A phosphoglycerate to inorganic phosphate ratio is the major factor in controlling starch levels in chloroplasts via ADP-glucose pyrophosphorylase regulation

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Received 1 February 1999

Abstract Purified barley leaf ADP-glucose pyrophosphorylase, a key enzyme of the starch synthesis in the chloroplast stroma, was analysed with respect to its possible regulation by factors defining the metabolic/effector status of the chloroplast during light and dark conditions. The enzyme required 3-phosphoglyceric acid for the maximal activity and was inhibited by inorganic phosphate. The optimal pH for the enzyme was at circa 7.0, regardless of the presence or absence of 3-phosphoglyceric acid, whereas the maximal activation by 3-phosphoglyceric acid was observed at pH 8.5 and higher. Changes in the concentration of Mg^{2+} and dithiothreitol had little or no effect on the enzymatic activity of AGPase. It has been directly demonstrated for the first time that a 3-phosphoglyceric acid/inorganic phosphate ratio, a crucial regulatory parameter, could be directly related to a defined activation state of the enzyme, allowing the prediction of a relative AGPase activity under given conditions. The predicted changes in the enzyme activity were directly correlated with earlier reported responses of starch levels to the 3-phosphoglyceric acid/inorganic phosphate ratio in chloroplasts. Consequences of this for the starch biosynthesis are discussed.

Key words: Allosteric effector; Light regulation; Photosynthesis; Starch synthesis

1. Introduction

ADP-glucose pyrophosphorylase (AGPase) catalyses the production of ADP-glucose which is the substrate for the starch biosynthesis in all plants [1–3]. Mutations and antisense approaches that eliminate most or all of the AGPase activity cause a severe reduction in the starch content [4–8], indicating that the enzyme is the major, if not the only, route for providing carbon skeletons for the starch formation. In photosynthetic tissues, AGPase is tightly regulated by 3-phosphoglycerate (PGA) and inorganic phosphate (Pi), which serve as enzyme activator and inhibitor, respectively [1,2,9]. Based on studies with starch deficient mutants of *Arabidopsis* [10], AGPase was proposed to exert a considerable but not absolute control on the rates of starch synthesis in leaves. Under certain conditions, other enzymes of the starch pathway (e.g. phosphoglucomutase) may also substantially contribute to the control of starch formation. It has been proposed [10] that AGPase utilises only a fraction of its maximal activity during the starch synthesis, which may imply a regulatory mechanism involving yet unidentified factors affecting the AGPase rate in vivo. Elucidating these factors may have far reaching implications for biotechnological approaches aimed at tailoring starch levels to a given agricultural or industrial need via manipulation of the AGPase gene expression [1,4,11].

Starch synthesis in leaves occurs only during photosynthesis, possibly reflecting an increased internal stromal concentration of PGA, the first stable product of photosynthesis in many plants, and lower levels of Pi, which is utilised during the photophosphorylation [12–14]. However, the diurnal metabolic/effector status of the chloroplast stroma depends also on other factors, including pH changes, the Mg^{2+} concentration and redox status [12]. Light-dependent changes in these parameters have all been implicated in the diurnal regulation of a number of chloroplast enzymes [12,15]. No comprehensive investigation on the effects of these factors has been carried out with regard to leaf AGPase.

In the present study, the barley leaf AGPase was analysed with respect to its possible regulation by factors defining the metabolic/effector status of the chloroplast during light and dark conditions. The mode of regulation by pH, Mg^{2+} and the redox status is presented, along with a characterisation of the effects of the PGA/Pi ratio, identified as the key factor coupling the activation state of AGPase with the rate of starch synthesis in leaves.

2. Material and methods

The enzyme was purified to homogeneity from leaves of barley (*Hordeum vulgare*, cv. Bomi), as described in [16]. The specific activity of the purified enzyme, when assayed in the direction of pyrophosphorolysis, was 74 U/mg protein. The AGPase was stored with 20% glycerol at −80°C.

The enzyme was assayed in the direction of the ADP-glucose formation (synthesis direction), using Mg-ATP and ^{14}C-labelled glucose-1-phosphate (glu-1-P) as substrates [16,17]. Reactions were initiated with the enzyme. All components of the reaction mixtures are given in the legends to the Figures and Table.

The protein content was determined using the Bio-Rad assay, with bovine serum as a standard.

3. Results and discussion

3.1. Regulation by pH

The AGPase activity was monitored in different assay pHs, ranging from 6.4 to 9.2 (Fig. 1). The enzyme was assayed by measuring the rate of ADP-glucose production, i.e. in a physiologically relevant direction of the reaction. The maximal activity of the enzyme was observed at pH 7.0, regardless of the presence or absence of PGA, but the activating effect of PGA was most evident at higher pH values, with a circa 18-fold activation at pH 9.0 (Fig. 1). Similar data were obtained for spinach AGPase [2], but not for the enzyme from green

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PII: S0014-5793(99)00346-4

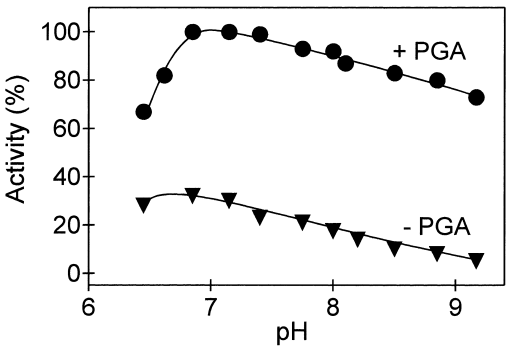

Fig. 1. Effects of pH and PGA on the activity of barley leaf AGPase. Assays contained 100 mM glycylglycine, 100 mM Mops, 7 mM $MgCl_2$, 2.5 mM ATP and 0.5 mM glu-1-P. Prior to assays, the pH of each reaction mixture was adjusted with 5 M NaOH. Where indicated, assays contained 2.5 mM PGA

algae where pH 8.5 was optimal for assays both in the absence and in the presence of PGA [18].

A diurnal change of the pH in chloroplast stroma is one of the most recognised factors defining the light/dark transition and it may potentially affect the activity of several photosynthetic enzymes [12]. However, based on data in Fig. 1, it appears unlikely that the pH regulation of AGPase represents any significant factor in the light-dependent control of the starch synthesis in plants. In fact, when considering the effect of pH aside from other regulatory factors (e.g. inhibition by Pi), the data suggest that AGPase should be more active at a lower pH, i.e. conditions prevalent in darkened chloroplasts [19]. In the dark, control of the AGPase activity is probably exerted via changes in the metabolite effector concentration and substrate availability rather than pH conditions.

3.2. Regulation by redox and magnesium

For several photosynthetic enzymes, dithiothreitol (DTT) is believed to mimick the effect of reduced thioredoxin(s), low molecular weight proteins, that regulate selected enzymes via redox control [12,15]. In the present study, DTT was tested as a possible effector of AGPase, using assay pH values that are characteristic of darkened (pH 7.0) and illuminated (pH 8.5) chloroplast stroma. It was expected that, provided that AGPase is responsive to redox control, assays would reveal DTT-induced changes in the AGPase activity. The assays were carried out for 15 min, which is usually a sufficient time for DTT-mediated reduction of thioredoxins-responsive enzymes [12]. At both pH values that were used in the present study, DTT decreased rather than increased the rate of the AGPase reaction, regardless of the presence or absence of PGA (Table 1). Also, a 30 min pre-incubation of the enzyme with 1 mM DTT before the assays had no effect on the AGPase activity (data not shown). The pre-incubation with DTT was also carried out for AGPase from crude leaf extracts to test for a possible direct effect of internal DTT-reduced thioredoxin(s) on the AGPase activity. No such an effect was detected (data not shown). Thus, the redox control seems unlikely to influence the starch formation via AGPase regulation. Although DTT is not a factor in regulating the AGPase activity, it may still have a stabilising effect on the enzyme.

Table 1
Effects of DTT, PGA and Mg^{2+} on the activity of barley leaf AGPase

Assay reagents	Activity (%)			
	pH 7.0		pH 8.5	
	$-Mg^{2+*}$	$+Mg^{2+}$	$-Mg^{2+*}$	$+Mg^{2+}$
Control (−PGA, −DTT)	100	105	31	24
+DTT	103	98	25	21
+PGA	350	370	280	285
+PGA+DTT	360	380	250	228

Reaction mixtures contained 100 mM Tes (pH 8.5) or 100 mM Mops (pH 7.0), 1 mM ATP, 0.5 mM glu-1-P and 1.5 mM $MgCl_2$. Where indicated, 1 mM PGA, 1 mM DTT and/or 3 mM $MgCl_2$ were added to the assays.
*Assays contained about 0.5 mM Mg^{2+}.

Similarly to DTT, changes in $[Mg^{2+}]$ had no apparent effect on the AGPase activity, regardless of the pH (Table 1). In chloroplast stroma, changes in $[Mg^{2+}]$ are intrinsically connected to the light-induced movement of protons across the thylakoid membrane. Depending on the experimental design, stromal levels of Mg^{2+} during light and dark conditions were estimated at 1–3 mM and 3–6 mM, respectively [20]. In the present studies, the levels of free magnesium were set at circa 0.5 mM ($-Mg^{2+}$) and 3.5 mM ($+Mg^{2+}$). Calculations of Mg^{2+} took into consideration the complexation of ATP and magnesium to form Mg-ATP, which is probably the true substrate of AGPase. Under assay conditions (1 mM ATP and 4.5 mM $MgCl_2$ for ($+Mg^{2+}$) conditions), virtually all ATP was in the form of Mg-ATP [21]. Although having apparently no effect on the AGPase activity, magnesium may be of importance in stabilising the conformation and/or aggregation states of AGPase subunits [22].

3.3. Regulation by the PGA/Pi ratio

Varying PGA at three varied fixed concentrations of Pi resulted in sigmoidal plots of the AGPase activity versus the PGA/Pi ratio (Fig. 2). An increasing concentration of PGA reversed the inhibition by Pi. For the three concentrations of Pi, the plots overlapped, with the highest activity observed at a PGA/Pi value of circa 1.5. The highest values were circa 25–30% higher than those obtained with PGA in the absence of Pi (100% activity in Fig. 2), confirming our earlier evidence [16] that Pi may act synergistically with PGA in activating the leaf enzyme. This may reflect a fine interplay between binding sites for PGA and Pi, leading to conformational changes fa-

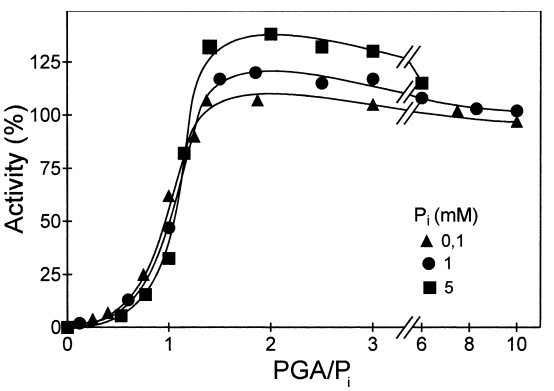

Fig. 2. The regulation of barley leaf AGPase by a PGA to Pi ratio. Pi concentrations were maintained at 0.2, 1 and 5 mM, whereas PGA was varied from 0 to 30 mM. The activity of 100% refers to that recorded for assays with 1 mM PGA in the absence of Pi. Assays contained 200 mM Tes (pH 8.0), 7 mM $MgCl_2$, 1 mM ATP, 0.5 mM glu-1-P and indicated concentrations of PGA and Pi.

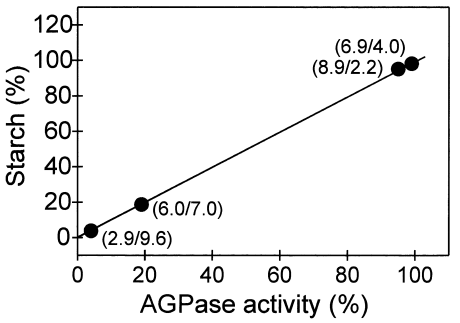

Fig. 3. Control of the starch synthesis via effects of the PGA/Pi ratio on the AGPase activity. Data for starch levels and the corresponding stromal PGA and Pi concentrations (allowing for calculation of PGA/Pi ratios) were taken from [24]. The calculated PGA/Pi ratios were correlated with the AGPase activity obtained in the present study (Fig. 2). Values in brackets correspond to stromal concentrations of PGA and Pi obtained in [24]. The value for 100% activity of AGPase was taken from Fig. 2 and it corresponds to the maximal activity at the PGA/Pi ratio when Pi was kept at 5 mM.

cilitating or restraining the access of a given effector [16,23]. The synergistic effect of PGA and Pi in activating AGPase, as found for the barley leaf enzyme, has not been reported for other AGPases, either of plant or bacterial origin.

The most important feature of this study is that the plots of the PGA/Pi ratio versus the AGPase activity roughly overlapped, regardless of individual concentrations of PGA or Pi (Fig. 2). Previously, the effect of PGA and Pi was studied in such a way as to vary either PGA or Pi in one or two concentrations of the second effector, resulting in a range of non-overlapping sigmoidal response curves [9,16]. In the present study, it was the PGA/Pi ratio that was varied over a wide range of values at three pre-set levels of Pi. The data in Fig. 2 may be regarded as a calibration curve to predict the relative activity of the enzyme. Any concentrations of PGA and Pi resulting in a PGA/Pi ratio below circa 1.3 would deem the enzyme prone to a very fine regulation. If, on the other hand, levels of both effectors result in PGA/Pi ratios above 1.3, this would have only a relatively small effect on catalysis (Fig. 2).

3.4. Control of the starch synthesis via regulation of AGPase

It has been noted that starch formation may directly depend on the PGA/Pi ratio in isolated chloroplasts [24]. The authors provided the chloroplasts with a defined concentration of Pi to estimate the kinetics of PGA and Pi changes in the stroma in relation to starch levels. To my knowledge, this is the only study where stromal levels of both PGA and Pi as well as the starch content were determined. In the present study, these data were plotted against the expected AGPase activity depending on the reported stromal PGA/Pi ratios and starch levels (Fig. 3). The resulting plot gave a straight line, where starch levels were directly correlated with the AGPase activity. This high correlation between the state of activation of AGPase and the PGA/Pi ratio versus the starch synthesis (Figs. 2 and 3) is unexpected given earlier data from studies on mutant *Arabidopsis* plants, where the AGPase activity apparently was in large excess over that required for starch formation [10]. However, in these earlier studies, the authors took the pyrophosphorolysis reaction as the measure of the V_{max} of AGPase towards the starch production. This is incorrect since the V_{max} in the synthesis direction (ADP-glucose production), i.e. physiologically relevant reaction, is circa 5–10-fold lower than the V_{max} of the pyrophosphorolysis reaction (glu-1-P formation) [16,17]. Thus, the conclusion that AGPase can not utilise more than 20% of its potential activity in vivo [10] needs to be revised. In fact, when accounting for the lower V_{max} of the synthesis versus pyrophosphorolysis reactions, the data obtained in [10] imply that the AGPase exerts an absolute control over the whole range of its activity on the starch production. It should be emphasised that, in the present study, AGPase was assayed by monitoring the rate of ADP-glucose formation, i.e. in the physiologically relevant direction of the reaction. In most papers on AGPase, the enzyme was assayed in the reverse (pyrophosphorolytic) direction only and such data do not necessarily reflect the actual properties of the enzyme in vivo.

The almost perfect fit for the AGPase activity versus starch levels (Fig. 3) strongly suggests that the PGA/Pi mode of regulation is the key, if not the only, mechanism controlling the starch formation under conditions reported in [24]. In certain cases (e.g. a high PGA/Pi ratio) other enzymes may exert some control in the starch synthesis [10], but the AGPase step must be certainly considered as the major factor at the PGA/Pi ratio range of 0.5–1.3. It has been proposed [10], based on work with starch deficient *Arabidopsis* mutants, that AGPase may represent the key limiting factor under conditions of low light (75 μmol/m^2/s), but not under high light exposure (600 μmol/m^2/s). In the latter case, AGPase was only partially controlling the rate of starch formation. The present data partially support these conclusions since, at high light, one would expect a high PGA/Pi ratio which would make AGPase fully active and not sensitive to further regulation. Under these conditions, other enzymes of the starch pathway, especially those having activities comparable to (or lower than) an overall rate of the starch synthesis, may become limiting. On the other hand, at low light, the PGA/Pi ratio is lower and AGPase is highly regulated by an even very small change in this ratio (Fig. 2). The data in [24] were obtained at a light intensity of circa 60–70 μmol/m^2/s, which should bring about a relatively low PGA/Pi ratio. This would result in an activation state of AGPase which is directly correlated with the rate of starch formation in vivo.

Assuming a direct relation between the activation state of AGPase and the starch synthesis (Fig. 3), the data in Fig. 2 theoretically allow one to estimate the stromal concentration of either PGA or Pi, provided that the rate of starch accumulation and stromal levels of one of these effectors are determined. Stromal levels of Pi are especially difficult to determine experimentally due to the fact that plants usually have a large amount of metabolically inactive Pi in vacuoles and the chloroplastic Pi itself may be to a large extent sequestered and made unavailable for metabolism [25]. The knowledge about the effects of the PGA/Pi ratio on starch, assuming that starch levels are directly related to the activation state of AGPase, should represent a useful tool for modelling photosynthetic responses in relation to starch and both PGA and Pi levels. It should be emphasised that the light/dark regulation of the AGPase activity via changes in the effector concentration must be considered separately from differential effects of the light/dark transition on the expression of AGPase genes in leaves [26]. The latter mode of regulation has been linked with the photoperiod-mediated changes in internal sugar/osmoticum status. Under experimental conditions, the sugar/osmoticum regulation of AGPase gene expression did not

lead to changes in an overall protein content nor in its enzymatic activity, suggesting a tight posttranscriptional control [26].

Acknowledgements: The author thanks Dr. M. Wilczynska for help with the preparation of Figures. This research was supported, in part, by grants from the Swedish Foundation For Strategic Research and from the Swedish Natural Science Research Council.

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