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Domain Swapping between a Cyanobacterial and a Plant Subunit ADP-Glucose Pyrophosphorylase

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ADP-glucose pyrophosphorylase (ADP-Glc PPase) catalyzes the regulatory step in the pathway for synthesis of bacterial glycogen and starch in plants. ADP-Glc PPases from cyanobacteria (homotetramer) and from potato (*Solanum tuberosum*) tuber (heterotetramer) are activated by 3-phosphoglycerate and inhibited by inorganic orthophosphate. To study the function of two putative domains, chimeric enzymes were constructed. PSSANA contained the N-terminus (292 amino acids) of the potato tuber ADP-Glc PPase small subunit (PSS) and the C-terminus (159 residues) of the *Anabaena* PCC 7120 enzyme. ANAPSS was the inverse chimera. These constructs were expressed separately or together with the large subunit of the potato tuber ADP-Glc PPase (PLS), to obtain homo- and heterotetrameric chimeric proteins. Characterization of these forms showed that the N-terminus determines stability and regulatory redox-dependent properties. The chimeric forms exhibited intermediate 3-phosphoglycerate activation properties with respect to the wild-type homotetrameric enzymes, indicating that the interaction between the putative N- and C-domains determines the affinity for the activator. Characterization of the chimeric heterotetramers showed the functionality of the large subunit, mainly in modulating regulation of the enzyme by the coordinate action of 3-phosphoglycerate and inorganic orthophosphate.

Keywords: ADP-glucose pyrophosphorylase — Cyanobacteria — Glycogen synthesis — Metabolic regulation — Potato tuber — Starch synthesis.

Abbreviations: ADP-Glc, ADP-glucose; ADP-Glc PPase, ADP-glucose pyrophosphorylase; ANA, C-terminus of *Anabaena* Pcc 7120 ADP-Glc PPase; BSA, bovine serum albumin; DTT, dithiothreitol; Glc1P, glucose 1-phosphate; 3-PGA, 3-phosphoglycerate; P_i, orthophosphate; PLS, large subunit of the potato tuber ADP-Glc PPase; PP_i, pyrophosphate; PSS, small subunit of the potato tuber ADP-Glc PPase.

Introduction

Starch and glycogen are α-1,4-glucans that constitute the main carbon and energy storage products of many organisms. In bacteria (synthesizing glycogen) and plants (accumulating starch), those polysaccharides are built up in a pathway that uses ADP-glucose (ADP-Glc) as the glucosyl donor molecule (Preiss and Sivak 1998, Sivak and Preiss 1998, Ballicora et al. 2003, Ballicora et al. 2004). The synthetic pathway is regulated at the level of ADP-Glc synthesis. This step, a reaction catalyzed by ADP-glucose pyrophosphorylase (ADP-Glc PPase; EC 2.7.7.27), uses glucose 1-phosphate (Glc1P) and ATP as substrates and releases ADP-Glc and pyrophosphate (PP_i) as products (Sivak and Preiss 1998, Ballicora et al. 2003). The reaction can be measured in both the forward (synthesis) and reverse (pyrophosphorolysis) direction. Most of the ADP-Glc PPases so far characterized are allosterically regulated by key intermediate metabolites of the major carbon assimilatory pathway of the organism (Preiss and Sivak 1998, Sivak and Preiss 1998, Ballicora et al. 2003, Ballicora et al. 2004). They have been classified into nine different classes based on their specificity for allosteric regulators (Ballicora et al. 2003, Ballicora et al. 2004). Enzymes from organisms performing oxygenic photosynthesis are included in class VIII as mainly activated by 3-phosphoglycerate (3-PGA) and inhibited by orthophosphate (P_i). The ratio of both effectors is a key determinant of the level of enzyme activity (Iglesias and Preiss 1992, Sivak and Preiss 1998). Enzymes from cyanobacteria and from potato tuber are both in this class.

The native bacterial enzymes, including those from cyanobacteria, are tetrameric (α_4) with only one type of subunit of molecular mass about 50 kDa (Iglesias and Preiss 1992, Sivak and Preiss 1998, Ballicora et al. 2004). Conversely, the plant enzymes are composed of two types of subunits. For convenience, they were named small (α, ~50–54 kDa) and large (β, ~51–60 kDa) even in cases where the difference is not more than ~1 kDa (Ballicora et al. 2003, Ballicora et al. 2004). The role of each subunit was revealed by heterologous expression in *Escherichia coli* of the genes encoding the mature small and large subunits of the potato tuber enzyme (Iglesias et al. 1993, Ballicora et al. 1995). The small subunit is catalytic and forms an active homotetramer (α_4) when expressed alone. The kinetic properties of the latter are similar to those of the heterotetrameric enzyme when assayed at saturating concentrations of the activator 3-PGA (Ballicora et al. 1995). However, the tetramer

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composed solely of small subunits (α_4) has defective regulatory properties. It is strictly dependent on the activator for the activity, with about 10-fold lower affinity for 3-PGA than the heterotetramer $\alpha_2\beta_2$ (Ballicora et al. 1995). The crystallographic structure of an allosterically inhibited form of this homotetrameric (α_4) plant enzyme has been solved (Jin et al. 2005). The large subunit alone has no detectable activity and its main role is altering the allosteric properties of the small subunit (Iglesias et al. 1993, Ballicora et al. 1995, Ballicora et al. 1998, Fu et al. 1998b, Frueauf et al. 2003). The emergence of two different subunits in the plant enzyme, each having a specific function, probably arose from the need for tissue-specific regulation (Krishnan et al. 1986, Smith-White and Preiss 1992, Sivak and Preiss 1998, Crevillén et al. 2003).

The ADP-Glc PPase from potato tuber has an intermolecular disulfide bridge that links the two small subunits by the Cys12 residue. The redox state of this residue is critical for the level of activity, the heat stability and the allosteric activation of the enzyme (Fu et al. 1998a, Ballicora et al. 1999, Ballicora et al. 2000). Thus, this enzyme is activated by reduction of the Cys12 disulfide linkage, which may be exerted chemically by dithiothreitol (DTT) (Fu et al. 1998a) or by reduced thioredoxin *f* and *m* from spinach leaves (Ballicora et al. 2000). The activation is reversed by oxidized thioredoxin. Also, reduction of the potato tuber enzyme increases both the affinity for 3-PGA and the fold activation exerted by the allosteric activator (Ballicora et al. 2000). This redox regulatory mechanism has been proposed to be operative in vivo (Fu et al. 1998a, Ballicora et al. 2000), which is supported by different experimental evidence (Tiessen et al. 2002, Geigenberger 2003, Hendriks et al. 2003, Geigenberger et al. 2005). Cys12 is conserved in the ADP-Glc PPases from plant leaves and other tissues (Ballicora et al. 1999). On the other hand, the heat stability of the potato tuber enzyme is dependent on the formation of this specific intersubunit disulfide bridge (Ballicora et al. 1999).

From an evolutionary point of view, cyanobacterial ADP-Glc PPases occupy a central position between the bacteria and plant enzymes with respect to structure and regulatory properties (Ballicora et al. 2004). Cyanobacterial and higher plant enzymes display similar specificity for allosteric regulators but differ in quaternary structure (Iglesias and Preiss 1992). Most probably, during evolution in photosynthetic eukaryotes, duplication and further divergence of the ADP-Glc PPase gene generated two different types of subunits (Ballicora et al. 2004). Recent mutagenesis studies explained the lack of activity of the large subunits from storage tissues (Ballicora et al. 2005). In establishing structure–function relationships between ADP-Glc PPases, a main question arises from the comparison between cyanobacterial and plant enzymes. What are the structural differences that determine the high affinity for the activator in the cyanobacterial homotetrameric enzyme and make it active in the absence of 3-PGA, when the plant small subunit requires the large subunit to exhibit a similar behavior?

The ADP-Glc PPase is predicted to comprise two domains. The C-domain contains the residues responsible for the activation and inhibition of the plant and cyanobacterial enzymes, whereas the N-domain contains the substrate site (Ballicora et al. 2004). In bacterial enzymes, the C-domain is responsible for the specificity of the activators but the N-domain also has residues responsible for the allosteric regulation (Ballicora et al. 2003). Recently, it has been shown that in the *E. coli* enzyme, these putative domains can be separated and expressed together to obtain a fully functional enzyme (Bejar et al. 2004). In the present work, we designed and constructed hybrid products between ADP-Glc PPases from *Anabaena* PCC 7120 and potato tuber (small subunit) to understand the role of the N- and C-terminal domains in determining allosteric properties. The N- and C-domains of these enzymes have been swapped and the kinetic and stability properties were studied.

Results

Among ADP-Glc PPases that are allosterically regulated by 3-PGA and P_i, we selected the enzyme from *Anabaena* (ANA) and the small subunit of the potato tuber ADP-Glc PPase (PSS) to swap the putative N- and C-domains because their properties have been well characterized (Iglesias et al. 1991, Iglesias et al. 1993, Ballicora et al. 1995, Gomez-Casati et al. 2000a, Frueauf et al. 2003). The two polypeptides are homologous (65% identity) with only a few small insertions in PSS (one of three residues and two of one residue). The main difference is that PSS is 17 amino acids longer in the N-terminus. We characterized the chimeric enzymes to establish the relevance of the different domains in determining the apparent affinity for allosteric regulators, as well as to analyze the interaction between subunits in heterotetrameric ADP-Glc PPases.

Expression and purification of chimeric ANAPSS and PSSANA ADP-Glc PPases

The specific activities in the crude extract of the PSSANA and ANAPSS chimeric enzymes were 0.04 and 0.07 U mg^{-1}, respectively; as compared with values of 0.19 U mg^{-1} for PSS and 0.27 U mg^{-1} for ANA (assayed at 2.5 mM 3-PGA in the pyrophosphorolysis direction). The wild-type and chimeric ADP-Glc PPases remained stable during the purification procedure. The enzymes were purified 40- (PSS), 36- (ANA), 50- (PSSANA) and 66-fold (ANAPSS), with total yields of 60, 55, 45 and 43%, respectively. Similar results were obtained when the different chimeric constructs were co-expressed with the potato large subunit (PLS). PSS + PLS was purified 50-fold to reach 20 U mg^{-1} specific activity. PSSANA + PLS and ANAPSS + PLS were purified to 3.5 and 5.2 U mg^{-1} after 49- and 42-fold purification, respectively. The partially purified enzymes (wild type and chimeric) were analyzed by gel filtration. They eluted as proteins of about 200–230 kDa, indicating tetrameric structures for all of them (not shown). In crude

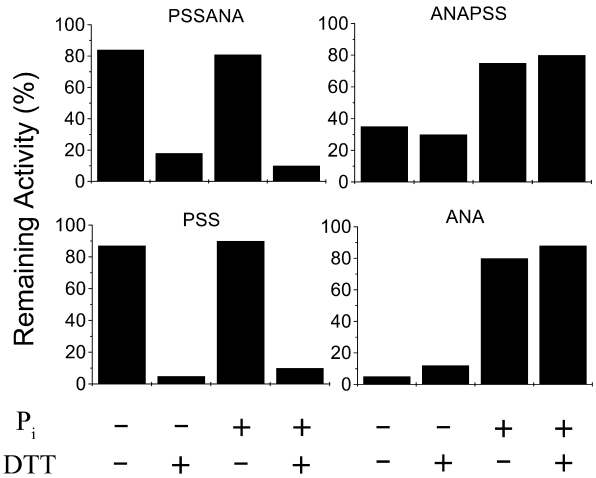

Fig. 1 Stability of different ADP-Glc PPase constructs. Thermal treatments to test the stability of the enzymes were performed as described in Materials and Methods.

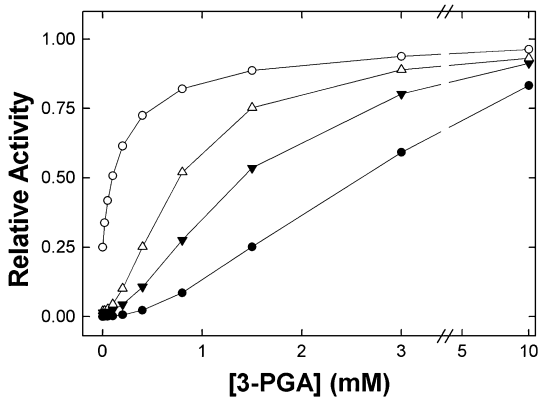

Fig. 2 Activation by 3-PGA in the pyrophosphorolysis direction of the wild-type and chimeric ADP-Glc PPases. The enzymes are ANA (open circles), PSS (filled circles), PSSANA (filled triangles) and ANAPSS (open triangles). The activity in each case was related to the V_{max} calculated from the plot for the respective enzyme: ANA, 9.7 U mg^{-1}; PSS, 11.4 U mg^{-1}; PSSANA, 2.5 U mg^{-1}; and ANAPSS, 4.8 U mg^{-1}.

extracts, 3-PGA enhanced the pyrophosphorolysis activity exhibited by PSSANA and ANAPSS 120- and 80-fold, respectively (see below). This effect of 3-PGA on the chimeric enzymes was intermediate with respect to the wild-type forms. ANA was activated about 4-fold by 3-PGA, whereas PSS is practically inactive in its absence (>500-fold activation) (Ballicora et al. 1995).

Thermal stability of chimeric ANAPSS and PSSANA

Heat stability is an important attribute of plant ADP-Glc PPases. The yield of some cereal crops grown in hot climates is possibly determined by the stability of the enzymes responsible for starch synthesis (Keeling et al. 1993, Singletary et al. 1994). Attempts have been pursued to improve the stability of the maize ADP-Glc PPase (Greene and Hannah 1998). Previous reports have shown that ADP-Glc PPases from potato tuber and *Anabaena* PCC 7120 are relatively stable to heat treatment (5 min at about 50–70°C), but under different conditions (Ballicora et al. 1999, Gomez-Casati et al. 2000b). The enzyme from cyanobacteria (ANA) is mainly stabilized by the inhibitor P_i (Gomez-Casati et al. 2000b). On the other hand, Cys12 in PSS needs to form an intersubunit disulfide bridge in order to confer heat stability to the plant enzyme (Ballicora et al. 1999). For that reason, the potato tuber ADP-Glc PPase is unstable in the presence of a reductant such as DTT (Ballicora et al. 1999). Conditions for thermal stability of the chimeric enzymes correlated with the corresponding N-terminus of each hybrid protein (Fig. 1). ANAPSS behaved as the ANA enzyme because it was stabilized by P_i and was insensitive to DTT (Fig. 1). Conversely, the thermal stability of PSSANA was not affected by P_i, and the enzyme was unstable in the presence of DTT (Fig. 1). These results indicate that the N-terminus of the cyanobacterial and potato tuber (PSS) enzymes determines the stability properties of the corresponding ADP-Glc PPase, and not the C-terminus. In addition, similar results were observed when PLS was co-expressed with each of the chimeric enzymes (data not shown), suggesting that the plant large subunit is not critical for the stability properties of the enzyme.

Regulatory properties of chimeric ADP-Glc PPases

Saturation curves for 3-PGA in the pyrophosphorolysis direction for both chimeric enzymes were intermediate between those obtained with the wild-type PSS and ANA proteins (Fig. 2). In agreement with previous reports (Iglesias et al. 1991, Iglesias et al. 1993, Ballicora et al. 1995), ANA was activated by 3-PGA about 4-fold, with hyperbolic kinetics and a relatively high affinity ($A_{0.5}$ = 0.15 mM). On the other hand, activation of PSS was sigmoidal, practically inactive in the absence of 3-PGA, and with a low affinity ($A_{0.5}$ = 4.8 mM) (Table 1). PSSANA and ANAPSS activation kinetics were in both cases sigmoidal and clearly distinct. They were activated nearly 80- and 50-fold, with $A_{0.5}$ values of 3.4 and 0.8 mM, respectively (Fig. 2 and Table 1). The chimeric enzymes were also co-expressed with the PLS and characterized (Table 1). As reported previously (Ballicora et al. 1995), PLS was modulatory because it altered the properties of the PSS. The heterotetrameric enzyme (PSS + PLS) was active in the absence of 3-PGA, with a higher affinity toward the activator (Table 1). The chimeric enzymes were also sensitive to modulation by PLS, which increased the apparent affinity for 3-PGA in the heterooligomer proteins that resulted from the co-expression. The $A_{0.5}$ values for PSSANA + PLS and ANAPSS + PLS were respectively lower than for PSSANA and ANAPSS (Table 1).

The behavior of chimeric enzymes in the synthesis direction was similar to what was observed in the pyrophosphorolysis direction (Table 2). In both cases, the hybrid proteins exhibited intermediate properties in terms of activation by 3-

Table 1 Kinetic parameters for 3-PGA activation in the pyrophosphorolysis direction

Construction	3-PGA		
	$A_{0.5}$ (mM)	n_H	Activation (-fold)
PSS	4.8 ± 0.5	3.3	≫500
PSS + PLS	0.021 ± 0.002	1.9	3
PSSANA	3.4 ± 0.3	2.0	130
PSSANA + PLS	0.84 ± 0.04	1.5	75
ANAPSS	0.79 ± 0.05	1.8	90
ANAPSS + PLS	0.11 ± 0.01	2.4	54
ANA	0.15 ± 0.01	1.0	4

Table 2 Kinetic parameters for 3-PGA activation in the synthesis direction

Construction	DTT (mM)	Activation by 3PGA		
		$A_{0.5}$ (mM)	n_H	Activation (-fold)
PSS	–	6.4 ± 0.6	2.8	≫500
	5	2.8 ± 0.2	1.8	≫500
PSS + PLS	–	0.24 ± 0.02	1.7	12
	5	0.08 ± 0.01	1.0	22
PSSANA	–	5.2 ± 0.3	2.0	60
	5	2.5 ± 0.2	2.5	100
PSSANA + PLS	–	6.3 ± 0.5	1.5	45
	5	2.7 ± 0.1	1.8	100
ANAPSS	–	1.0 ± 0.1	2.4	80
	5	0.85 ± 0.06	1.6	75
ANAPSS + PLS	–	0.84 ± 0.05	3.3	65
	5	0.81 ± 0.04	2.5	80
ANA	–	0.061 ± 0.003	1.0	12
	5	0.055 ± 0.003	0.9	16

Assays were performed as described in Materials and Methods with or without 5 mM DTT, as indicated. The enzyme was incubated for 5 min in the respective assay medium before the reaction was started with the substrate [^{14}C]Glc1P.

PGA compared with the wild-type enzymes. The only difference was that the properties of heterotetrameric chimeric enzymes containing PLS were not significantly different from those of the homotetramer constructs (Table 2). It is worth pointing out that changes in affinity for 3-PGA of heterotetrameric PSS + PLS compared with PSS are higher in the pyrophosphorolysis direction. The heterotetrameric enzyme (PSS + PLS) from potato tuber exhibited an affinity for 3-PGA about 220-fold higher than PSS in the pyrophosphorolysis direction, whereas in the ADP-Glc synthesis direction such a change was one order of magnitude lower (Tables 1, 2).

DTT modified the activation by 3-PGA of the chimeric enzymes having the N-terminus from the potato tuber small subunit (Table 2). DTT increased the fold activation by 3-PGA

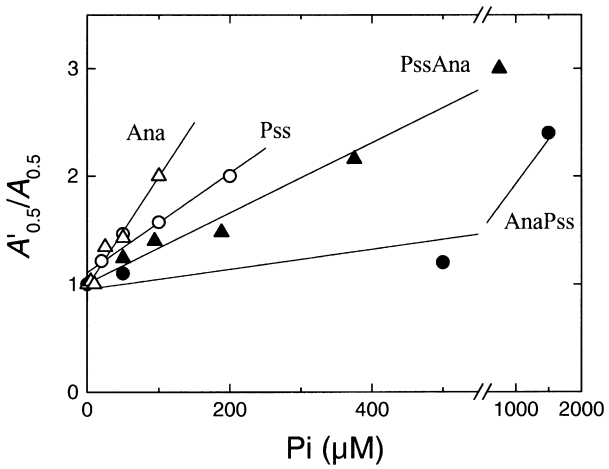

Fig. 3 Effect of P_i on the activation by 3-PGA. $A'_{0.5}$ and $A_{0.5}$ are the activation parameters for 3-PGA in the presence and absence of Pi, respectively.

and reduced the $A_{0.5}$ for the enzymes PSS, PSS + PLS, PSSANA and PSSANA + PLS (Table 2). Conversely, no significant changes were observed for the enzymes ANA, ANAPSS and ANAPSS + PLS (Table 2). It has been determined that the reduction of Cys12 in the small subunit of the potato tuber ADP-Glc PPase is relevant for the activation of the enzyme (Fu et al. 1998a) through a regulatory mechanism involving post-translational modification by thioredoxin (Ballicora et al. 2000). This regulation via enzyme reduction is exerted in the plant enzyme but not in the bacterial enzyme, since the latter lacks the equivalent cysteine residue (Fu et al. 1998a). In fact, in the N-terminus, the ANA enzyme has only one cysteine at position 45. Although this residue is conserved in PSS (Cys62), it seems not to play a functional role in enzyme regulation nor in protein stability. Our results indicate that the N-terminus in the chimeric enzymes is functional to confer not only stability to the proteins but also catalytic and regulatory properties to the different hybrid enzymes. The redox regulation can occur even in the presence of a 'foreign' regulatory C-domain from an enzyme that is not originally redox regulated.

To characterize further the regulatory properties of the chimeric ADP-Glc PPases, we studied how the inhibitor P_i affected the kinetic parameters of activation by 3-PGA. Fig. 3 shows how P_i increased the $A_{0.5}$ values for the allosteric activator in the different constructs. The affinity for 3-PGA was reduced by the presence of different levels of P_i in the assay medium, due to the sensitivity of the different enzymes, in decreasing order: ANA > PSS > PSSANA > ANAPSS (Fig. 3).

To quantify better the interaction between both allosteric effectors, we defined the parameter I_D (Materials and Methods) as a measurement of the apparent affinity for the inhibitor related to its interaction with the activator. The lower the I_D value, the lower the concentration of inhibitor needed to reduce by 2-fold the apparent affinity towards the activator. Interest-

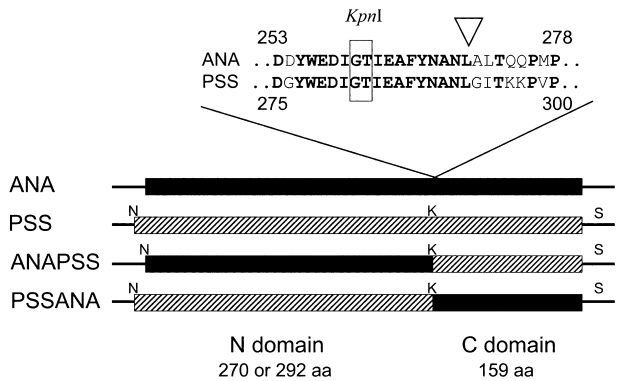

Fig. 4 Chimeric enzymes for domain swapping. Constructs ANAPSS and PSSANA were assembled as described in Materials and Methods. ANA and PSS represent the genes for the ADP-Glc PPase from *Anabaena* and the small subunit of the potato tuber enzyme, respectively. Restriction sites *Nco*I, *Kpn*I and *Sac*I are depicted as N, K and S, respectively. The box marked as *Kpn*I in the upper inset illustrates where a silent mutation was introduced to obtain a compatible restriction site. A white triangle indicates where swapping occurs.

ingly, PLS played a relevant role in coordinating regulation of ADP-Glc PPase, as I_D values for PSS, PSSANA and ANAPSS were significantly lower in the respective heterotetramers containing the large subunit (Table 3). These results are in good agreement with the proposed involvement of this subunit in the modulation of the enzyme activity (Smith-White and Preiss 1992, Ballicora et al. 1995, Frueauf et al. 2003, Ballicora et al. 2004).

Discussion

ADP-Glc PPases from different sources seem to have evolved from a common ancestor and probably share a similar three-dimensional structure (Smith-White and Preiss 1992, Frueauf et al. 2001, Ballicora et al. 2003, Ballicora et al. 2004). Secondary structure prediction, alignment with other sugar nucleotide pyrophosphorylases and site-directed mutagenesis studies identified the central region of the protein as the substrate-binding and catalytic domain (Frueauf et al. 2001, Ballicora et al. 2003, Ballicora et al. 2004). It is expected that the N- (20–30 residues) and C-termini (~150 residues) determine the distinctive regulatory properties exhibited by the different classes of ADP-Glc PPases. In addition, studies on the characterization of chimeric enzymes from *E. coli* and *Agrobacterium tumefaciens* have shown that regulation in bacterial enzymes is exerted by the combined arrangement and interaction between the N- and the C-termini (Ballicora et al. 2002). In the enzyme from eukaryotes, the presence of a second subunit establishes a higher degree of complexity in determining the structure–function relationship. In addition, the enzyme from potato tuber is regulated at an additional level by a post-translational redox mechanism involving a disulfide bridge

Table 3 Effect of P_i on the apparent affinity for 3-PGA

Construction	P_i I_D (μM)
ANA	97
PSS	239
PSSANA	335
ANAPSS	1,038
PSS + PLS	87
PSSANA + PLS	225
ANAPSS + PLS	174

Activation curves for 3-PGA were performed in the ADP-Glc synthesis direction at different concentrations of P_i. Values for I_D were the concentration of P_i necessary to increase the $A'_{0.5}$ value for 3-PGA2-fold.

(Ballicora et al. 1995, Fu et al. 1998a, Ballicora et al. 2000, Ballicora et al. 2003, Ballicora et al. 2004).

Chimeric constructs between the enzymes from two different plants, which are sufficiently similar to form hybrids despite the fact that they were separated by speciation, were used to study the structure–function relationship (Cross et al. 2004, Boehlein et al. 2005). In the present study, we showed the compatibility between domains of the enzyme forms from a photosynthetic prokaryote and a photosynthetic eukaryote. The characteristics of ADP-Glc PPases from cyanobacteria and potato tuber make them suitable targets to determine the structural basis of their respective similarities and differences. We swapped the putative regulatory domain, exchanging 159 amino acids of the C-terminus between the small subunit of the potato tuber and the *Anabaena* enzymes. These constructs were expressed alone or co-expressed with the potato tuber large subunit, to obtain homotetrameric (PSS, ANA, PSSANA and ANAPSS) and heterotetrameric (PSS + PLS, PSSANA + PLS and ANAPSS + PLS) enzymes. All these proteins were partially purified and their regulatory properties studied. The chimeric enzymes showed distinctive responses to the activator 3-PGA and to the inhibitor P_i, allowing evaluation of the role of the N- and C-terminus as well as of the large subunit in determining the regulatory properties.

We studied the conditions necessary to stabilize the chimeric ADP-Glc PPases against thermal treatments, establishing the contribution of the different domains to the maintenance of an active protein. The chimeric enzymes containing the N-terminus from the potato tuber small subunit required the absence of DTT in the medium to be stable to a thermal treatment of 60°C during 5 min. In contrast, the stability of the chimeric proteins containing the N-terminus from *Anabaena* was independent of the presence or absence of DTT, but required P_i in the medium. These results agree with previous reports showing that the formation of a disulfide bridge between Cys12 residues of the small subunits of the potato tuber enzyme is relevant to stabilize the enzyme to heat treatment (Ballicora et al. 1999). Also in agreement is a recent report dealing with the construction of hybrid proteins between heat-stable (potato

tuber) and heat-labile (maize endosperm) ADP-Glc PPases (Linebarger et al. 2005). Heat-stable enzymes have a conserved amino acid motif at the N-terminus of the small subunit (QTCL, C corresponding to Cys12 in the potato tuber enzyme), which is absent in the heat-labile enzymes. In this recent work (Linebarger et al. 2005), the authors demonstrated that insertion of the specific motif in the maize endosperm enzyme increases its stability to heat treatment >70-fold. On the other hand, it has been demonstrated previously that ADP-Glc PPase from *Anabaena* is stabilized by allosteric effectors, mainly by the inhibitor P_i (Gomez-Casati et al. 2000b). Thus, our results also suggest that the N-terminus in the cyanobacterial enzyme plays a role in P_i binding despite the fact that the identified residues for P_i inhibition are in the C-terminus (Sheng and Preiss 1997, Frueauf et al. 2002). However, it is possible that the P_i involved in stabilizing the cyanobacterial enzyme is not related to the regulatory site or that the inhibitory effect results from the interaction between the N- and the C-domains. Interestingly, in the work by Linebarger et al. (2005), insertion of the QTCL motif into a chimeric ADP-Glc PPase (hybrid between the maize endosperm and the potato tuber enzyme) not only increased the heat stability by >300-fold but also nullified the P_i insensitivity of the chimeric enzyme.

A main characteristic of the homotetrameric PSS is its requirement for 3-PGA for activity and the low affinity for the allosteric activator (Ballicora et al. 1995, Ballicora et al. 2004). The heterotetramer PSS + PLS is active in the absence of 3-PGA and possesses a high affinity for the effector, suggesting a main role for the large subunit in modifying the kinetic properties (Ballicora et al. 2004). Conversely, the enzyme from cyanobacteria is active even in the absence of 3-PGA and is activated with a relatively low $A_{0.5}$ value (Iglesias et al. 1991, Ballicora et al. 2003, Ballicora et al. 2004). We found that the chimeric enzymes exhibited intermediate properties with respect to the activation by 3-PGA. Both PSSANA and ANAPSS exhibited a basal (low) activity and were sensitive to 3-PGA activation with lower $A_{0.5}$ values than those corresponding to PSS. The increase in affinity toward 3-PGA was more important in ANAPSS. As a whole, these results reinforce an important function of the N-terminus for regulation of ADP-Glc PPases by 3-PGA, even when residues involved in the binding of this effector were localized towards the C-domain (Ballicora et al. 2003, Ballicora et al. 2004). Also, it is tempting to speculate that as found for the enzyme from bacteria (Ballicora et al. 2002), regulation in cyanobacteria and plants is exerted by the combined arrangement and interaction between the N- and C-terminus. The chimeric enzymes with heterotetrameric structure were found to have relatively higher affinity toward 3-PGA when assayed in the pyrophosphorolysis direction, indicating that the large subunit was functional in modulating the regulatory properties of the chimeric proteins. In addition, the chimeric heterotetramers exhibited higher sensitivity to the cross-talk existing between the allosteric activator and inhibitor of the enzyme when compared with the respective homotetrameric enzyme. In the characterization of the heterotetramers, we cannot ignore the fact that samples contain variable percentages of homo-oligomers, as a consequence of the distinct velocities of expression of the subunits, as observed previously (Crevillén et al. 2003). However, we assume in each case that most of the purified enzyme characterized is heterotetrameric on the basis of the relatively large differences in regulatory and kinetic properties with respect to homotetramers. A similar criterion was followed in the previous characterization of the potato tuber enzyme (Iglesias et al. 1993, Ballicora et al. 1995).

ADP-Glc PPase from potato tuber has been characterized as an enzyme of the starch biosynthetic pathway which is subject to post-translational regulation by thioredoxin (Ballicora et al. 2000). The enzyme undergoes activation by reduction of an intermolecular disulfide bridge that links the two small subunits by the Cys12 residue (Fu et al. 1998a, Ballicora et al. 2000). Oxidized thioredoxin reverses the activation. Cys12 is conserved in ADP-Glc PPases from leaves and other tissues, except for the monocot endosperm enzymes, but such a residue is absent in the protein from bacteria (Ballicora et al. 1999). This mechanism of activation of the enzyme has been demonstrated to be operative in vivo, being relevant to determine carbon partitioning in potato tuber and other plant tissues and species in response to light and sugars (Tiessen et al. 2002, Geigenberger 2003, Hendriks et al. 2003, Geigenberger et al. 2005). Post-translational regulation of the potato tuber enzyme is complementary to activation by 3-PGA. In fact, a main kinetic characteristic distinguishing the reduced (activated) ADP-Glc PPase is its higher affinity toward 3-PGA (Ballicora et al. 2000). Chimeric ADP-Glc PPases containing the N-terminus from the potato tuber small subunit exhibited a higher affinity and a nearly 2-fold higher activation by 3-PGA when assayed in the presence of DTT. This effect of the reducing agent was not observed in the chimeric constructs containing the N-terminus from *Anabaena* PCC 7120. Our results suggest that the N-terminal domain from the potato tuber small subunit was responsive to the reductive activation of the chimeric enzymes even in the presence of the C-terminus (and its regulatory sites) of an enzyme that is not modulated by reduction. These results reinforce the idea that the mechanism of activation of ADP-Glc PPase by reductive modification is exclusively exerted by the N-terminus of the small subunits, without involvement of other domains in the heterotetrameric enzyme. Allosteric regulation might be shared between N- and C-domains, but redox regulation is determined by the N-terminus.

An important conclusion from the presented results is that the domains from ANA and PSS are compatible and exchangeable to render a functional enzyme, despite the fact that one is from cyanobacteria and the other from plants. The interaction between their domains is also compatible. Conversely, attempts to construct fully functional chimeric enzymes between the *E. coli* and the cyanobacterial enzyme have failed (C. Bejar, M. A. Ballicora and J. Preiss, unpublished results). This is further evidence that small subunits evolved from a cyanobacterial enzyme.

Materials and Methods

Materials

[^{32}P]PP_i was purchased from NEN Life Science Products (currently PerkinElmer Life and Analytical Sciences, Inc., Wellesley, MA, USA). [^{14}C]Glc1P was obtained from ICN Pharmaceuticals Inc. (currently MP Biomedicals, Irvine, CA, USA). DNA sequencing and oligonucleotide synthesis were performed by the Macromolecular Facility at Michigan State University. The Mono Q HR 5/5 and Phenyl-Superose columns were acquired from Amersham Pharmacia Biotech (currently GE Healthcare Bio-Sciences Corp., Piscataway, NJ, USA).

Enzyme assay

Pyrophosphorolysis direction. Formation of [^{32}P]ATP from [^{32}P]PP_i was determined as described (Shen and Preiss 1964). The reaction mixture was incubated at 37°C and contained 80 mM HEPES (pH 7.5), 7 mM $MgCl_2$, 1.5 mM [^{32}P]PP_i (1,500–2,500 cpm nmol^{-1}), 2 mM ADP-Glc, 4 mM NaF and 0.05 mg ml^{-1} bovine serum albumin (BSA), plus enzyme in a total volume of 0.25 ml. The amounts of activator and/or further additions are indicated in the respective experiments.

Synthesis direction. Formation of [^{14}C]ADP-Glc from [^{14}C]Glc1P was determined as described previously (Ghosh and Preiss 1966). The reaction mixture were incubated at 37°C and consisted of 100 mM HEPES (pH 7.5), 6 mM $MgCl_2$, 0.5 mM [^{14}C]Glc1P (~1,000 cpm nmol^{-1}), 1 mM ATP, 1.5 U ml^{-1} pyrophosphatase and 0.2 mg ml^{-1} BSA, plus enzyme in a total volume of 0.20 ml. The amount of activator and/or other additions is indicated in the respective experiments.

One unit of enzyme activity in the above assays is equal to 1 µmol of product, either [^{32}P]ATP or [^{14}C]ADP-Glc, formed per min at 37°C.

Kinetic characterization

The kinetic data were plotted as initial velocity (nmol min^{-1}) vs. substrate or effector concentration (mM). The kinetic constants were acquired by fitting the data to the Hill equation with the non-linear least square algorithm provided by the program Origin™ 5.0. The Hill coefficient, n_H, and the kinetic constants, $S_{0.5}$, $A_{0.5}$ and $I_{0.5}$, which correspond to the concentration of substrate, activator or inhibitor giving 50% of the maximal velocity, activation or inhibition, respectively, were calculated from the Hill plots. To quantify better the interaction between allosteric effectors (P_i and 3-PGA), we defined the parameter I_D as the concentration of inhibitor (P_i) needed to 'double' the $A_{0.5}$ for 3-PGA. The lower the I_D value, the lower the concentration of inhibitor needed to reduce by 2-fold the apparent affinity toward the activator. In this way, I_D is a measurement of the apparent affinity for the inhibitor and its interaction (reciprocal) with the activator. For enzymes with hyperbolic kinetics and a single-site competitive inhibition model, the concentration of inhibitor that doubles the apparent affinity of the other ligand equals K_i (Segel 1975). Kinetic constants were the mean of at least two independent sets of data and they were reproducible within ±10%.

Construction of chimeric enzymes

Plasmids pML10 and pAnaE3a encode the PSS and ANA, respectively (Charng et al. 1992, Ballicora et al. 1995) and were used to construct hybrid genes (Fig. 4). The PSS gene has a *Kpn*I site that was used for exchanging fragments. The *Kpn*I site falls in residues GT of the region Y^{277}WEDIGTIEAFYNANL^{292} that is conserved in both enzymes (Fig. 4). To exchange the N- and C-regions between the ANA and PSS genes, both fragments of the ANA gene were amplified by PCR. To insert them into pML10 and replace the homologous regions in PSS, we engineered compatible *Nco*I and *Kpn*I restriction sites for the N-terminus, and *Kpn*I and *Sac*I for the C-terminus since they are not present in the ANA gene. To create these sites, silent mutations were introduced by PCR amplification of the N- and C-terminal fragments of ANA. PCR fragments of the N- and C-region of ANA, containing the respective engineered *Nco*I–*Kpn*I and *Kpn*I–*Sac*I restriction sites, were purified by agarose gel electrophoresis. Later, they were digested with the respective enzymes and subcloned into pML10, replacing the homologous regions in PSS. The plasmid bearing the chimeric gene (pPSSANA) codes for a protein (PSSANA) containing 292 amino acids from the N-terminus of PSS and 159 amino acids from the C-terminus of the enzyme from *Anabaena* PCC 7120. The inverse construct, the plasmid pANAPSS, encodes ANAPSS.

Mutant primers utilized in the PCR to modify the *Anabaena* gene were: 5′-TTCGATGGTACCAATATCTTCCCAGTAGTC-3′, to introduce the *Kpn*I site for chimeric PSSANA; 5′-AAATCCCATGGCCAAAAAAGTCTTAGCAATTATTCT-3′, to introduce the *Nco*I site for chimeric PSSANA; 5′-GATATTGGTACCATCGAAGCTTTTTATA-3′ for engineering a *Kpn*I site for ANAPSS; and 5′-TAATATGAGCTCTGTCATTAGTCTAGGG-3′ to introduce a *Sac*I site for chimeric ANAPSS. All the mutations introduced were silent. The coding regions of the final products were sequenced to confirm the absence of unwanted mutations.

Expression and partial purification of the enzymes

Plasmids pPSSANA and pANAPSS were transformed into *E. coli* AC70R1-504 cells (devoid of endogenous ADP-Glc PPase activity) alone or together with the compatible plasmid pMON17336 that encodes the PLS (Iglesias et al. 1993, Ballicora et al. 1995). Chimeric enzymes PSSANA, PSSANA + PLS, ANAPSS and ANAPSS + PLS were expressed and purified as described previously for PSS with minor modifications (Ballicora et al. 1995). In addition, wild-type proteins PSS, PSS + PLS and ANA were expressed from the respective original plasmids, purified and analyzed in parallel for comparative purposes. In all cases, cells were grown at 37°C up to an OD_{600} of ~0.6 and induced with 1 mM isopropyl-β-D-thiogalactopyranoside for 4 h at room temperature. For chimeric enzymes containing PLS, the co-expression of this subunit was induced with a further addition of 5 µg ml^{-1} nalidixic acid. Cells were harvested by centrifugation and stored frozen at –80°C.

All protein purification steps were carried out at 0–5°C. Cells harboring the wild-type or chimeric enzymes were resuspended (~1 g cells per 3 ml) and sonicated in the presence of 50 mM HEPES (pH 8.0), 5 mM $MgCl_2$, 0.1 mM EDTA and 10% sucrose (w/v) (buffer A). The sonicated suspensions were centrifuged at 12,000×*g* for 15 min. An ammonium sulfate cut (30–70%) of the supernatants was performed, followed by centrifugation of the samples for 20 min at 15,000×*g*. The 30–70% ammonium sulfate pellets were resuspended in buffer A and desalted on Bio-Rad 10 DG chromatography columns equilibrated with the same buffer. The desalted samples were applied individually to a Mono Q HR 5/5 (FPLC, Pharmacia) column equilibrated with buffer A. The column was washed with four bed volumes of buffer A, and the samples were eluted with a linear KCl gradient (20 column volumes, 0–0.5 M) in buffer A. Gel filtration to evaluate the quaternary structure of the enzymes was performed on a Superose 12 column on an FPLC system (Pharmacia). The protein concentration was determined was described (Frueauf et al. 2003).

Thermal treatments

Thermal treatment of ADP-Glc PPase was performed essentially as previously described (Ballicora et al. 1999, Gomez-Casati et al. 2000b). The partially purified enzymes (about 0.6 mg ml^{-1}) were incubated in buffer A, with the additions specified for each condition, at 60°C during 5 min. Thermal treatment was stopped by transferring the incubation tube to an ice bath. After 5 min, samples were centrifuged at 12,000×*g* for 5 min at 4°C and an aliquot was withdrawn from the supernatant and assayed for activity immediately after centrifugation.

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