates ultrasensitively as a function of the ADPglucose pyrophosphorylase (ADPGlcPPase) allosteric effectors, 3-phosphoglycerate and Pi, in permeabilized cells of the cyanobacterium *Anabaena* PCC 7120. In vitro data previously showed that the ultrasensitive behavior of ADPGlcPPase depends upon cross-talk between the two allosteric effectors, the enzyme's response being additionally modulated by molecular crowding [D.F. Gómez Casatiet al. (2000) Biochem J 350:139–147]. In the present work we show, experimentally and with a mathematical model, that α-1,4-glucan synthesis is also ultrasensitive in cells due to the propagation of the switch-like behavior of ADPGlcPPase to the synthetic pathway. Amplifications of up to 20-fold in storage-polysaccharide synthesis can be achieved with a modest 6.7-fold increase in 3-phosphoglycerate in the presence of 5 mM Pi in contrast to the 30-fold necessary in its absence. This is the first time that this phenomenon has been reported to occur in the glycogen synthetic pathway of a photosynthetic prokaryote. The implications of the results for plant cell physiology during light–dark transitions are discussed.

Keywords ADPglucose pyrophosphorylase · *Anabaena* · Glycogen/starch synthesis · Molecular crowding · Permeabilized cyanobacteria · Ultrasensitivity (of enzymes)

Abbreviations ADPGlc: ADPglucose · ADPGlcPPase: ADPGlc pyrophosphorylase · GLY: glycogen · G1P: glucose-1-phosphate · ODE: ordinary differential equation · PEG: polyethylene glycol · 3PGA: 3-phosphoglycerate

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Introduction

Biological systems must continuously adapt to changing environmental stimuli. A plant cell's response to changeable light conditions is particularly important, since this environmental cue induces a different energetic status, which, in turn, leads to the accumulation or degradation of reserve polysaccharides. Starch and glycogen are major storage products of photosynthesis in plants and bacteria, respectively (Preiss and Sivak 1998a, 1998b; Sivak and Preiss 1998). At least 30% of the carbon photoassimilated by plants is channelled to starch under optimal conditions of light, water, temperature and CO_2 concentration (Heldt et al. 1977). Thus, knowing how starch levels are controlled and regulated in plants could have a tremendous potential impact on the primary productivity of Earth.

Glycogen and starch synthesis in bacteria and plants, respectively, have a common pathway involving: (i) production of the glucosyl donor, ADPglucose (ADPGlc), via ADPGlc pyrophosphorylase (ADPGlcPPase; EC 2.7.7.27); (ii) elongation of an α-1,4-glucan chain catalyzed by glycogen/starch synthase; and (iii) ramification through α-1,6 linkages of the growing polysaccharide chain (Preiss and Sivak 1998b; Sivak and Preiss 1998). In addition, in bacteria and plants the production of sugar nucleotides, catalyzed by ADPGlcPPase: G1P + ATP ⇔ ADPGlc + PPi, constitutes the regulatory step in the biosynthetic route (Preiss 1991; Preiss and Sivak 1998a, 1998b; Sivak and Preiss 1998).

ADPGlcPPases from different sources are regulated by effectors derived from the dominant carbon assimilation route in the respective organism (Iglesias and Preiss 1992; Sivak and Preiss 1998). The enzyme from bacteria is activated by fructose-1,6-bisphosphate, fructose-6-phosphate, or pyruvate, main intermediates of

glycolytic metabolism. ADPGlcPPases from organisms performing oxygenic photosynthesis (cyanobacteria, green algae, and higher plants) are allosterically regulated by two key metabolites of the photosynthetic process: 3-phosphoglycerate (3PGA; activator), the primary product of CO_2 fixation by the Calvin cycle; and Pi (inhibitor) (Iglesias et al. 1991; Preiss 1991; Iglesias et al. 1994; Preiss and Sivak 1998b; Sivak and Preiss 1998). Thus, the level of polysaccharide accumulated in photosynthetic tissues is determined by the 3PGA/Pi ratio (Preiss 1991; Iglesias and Preiss 1992; Preiss and Sivak 1998b; Sivak and Preiss 1998). Recent reports on studies carried out in vitro have shown that the cross-talk between 3PGA and Pi, under polyethylene glycol (PEG)-elicited molecular crowding, induces an ultrasensitive behavior of cyanobacterial ADPGlcPPase that would be critical for the physiological regulation of the enzyme (Gómez Casati et al. 1999, 2000).

Ultrasensitivity is a type of amplification by which the percentage change in the response of a biological system increases several-fold in a narrow variation range of the stimulus (Goldbeter and Koshland 1982; Koshland et al. 1982; Koshland 1987, 1998). One main advantage of ultrasensitivity is to allow increases of, for example, a flux by several-fold after fluctuations in substrate or effector concentration slightly change over the background. The latest findings show the occurrence of ultrasensitive responses in several enzymatic systems and signalling cascades operating under intracellular conditions (Ferrell and Machleder 1998; Hardie et al. 1999; Cluzel et al. 2000; Aon et al. 2001). It is increasingly evident that (supra)molecularly organized and crowded environments may provide an additional level of regulation of protein dynamics, such as their potentiality to function as switch-like devices. The powerful combination of cooperativity and sensitivity amplification allows sharp, digital-like responses of target enzymes (Koshland 1987; Bray 1995; Aon and Cortassa 1997; Aon et al. 2001). Now, several findings show that physicochemical conditions resembling those prevailing in the cell may either induce or modulate the ultrasensitive responses of enzymes (Aon and Cortassa 1997; Aon et al. 2000; Aon et al. 2001).

In the present work we investigated if the ultrasensitivity exhibited by ADPGlcPPase in vitro, under conditions of molecular crowding (Gómez Casati et al. 1999, 2000), also occurs in cells. In addition, the objective was to analyze if such ultrasensitivity can further propagate into storage-polysaccharide synthesis, thus determining a mechanism for the fine regulation of the metabolic pathway. We found that the accumulation of glycogen in permeabilized cells (in situ) of *Anabaena* PCC 7210 is ultrasensitive to the ADPGlcPPase allosteric effectors, 3PGA and Pi. Based on in vitro and in situ kinetic data, a mathematical model of the glycogen synthetic pathway was developed, which is able to reproduce the ultrasensitivity observed in cells.

Materials and methods

General

Reagents

Radioactive material was from DuPont NEN. Alkaline phosphatase, inorganic pyrophosphatase, glucose-1-phosphate (G1P), ATP, and 3PGA were from Sigma Chemical Co. (St. Louis, Mo., USA). All other reagents were of the highest quality available.

Permeabilization of cyanobacterial cells and assay of α-1,4-glucan synthesis

Cells of *Anabaena* PCC 7120 were grown autotrophically in BG-11 medium (Castenholz 1988). The permeabilization procedure was performed as recently described (Gómez Casati et al. 2001). After permeabilization, the incorporation of radioactivity onto glycogen was quantified in the presence of different concentrations 3PGA and Pi, using [^{14}C]G1P or [^{14}C]ADPGlc as substrates, as also described by Gómez Casati et al. (2001).

Synthesis of [^{14}C]glucan from [^{14}C]G1P and ATP was measured in an assay mixture of 0.2 ml final volume containing (unless otherwise specified) 16 μmol Mops–KOH buffer (pH 7.5), 1.5 μmol $MgCl_2$, 0.5 μmol ATP, 0.1 μmol α-[^{14}C]G1P (specific activity 9.9×10^6 cpm μmol^{-1}) and 0.15 U of inorganic pyrophosphatase. The reaction was initiated by addition of permeabilized cells and stopped by heating the mixture in a boiling water bath for 2 min, followed by vortexing, and cooling on ice for 3 min. Labelled glycogen was extracted and purified as previously described (Aon and Curtino 1984) and then radioactivity quantified. Incorporation of [^{14}C]glucose into glycogen from [^{14}C]ADPGlc was measured under the same conditions, except that reaction mixtures contained 0.2 μmol [^{14}C]ADPGlc (specific activity 7.0×10^6 cpm μmol^{-1}) instead of [^{14}C]G1P and ATP.

In vitro assay of ADPGlcPPase

The activity of purified ADPGlcPPase was determined by measuring [^{14}C]ADPGlc synthesis from [^{14}C]G1P, as described elsewhere (Ghosh and Preiss 1966; Gómez Casati et al. 1999, 2000). The standard reaction mixture contained 20 μmol Mops–KOH buffer (pH 7.5), 1 μmol $MgCl_2$, 0.35 μmol ATP, 0.1 μmol [^{14}C]G1P (approx. 1.0×10^6 cpm μmol^{-1}), 0.3 U of inorganic pyrophosphatase, and 50 μg of BSA in a final volume of 0.2 ml. Assays were initiated by addition of enzyme, incubated 10 min at 37 °C, and stopped by heating in a boiling water bath for 30 s. Assays under crowded conditions were identical, except that BSA in the media was raised to 66 mg and reactions were stopped by diluting reaction mixtures 4-fold and heating in a boiling water bath for 1 min.

Kinetic studies in situ

The incorporation of ^{14}C into glucans using [^{14}C]G1P as substrate, was determined at different concentrations of 3PGA and Pi. Kinetic parameters were determined as previously (Gómez Casati et al. 1999, 2000) and they represent the mean of triplicates from at least four independent experiments with ±10% reproducibility.

Under in vitro conditions, ADPGlcPPase irreversibility was ascertained by the presence of pyrophosphatase. In situ, α-1,4 glucan synthesis from G1P proceeds linearly with time (Gómez Casati et al. 2001), strongly suggesting that ADPGlcPPase is working irreversibly in permeabilized cells. The latter is probably due to the fact that the enzyme products, PPi and ADPGlc, are substrates of pyrophosphatase and glycogen synthase, respectively, both catalyzing irreversible reactions. Taken together, these results led us to consider ADPGlcPPase as irreversible in our model (see Eq. 4 below).

The ultrasensitive response of polysaccharide accumulation was quantified through the amplification factor, A_s (see below).

Mathematical modelling of the glycogen synthetic pathway in cyanobacteria

Glycogen synthetic pathway

Carbon metabolism from G1P toward storage-polysaccharide (cyanobacterial glycogen) synthesis and subsequent degradation, was modelled with the following reaction scheme:

$$\begin{aligned} &\mathrm{ATP} + \mathrm{G1P} \xrightarrow{\mathrm{ADPGlcPPase}} \mathrm{ADPGlc} + \mathrm{PPi} \\ &\mathrm{ADPGlc} + \mathrm{GLY}_n \xrightarrow{\mathrm{GLYsy}} \mathrm{GLY}_{n+1} + \mathrm{ADP} \\ &\mathrm{GLY} \xrightarrow{\mathrm{GLYdeg}} \end{aligned} \quad (1)$$

When translated into mathematical terms, Eq. 1 shows a two zordinary differential equations (ODEs) system representing the dynamics of ADPGlc and glycogen (GLY), i.e. the state variables. Each ODE is essentially a balance between synthetic and degradation terms, as follows:

$$\frac{\mathrm{dADPGlc}}{\mathrm{d}t} = V_{\mathrm{AGPase}} - V_{\mathrm{GLYsy}} \quad (2)$$

$$\frac{\mathrm{dADPGlc}}{\mathrm{d}t} = V_{\mathrm{AGPase}} - V_{\mathrm{GLYsy}} \quad (3)$$

where V_{AGPase}, V_{GLYsy} and V_{GLYdeg} stand for the rate equations ruling the reactions in Eq. 1, V_{AGPase} represents the reaction rate catalyzed by ADPGlcPPase, and V_{GLYsy}, V_{GLYdeg} the glycogen synthesis and degradation rates, respectively.

ADPGlcPPase-catalyzed step

Recent work (Gómez Casati et al. 1999, 2000) has demonstrated the ultrasensitive response of V_{AGPase} toward its allosteric activator 3PGA, elicited by Pi and PEG-induced molecular crowding. The ultrasensitive behavior of ADPGlcPPase was apparent under zero- or first-order conditions with respect to its substrates, G1P and ATP. The rate expression for V_{AGPase}, based on the equation originally proposed by Petterson and Ryde-Petterson (1988) but modified to account for ultrasensitivity, reads:

$$V_{\mathrm{AGPase}} = \frac{V_{\mathrm{MAGP}}\mathrm{G1PATP}}{\left(\mathrm{G1P} + S_{0.5}^{\mathrm{G1P}}\right)\left(\mathrm{ATP} + S_{0.5}^{\mathrm{ATP}} + \frac{\left(I_{0.5}^{\mathrm{P}}\right)^{n_{\mathrm{H}}} P_i^{(n_{\mathrm{H}}+1)}}{\left(A_{0.5}^{\mathrm{3PGA}}\right)^{n_{\mathrm{H}}} \mathrm{3PGA}^{n_{\mathrm{H}}}}\right)} \quad (4)$$

with V_{MAGP}, $S_{0.5}^{\mathrm{G1P}}$, $S_{0.5}^{\mathrm{ATP}}$, $A_{0.5}^{\mathrm{3PGA}}$, $I_{0.5}^{\mathrm{P}}$ and n_{H} being the maximal velocity (V_{max}); the constant for G1P, ATP, and 3PGA giving half V_{max}; the constant for Pi giving 50% inhibition; and the Hill coefficient of the ADPGlcPPase reaction, respectively.

The second factor in the denominator accounts for the effect of Pi through the inhibition constant $I_{0.5}^{\mathrm{P}}$ together with the activation by 3PGA given by $A_{0.5}^{\mathrm{3PGA}}$. The Hill coefficient, n_{H}, and $A_{0.5}^{\mathrm{3PGA}}$ are modified through the cross-talk between 3PGA and Pi concentrations, which, combined with PEG-induced molecular crowding, accounts for the ultrasensitive behavior of ADPGlcPPase (Eqs. 5 and 6). Indeed, n_{H} and $A_{0.5}^{\mathrm{3PGA}}$ were shown to vary with Pi concentration (Gómez Casati et al. 2000). According to experimental evidence, n_{H} was assumed to have a component in the absence of Pi and to change as a function of its concentration, following a saturation curve (Gómez Casati et al. 2000):

$$n_{\mathrm{H}} = n_{\mathrm{o}} + n_{\mathrm{v}}\left(\frac{\mathrm{Pi}}{K_{\mathrm{i}}^{\mathrm{P}} + \mathrm{Pi}}\right) \quad (5)$$

with n_{o}, n_{v} and $A_{0.5}^{\mathrm{3PGA}}$ as the Hill coefficient in the absence of Pi, the maximal increase recorded in n_{H}, and the Pi concentration at which 50% of the maximal increase in n_{H} was registered, respectively.

The half-maximal activation constant, $A_{0.5}^{\mathrm{3PGA}}$, follows a Pi-dependent linear increase (Gómez Casati et al. 2000):

$$A_{0.5}^{\mathrm{3PGA}} = A_{0.5\mathrm{o}}^{\mathrm{3PGA}} + \mathrm{SpPi} \quad (6)$$

being $A_{0.5\mathrm{o}}^{\mathrm{3PGA}}$ the half-maximal activation constant for 3PGA in the absence of Pi, and Sp the slope of the $A_{0.5}^{\mathrm{3PGA}}$ variation as a function of Pi concentration (Gómez Casati et al. 2000).

Glycogen synthesis and degradation

Glucan synthesis in permeabilized cyanobacteria was measured after Gómez Casati et al. (2001), and it follows Michaelis–Menten kinetics with respect to ADPGlc and GLY (V_{GLYsy}):

$$V_{\mathrm{GLYsy}} = \frac{V_{\mathrm{MGS}}\mathrm{GLYADPGlc}}{K_{\mathrm{M}}^{\mathrm{GLY}}K_{\mathrm{M}}^{\mathrm{ADPG}} + K_{\mathrm{M}}^{\mathrm{ADPG}}GLY + K_{\mathrm{M}}^{\mathrm{GLY}}\mathrm{ADPGlc} + \mathrm{GLYADPGlc}} \quad (7)$$

Glycogen degradation (V_{GLYdeg}) was assumed to follow Michaelis–Menten kinetics:

$$V_{\mathrm{GLYdeg}} = \frac{V_{\mathrm{MGD}}\mathrm{GLY}}{\mathrm{GLY} + K_{\mathrm{M}}'^{\mathrm{GLY}}} \quad (8)$$

with V_{MGS} and V_{MGD} as V_{max} for glycogen synthesis and degradation, respectively. $K_{\mathrm{M}}^{\mathrm{ADPG}}$, $K_{\mathrm{M}}^{\mathrm{GLY}}$ and $K_{\mathrm{M}}'^{\mathrm{GLY}}$ are the half-saturation constants for ADPGlc and glycogen for V_{GLYsy} and V_{GLYdeg}, respectively.

Amplification factor

Sensitivity amplification was quantified through the amplification factor, A_s, as defined elsewhere (Koshland et al. 1982), and previously applied (Gómez Casati et al. 1999, 2000) as follows:

$$A_{\mathrm{s}} = \frac{\frac{V_{\mathrm{AGPase}}(\mathrm{f}) - V_{\mathrm{AGPase}}(1\%)}{V_{\mathrm{AGPase}}(1\%)}}{\frac{\mathrm{3PGA_{(f)}} - \mathrm{3PGA_{(1\%)}}}{\mathrm{3PGA_{(1\%)}}}} \quad (9)$$

where V_{AGPase}(f) is the rate displayed by the enzyme at a concentration corresponding to $\mathrm{3PGA_{(f)}}$ and $V_{\mathrm{AGPase}}(1\%)$ is the reaction rate with respect to a $\mathrm{3PGA_{(1\%)}}$ concentration corresponding to 1% of the V_{max} of the enzyme under a given experimental condition, i.e. for a particular set of substrate and effector concentrations.

Simulation parameters

Experimentally obtained model parameters are shown in Table 1 as indicated for each condition analyzed. ATP and G1P concentrations utilized in the simulations were taken from in vitro (Gómez Casati et al. 1999, 2000), in situ (Gómez Casati et al. 2001), or from those measured intracellularly (Kaiser and Bassham 1979; Preiss

Table 1 Ultrasensitive behavior in the glycogen biosynthetic pathway in situ. Incorporation of [^{14}C]Glc from [^{14}C]G1P and ATP in permeabilized cells of *Anabaena* PCC 7120 was determined at different concentrations of Pi and 3PGA. Values of 3PGA ratio correspond to the experimental results obtained in at least four independent experiments with triplicates in each of them

Condition Pi (mM)	$\mathrm{3PGA_{0.9}}$/ $\mathrm{3PGA_{0.01}}$	$\mathrm{3PGA_{0.9}}$/ $\mathrm{3PGA_{0.1}}$	n_{H}	A_{s}	
				0.01[a]	0.01[b]
0	313	30	2.0	3.1	n.d.[c]
1	186	33	2.2	5.7	6.1
5	80	6.7	3.1	20	17

[a] A_{s} obtained experimentally
[b] A_{s} obtained using the mathematical model
[c] Not determined

1982). The in situ values of V_{max} for the ADPGlcPPase and glycogen synthase steps were taken from initial rate curves as a function of G1P or ADPGlc as variable substrate, respectively (Table 1). ATP and G1P concentrations used in the simulations were saturating ones and around $S_{0.5}^{G1P}$, respectively, as happens in cells.

Mathematical procedures

The temporal evolution of state variables in the ODEs system (Eqs. 2, 3) was obtained by numerical integration with the ScoP package (Duke University) using the ADAMS' predictor–corrector method.

Stability and bifurcation analysis

The model was subjected to stability analysis with the software AUTO (Doedle 1986) running in a linux environment on a desktop computer. The steady values of the state variables are obtained with AUTO, in this way allowing us to analyze the sensitivity of the model to different parameters. The behavior of the model was studied as a function of 3PGA, Pi, $I_{0.5}^{P}$, V_{MGS}, and V_{MAGP}.

Results

We recently developed a procedure for the permeabilization of *Anabaena* PCC 7120, rendering cells that retain the activity of enzymes involved in glycogen synthesis (Gómez Casati et al. 2001). Label incorporation into α-1,4-glucan can be measured in permeabilized cyanobacterial cells, either from [^{14}C]G1P or [^{14}C]ADPGlc, the system allowing the evaluation of the in situ behavior of ADPGlcPPase or glycogen synthase, respectively (Gómez Casati et al. 2001). As expected from previous results, synthesis of α-1,4-glucan from ADPGlc was shown to be insensitive to 3PGA, whereas this metabolite highly affected the activity of ADPGlcPPase determined in situ (Gómez Casati et al. 2001).

That α-1,4-glucan synthesis from G1P in permeabilized cyanobacterial cells behaves ultrasensitively with respect to the allosteric activator of ADPGlcPPase (3PGA) is shown in Fig. 1. Indeed, a comparative study of the stimulus–response curves plotted as v/V_{max} versus logarithmic concentrations of 3PGA, revealed that the ultrasensitive behavior of α-1,4-glucan synthesis from G1P in situ is closer to the response of purified ADPGlcPPase assayed in vitro under BSA-elicited crowding than to the non-crowded condition (Fig. 1). Thus, $3PGA_{0.9}/3PGA_{0.1}$ ratios calculated from Fig. 1 give values of 81 or 9 for ADPGlcPPase assayed in vitro under non-crowded or crowded conditions, respectively, whereas glycogen synthesis in situ requires the 3PGA concentration to be increased 30-fold for ADPGlcPPase to attain 90% of its V_{max} (Table 1). Accordingly, significant amplification factors, A_s, are shown by the enzyme either in situ or when crowded in vitro, unlike the in vitro non-crowded condition (Fig. 1, inset).

Table 1 shows the quantitative characterization of ultrasensitive glycogen synthesis from G1P in permeabilized cells of cyanobacteria as affected by 3PGA and Pi, the allosteric effectors of ADPGlcPPase. In the presence of 1 mM or 5 mM Pi, substantially lower increases in the amount of 3PGA are needed to raise the maximal velocity for the polysaccharide synthesis from 1% or 10% to 90% than in the absence of Pi (Table 1). Values of A_s as high as 20 are achieved in the presence of 5 mM Pi (Table 1).

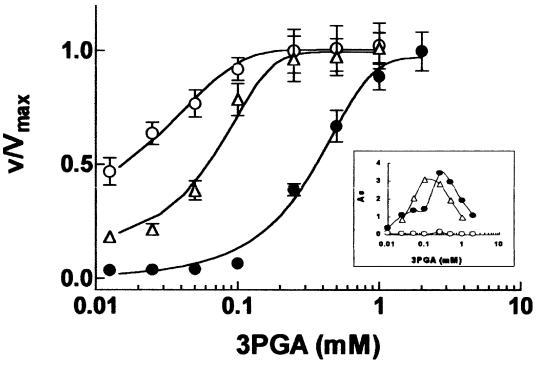

Fig. 1 Stimulus–response curves for ADPGlc synthesis catalyzed by purified cyanobacterial ADPGlcPPase and for α-1,4-glucan synthesis in permeabilized cells. ADPGlcPPase was assayed in vitro under conditions that were non-crowded (*empty circles*) or crowded by the presence of 33% (w/v) BSA (*filled circles*), and incorporation of radioactivity into α-1,4-glucan from [^{14}C]G1P was measured in permeabilized cells of *Anabaena* PCC 7120 (in situ, *triangles*). The *inset* shows the amplification factor, A_s, under each condition. V_{max} is the maximal velocity for the synthesis of ADPGlc or glucan under the respective experimental conditions, whereas v is the actual velocity value at the corresponding 3PGA concentration. The respective values of V_{max} are: 40 μmol min^{-1} mg^{-1} protein (in vitro, non-crowded); 66 μmol min^{-1} mg^{-1} protein (in vitro, crowded); and 0.015 μmol min^{-1} ml^{-1} permeabilized cells (in situ)

Glucan synthesis from G1P proceeds in an ultrasensitive manner as a function of the relative levels of 3PGA and Pi in permeabilized cyanobacterial cells (Fig. 2). The ultrasensitive behavior is evidenced by $3PGA_{0.9}/3PGA_{0.1}$ ratios of 33 and 6.7, as calculated from the kinetics in Fig. 2A assayed in situ in the presence of 1 mM or 5 mM Pi, respectively (Table 1). Furthermore, maximal A_s values of about 6 or 20 were experimentally determined for the activating effect of 3PGA measured in the presence of 1 mM or 5 mM exogenous concentrations of Pi, respectively (Fig. 2B).

The curves drawn through the experimental points in Fig. 2 belong to the simulation results obtained with a mathematical model of the glycogen synthetic pathway. This mathematical model (see *Materials and methods*) reproduces the incorporation of radioactivity from [^{14}C]G1P into α-1,4-glucan and the A_s as a function of the levels of 3PGA and Pi, at a qualitative and quantitative level, respectively (Fig. 2). The 3PGA/Pi ratio at which the triggering of ADPGlcPPase and glucan synthesis ultrasensitive behavior occurs, is quantitatively simulated (Fig. 2A, B; Table 1). Seemingly, the model is able to attain up to 17-fold maximal A_s compared with the 20-fold obtained experimentally in the presence of 5 mM Pi (Fig. 2B, Table 1). Thus, experimentally and theoretically, it is shown that the ultrasensitive response of the ADPGlcPPase rate translates into glucan

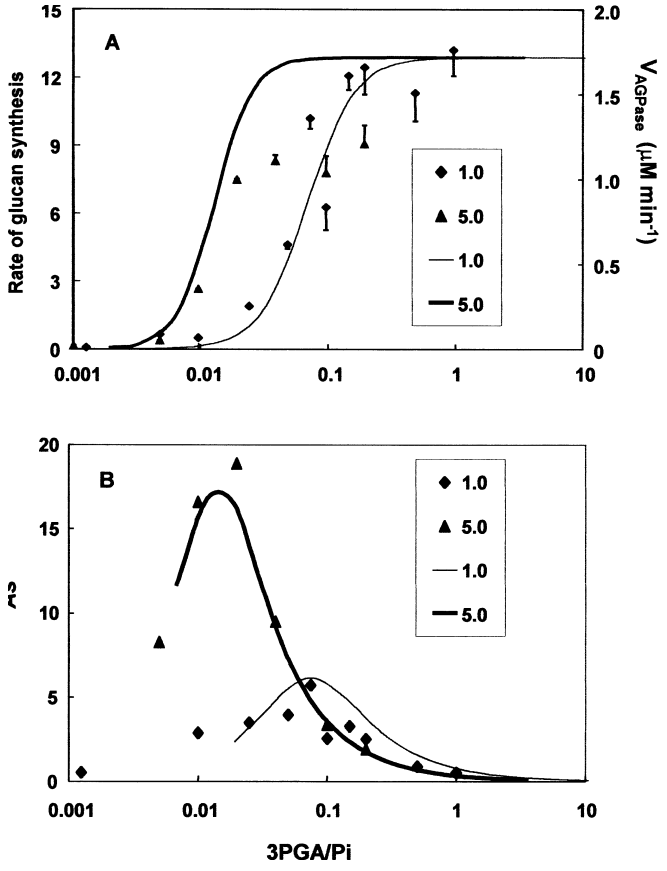

Fig. 2A, B Ultrasensitive α-1,4-glucan synthesis in permeabilized *Anabaena* cells as a function of 3PGA and Pi. The plotted experimental data (*diamonds*, *triangles*) show the incorporation of radioactivity into glucans (**A**) and the amplification factor, A_s (**B**), at varying ratios of 3PGA/Pi, in the presence of 1 mM or 5 mM of exogenous Pi concentrations in the cell suspension. Incubation conditions, sample processing and calculation of A_s were as described in *Materials and methods*. Simulations of the experimental results, using the mathematical model and parameter values as in Table 2, are shown by the curves. Modelling and experimental data related to A_s values may be both qualitatively and quantitatively compared whereas V_{AGPase} values are only comparable in qualitative terms since experimental points are expressed as relative activity instead of on a volumetric basis as in the model. The relative activity in situ is defined with respect to the maximal velocity of radioactivity incorporation into α-1,4-glucans from G1P at 30 °C (Gomez-Casati et al. 2001). The numbers in the key to symbols denote mM concentrations of Pi used in each case

steady-state levels as a function of 3PGA at varying concentrations of Pi (Fig. 2A).

The relative rate of α-1,4-glucan synthesis by permeabilized cells was dependent upon Pi concentrations within the range 1–10 mM, in the presence of low (0.05–0.1 mM) 3PGA levels (Fig. 3). The mathematical model also simulates the glucan synthesis (ADPGlcPPase velocity) dependence on Pi at different levels of 3PGA, as determined experimentally using permeabilized cyanobacterial cells (Fig. 4). At concentrations higher than 0.5 mM of the enzyme allosteric activator, the rate of glucan synthesis becomes insensitive to Pi (Figs. 3, 4). Moreover, model results further suggest that below

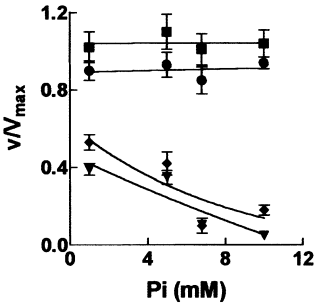

Fig. 3 Synthesis of α-1,4-glucan in situ as a function of Pi concentration in the presence of varying amounts (1 mM, *squares*; 0.5 mM, *circles*; 0.1 mM, *diamonds*; or 0.05 mM, *triangles*) of 3PGA. The normalized rates of glucan synthesis achieved in situ at varying Pi concentrations in the presence of high or low levels of 3PGA are represented. V_{max} corresponds to the maximal velocity of glucan synthesis achieved at each Pi concentration in the presence of saturating amounts of 3PGA (2.5 mM)

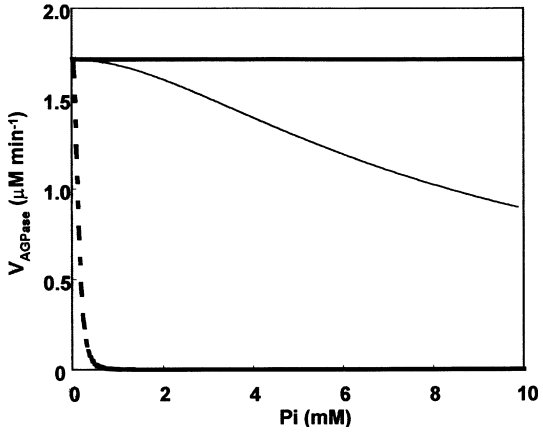

Fig. 4 Dependence of V_{AGPase} on Pi concentration in the presence of different 3PGA levels. Each curve was simulated at the 3PGA concentration of 0.05 (*dashed line*), 0.1 (*thin solid line*), or 0.5 (*wide solid line*) mM with the set of parameters corresponding to in situ conditions as specified in Table 2

1 mM Pi the amount of storage polysaccharide is strongly dependent on Pi levels at 3PGA concentrations lower than 0.05 mM (Fig. 4).

Discussion

In the present work we demonstrate that the glycogen synthetic pathway behaves ultrasensitively toward the ratio of the two allosteric effectors, 3PGA and Pi, of ADPGlcPPase in permeabilized cells of cyanobacteria. The ultrasensitive behavior of ADPGlcPPase is triggered under conditions of protein-elicited (similar to PEG-elicited, see Gómez Casati et al. 1999, 2000) crowding that would mimic the situation of the enzyme in permeabilized cells (Fig. 1).

A mathematical model that takes into account the ADPGlcPPase and glycogen synthetic and degradation steps, reproduces quantitatively or qualitatively the

Table 2 Model parameters used in the simulations

Parameter	In situ[b]
V_{MAGP}[a]	3.3
V_{MGS}	84
V_{MGD}	28
$S_{0.5}^{G1P}$(mM)	0.03
$S_{0.5}^{ATP(mM)}$	0.2
$I_{0.5}^{P}$(mM)	0.01
ATP (mM)	1.0
G1P (mM)	0.05
K_M^{ADPG}(mM)	0.053
K_M^{GLY}(mM)	0.001
K'^{GLY}_M(mM)	0.1
$A_{0.5o}^{3PGA}$ (mM)	0.086
Sp	0.043
N_o	1.5
N_v	1.75
K_i^P(mM)	1.0

[a]Concentration units have been chosen from experimental measurements according to the volume of the incubation mixture in each case

[b]The parameter values presented in this table correspond to the experimental results obtained in at least two independent experiments with triplicates in each of them (Gómez Casati et al. 1999, 2000,2001). Results were reproducible within ± 10% of the informed values

experimental data in permeabilized cells at three main levels: (i) the shape of the V_{AGPase} rate curve(Fig. 2A); (ii) the A_s (Fig. 2B), as a function of the ratio 3PGA/Pi; and (iii) the 3PGA/Pi ratio at which the ultrasensitive behavior of the enzyme is triggered (Fig. 2A). Thus, experimentally and theoretically, the ultrasensitive behavior of V_{AGPase} results in an ultrasensitive response by α-1,4-glucan steady-state levels as a function of 3PGA at varying concentrations of Pi (Fig. 2).

The model results indicate that ADPGlcPPase must be controlling the rate of the glycogen flux and glycogen synthase, operating with first-order kinetics with respect to ADPGlc for propagation of the ultrasensitive response resulting in the amplification of glycogen synthesis. The practical importance of our findings concerns the engineering of plants. Our results indicate that only when ADPGlcPPase is a main rate-controlling step can starch synthesis be amplified according to the 3PGA/Pi ratio (Fig. 2). Under these conditions, increasing the levels of the enzyme activity by molecular biological methods becomes worthwhile (Stark et al. 1992).

The ultrasensitive phenomenon described is very important for plant cell physiology since it responds to 3PGA, the primary product of CO_2 fixation, and is elicited by intracellular Pi concentrations, the ADPGlcPPase allosteric inhibitor (Preiss 1982; Iglesias and Preiss 1992; Stark et al. 1992; Preiss and Sivak 1998a, 1998b; Sivak and Preiss 1998). Indeed, ADPGlcPPase operates at high Pi levels inside the cell (Pi may reach 5–7 mM intracellularly during dark periods; Heldt et al. 1977). Thus, ultrasensitivity allows the enzyme to respond efficiently to small changes in 3PGA concentration against a background of high Pi.

Two other main advantages conferred by ultrasensitivity to storage-polysaccharide synthesis may be pointed out: (i) quick deactivation following significant amplification, thus hindering ATP wastage; and (ii) filtering out of small stimuli, making the ultrasensitive response more specific with respect to variation in the level of effectors. Certainly, the rate of glucan synthesis may abruptly change from 10% to 90% over a very modest range of allosteric effector concentrations (Fig. 2). The latter is in agreement with maximal amplifications achieved in cyanobacterial cells at very low 3PGA/Pi ratios (Fig. 1). Thus, sensitivity amplification in the storage-polysaccharide pathway may well serve to preserve the energetic economy of the system in the absence of light. When the light comes on again the system would be ready to be turned on to optimal functioning in response to a small change in 3PGA concentration over the background level.

A long-standing observation in the literature is that storage-polysaccharide levels (e.g. starch) are sensitive to Pi concentration, i.e. decreasing steady-state levels of the polysaccharide as Pi increases (Heldt et al. 1977). Levels of synthesized glucans were only sensitive (i.e. decreasing) to Pi in the presence of low (0.05 mM) 3PGA concentration. The mathematical model reproduces the expected relationship between the rate of ADPGlcPPase activity versus Pi at low 3PGA concentrations (i.e. in the range 1–100 μM 3PGA; Fig. 4). These data are partly in agreement with reported experimental evidence (Heldt et al. 1977) since at high concentrations of the allosteric activator (> 0.5 mM 3PGA) glucan levels remained high and insensitive to Pi concentration (Figs. 3, 4). Because levels of 3PGA of 4 mM or higher are usually found in photosynthesizing chloroplasts, it is expected that regulation by ultrasensitivity is not operative in conditions of full light, with ADPGlcPPase exhibiting high activity and insensitivity to Pi inhibition.

Although our results have been obtained in cyanobacteria (which accumulate glycogen), several lines of evidence suggest that they may be extended to starch synthesis by cells performing oxygenic photosynthesis (Preiss 1982; Iglesias et al. 1991; Iglesias and Preiss 1992; Preiss and Sivak 1998b; Sivak and Preiss 1998). Very recently, the ultrasensitive response has been shown in a wide range of biological processes (Aon et al. 2001) such as the mitogen-activated protein kinase cascade (Ferrell and Machleder 1998), the AMP-activated protein kinase cascade (Hardie et al. 1999), pheromone detection in mammalian neurons (Leinders-Zufall et al. 2000), and the response of bacterial flagellar motors to chemotactic signalling proteins (Cluzel et al. 2000). With the results presented herein, storage-polysaccharide synthesis in photosynthetic cells could also be considered as a process that shows an ultrasensitive response to regulators.

Synthetically, the ultrasensitive mechanism of amplification of glycogen synthesis may be important for

regulating the accumulation of reserve polysaccharides in photosynthetic cells, especially during transitions from light to darkness. This is the first time that this phenomenon has been reported to occur in the glycogen synthetic pathway of a photosynthetic prokaryote.

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