

# Characterization of Chimeric ADPGlucose Pyrophosphorylases of *Escherichia coli* and *Agrobacterium tumefaciens*. Importance of the C-Terminus on the Selectivity for Allosteric Regulators<sup>†</sup>

Miguel A. Ballicora,<sup>‡</sup> Juliana I. Sesma,<sup>§</sup> Alberto A. Iglesias,<sup>§</sup> and Jack Preiss<sup>\*,‡</sup>

Department of Biochemistry, Michigan State University, East Lansing, Michigan 48824, and Instituto Tecnológico de Chascomús (IIB-INTECH), Camino Circunv. Laguna km 6, CC 164, Chascomús 7130, Buenos Aires, Argentina

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**ABSTRACT:** ADPGlucose pyrophosphorylase catalyzes the regulatory step in the pathway for bacterial glycogen synthesis. The enzymes from different organisms exhibit distinctive regulatory properties related to the main carbon metabolic pathway. *Escherichia coli* ADPGlucose pyrophosphorylase is mainly activated by fructose 1,6-bisphosphate (FBP), whereas the *Agrobacterium tumefaciens* enzyme is activated by fructose 6-phosphate (F6P) and pyruvate. Little is known about the regions determining the specificity for the allosteric regulator. To study the function of different domains, two chimeric enzymes were constructed. “AE” contains the N-terminus (271 amino acids) of the *A. tumefaciens* ADPGlucose pyrophosphorylase and the C-terminus (153 residues) of the *E. coli* enzyme, and “EA”, the inverse construction. Expression of the recombinant wild-type and chimeric enzymes was performed using derivatives of the pET24a plasmid. Characterization of the purified chimeric enzymes showed that the C-terminus of the *E. coli* enzyme is relevant for the selectivity by FBP. However, this region seems to be less important for the specificity by F6P in the *A. tumefaciens* enzyme. The chimeric enzyme AE is activated by both FBP and F6P, neither of which affect EA. Pyruvate activates EA with higher apparent affinity than AE, suggesting that the C-terminus of the *A. tumefaciens* enzyme plays a role in the binding of this effector. The allosteric inhibitor site is apparently disrupted, as a marked desensitization toward AMP was observed in the chimeric enzymes.

The reaction catalyzed by ADPGlucose pyrophosphorylase (ADPGlc PPase)<sup>1</sup> (EC 2.7.7.27),  $\text{Glc-1-P} + \text{ATP} \leftrightarrow \text{ADPGlc} + \text{PP}_i$ , is the first committed step in the pathway for the synthesis of storage polysaccharides in bacteria and plants, namely, glycogen and starch, respectively (1, 2). Most of the ADPGlc PPases so far characterized are allosterically regulated by small effector molecules that are intermediates of the major carbon assimilatory pathway in the organism (2–5). For instance, many of the enzymes from bacterial origin are activated by metabolites of glycolysis (the classical Embden–Meyerhof metabolism) or the Entner–Doudoroff pathway, such as F6P, FBP, or pyruvate, and inhibited by AMP, ADP, and/or  $\text{P}_i$  (2–4). On the other hand, ADPGlc PPases from photosynthetic organisms (cyanobacteria, green algae, and higher plants) have, as main allosteric regulators 3PGA (activator) and  $\text{P}_i$  (inhibitor), key intermediates of carbon photoassimilation (5). The native ADPGlc PPase is a tetramer of about 200 kDa, formed by only one type of subunit ( $\alpha_4$ ) in bacteria, and in plants is two different subunits

( $\alpha_2\beta_2$ ) (3, 5). The two subunits in the plant enzyme have different functions:  $\alpha$  is the catalytic subunit and  $\beta$  is the regulatory subunit (5–7).

Bacterial ADPGlc PPases have been grouped into seven different classes (1, 8). The regulatory effectors of the enzymes from bacteria having glycolysis as their predominant pathway in class I (e.g., enteric bacteria) have FBP as the activator and AMP as inhibitor (2, 4, 8). Classes II and III also contain ADPGlc PPases from glycolytic bacteria such as *Micrococcus luteus* and *Serratia marcesens*, respectively (1, 8). The enzymes from group II are activated by FBP and F6P and inhibited by AMP and ADP, whereas those from class III have no activator and are inhibited by AMP. Class IV is a group of enzymes activated by F6P and pyruvate and found in bacteria, typically *Agrobacterium tumefaciens*, using the Entner–Doudoroff pathway. *Rhodobacter sphaeroides* and other bacteria capable of utilizing both glycolysis and the Entner–Doudoroff metabolism contain ADPGlc PPases of class V, which are sensitive to activation by FBP, F6P, and pyruvate (1, 8). In class VI, ADPGlc PPases are activated only by pyruvate, which is a central metabolite in metabolic pathways used by anoxygenic photosynthetic prokaryotes such as *Rhodospirillum rubrum* that cannot metabolize glucose. Finally, class VII includes the enzymes from cyanobacteria and plants that are activated by 3PGA and inhibited by  $\text{P}_i$  (1, 3, 5, 8). Only ADPGlc PPases from sporulating bacteria of the genus *Bacillus* escape from the

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<sup>\*</sup> To whom correspondence should be addressed. Telephone: 517-353-3137. Fax: 517-353-9334. E-mail: preiss@msu.edu.

<sup>‡</sup> Michigan State University.

<sup>§</sup> Instituto Tecnológico de Chascomús.

<sup>1</sup> Abbreviations: ADPGlc PPase, ADPGlucose pyrophosphorylase;  $\text{PP}_i$ , pyrophosphate; Glc-1-P, glucose 1-phosphate; FBP, fructose 1,6-bisphosphate; F6P, fructose 6-phosphate; 3PGA, 3-phosphoglycerate;  $\text{P}_i$ , orthophosphate; BSA, bovine serum albumin.

above classification, as they seem to have no regulatory properties (9).

The three-dimensional structure of an ADPGlc PPase has not yet been solved by X-ray crystallography. Many structural studies have been carried out using other approaches to establish structure-to-function relationships, and recently a prediction of the secondary structure of different ADPGlc PPases was found to be similar (10). However, little is known about the structural basis for the regulator specificity of the enzymes grouped in the different classes described above, and the characterization of domains with distinctive regulatory functions has not been performed.

Chemical modification and site-directed mutagenesis studies have identified the role of key amino acid residues for the substrate binding, catalysis, or regulation of the *Escherichia coli* enzyme. Azido-based photoaffinity analogues of ATP and ADPGlc were used to identify Tyr-114 as involved in the binding of the adenine nucleotide substrate (11). Mutagenesis of the Lys-195 residue produced enzymes with drastically decreased affinity toward Glc-1-P (12), and the essential role for catalysis of Asp-142 was recently demonstrated (10). All of these data agree with the hypothesis that the activity of ADPGlc PPase is exerted by a domain localized in the central region of the protein, with the N- and C-termini probably playing roles in the regulation of the enzyme.

Different studies have shown that the N-terminus contains residues involved in the regulation of ADPGlc PPases from two of the above classes. Thus, in the *E. coli* enzyme (class I), Lys-39 was identified in the binding of the activator FBP by site-directed mutagenesis (13), and truncation of 11 amino acids made it insensitive to activation (14–16). In addition, the functional role of some arginine residues in the N-terminus of the *A. tumefaciens* ADPGlc PPase (class IV) was suggested as important for the activation by F6P and pyruvate (17). On the contrary, no evidence has been reported showing that the C-terminus takes part in the regulation or catalysis of ADPGlc PPases from heterotrophic bacteria. In contrast, in the enzyme from oxygenic photosynthetic organisms (including cyanobacteria), residues located at the C-terminus were identified as playing a key role in the binding of the activator 3PGA (18–20).

In the present work we constructed and characterized chimeric proteins, hybrid products between the enzymes of two different regulatory classes, from *E. coli* and *A. tumefaciens* to study the role of the N- and C-terminus in catalysis and regulation of bacterial ADPGlc PPase. For the first time a specific contribution for the C-terminus in allosteric regulation of nonphotosynthetic bacterial ADPGlc PPase is presented.

## EXPERIMENTAL PROCEDURES

**Materials.** [ $^{32}$ P]PP<sub>i</sub> was purchased from NEN Life Science Products. [ $^{14}$ C]Glc-1-P was obtained from ICN Pharmaceuticals Inc. Glc-1-P, ATP, ADPGlc, FBP, AMP, and inorganic pyrophosphatase were purchased from Sigma Chemical Co. Oligonucleotides were synthesized and purified by the Macromolecular Facility at Michigan State University. BL21-(DE3) cells and Perfect protein markers were obtained from Novagen, Inc. The Mono Q HR 5/5 and phenyl-Superose columns were acquired from Amersham Pharmacia Biotech.

**Enzyme Assay. (Assay A) Pyrophosphorolysis.** Formation of [ $^{32}$ P]ATP from [ $^{32}$ P]PP<sub>i</sub> in the direction of pyrophosphorolysis at 37 °C was determined by the method of Shen and Preiss (21). The reaction mixtures contained 80 mM HEPES (pH 8.0), 7 mM MgCl<sub>2</sub>, 1.5 mM [ $^{32}$ P]PP<sub>i</sub> (1500–2500 cpm/nmol), 2 mM ADPGlc, 4 mM NaF, and 0.05 mg/mL bovine serum albumin (BSA), plus enzyme in a total volume of 250  $\mu$ L and the amount of activator and/or inhibitor indicated in the respective experiment.

**(Assay B) Synthesis.** Formation of [ $^{14}$ C]ADPGlc from [ $^{14}$ C]-Glc-1-P in the synthesis direction at 37 °C was determined by the method of Preiss et al. (22). The reaction mixtures consisted of 100 mM HEPES (pH 8.0), 6 mM MgCl<sub>2</sub>, 0.5 mM [ $^{14}$ C]Glc-1-P (~1000 cpm/nmol), 1 mM ATP, 0.0015 unit/ $\mu$ L pyrophosphatase, and 0.2 mg/mL BSA, plus enzyme in a total volume of 200  $\mu$ L. The amount of activator and/or inhibitor is indicated in the respective experiment.

One unit of enzyme activity in the above assays is equal to 1  $\mu$ mol of product, either [ $^{32}$ P]ATP or [ $^{14}$ C]ADPGlc, formed per minute at 37 °C.

**Kinetic Characterization.** The kinetic data were plotted as initial velocity (nmol/min) versus substrate or effector concentration (mM). The kinetic constants were acquired by fitting the data with a nonlinear least-squares formula and the Hill equation using 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. The standard deviations of these parameters were also calculated by this software. Kinetic constants are the mean of at least two independent sets of data, and they are reproducible within  $\pm 10\%$ .

**Protein Assay.** Protein concentration during enzyme purification was measured by using bicinchoninic acid reagent (23) from Pierce Chemical Co., with BSA as the standard. Protein concentration of the purified enzymes was determined by UV absorbance at 280 nm using an extinction coefficient of 1.0.

**Construction of Chimeric Proteins Using the Enzymes from *E. coli* and *A. tumefaciens*.** Chimeric enzymes between the ADPGlc PPase from *A. tumefaciens* and *E. coli* were constructed from plasmids pATUI and pOP12, respectively (24, 25). It was necessary to engineer a *Kpn*I site in both genes at the conserved sequence YWRDVG<sup>279</sup> (Figure 1) and eliminate another *Kpn*I site present in the N-terminus of the *E. coli* enzyme. This was performed by “combined chain reaction” (CCR) (26), since this technique allows the mutagenesis of several sites at the same time. The *E. coli* gene was amplified by CCR from the plasmid pOP12 (24), introducing also an *Nde*I site at the N-terminus and a *Sac*I site at the C-terminus using mutated primers. After the amplification, the fragment was separated by electrophoresis, digested with *Nde*I and *Sac*I, and subcloned into pET24a (Novagen) to obtain the plasmid pETEC, which expresses the wild-type *E. coli* enzyme. Plasmid pETAT expresses the wild-type *A. tumefaciens* enzyme and was constructed in a similar way, amplifying the coding region from pATUI (25). The plasmids pETAE and pETEA were constructed by exchanging the fragments obtained after digestion of pETEC and pETAT with *Kpn*I and *Sac*I. pETAE and pETEA express the chimeric enzymes “AE” and “EA”, respectively. AE

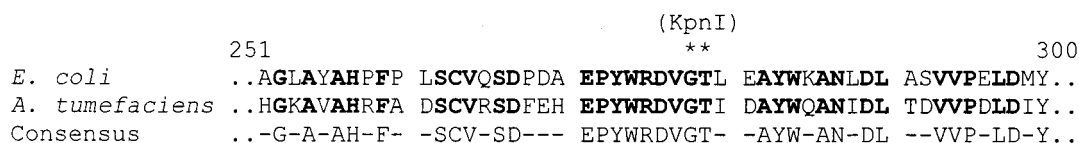


FIGURE 1: Alignment of the ADPGlc PPases from *A. tumefaciens* and *E. coli*. Conserved residues in both enzymes are depicted in bold. Asterisks indicate the position on the translated protein where a *KpnI* site was engineered in both genes by mutagenesis as described in Experimental Procedures. The modification on the DNA was silent in order to maintain the sequence Gly-Thr in the wild-type and chimeric enzymes.

contains the N-terminus (271 amino acids) of the *A. tumefaciens* enzyme and the C-terminus (153 residues) of the *E. coli* enzyme, and EA is the inverse construct.

Mutant primers utilized in the CCR reaction to modify the *E. coli* gene were as follows: 5' AGG AGT TAC ATA TGG TTA GTT TAG AGA A 3', to introduce an *NdeI* site at the N-terminus; 5' GAG GAC GTG GCA CCC GCC TGA 3', to eliminate a *KpnI* site in the N-terminus; 5' ATG TGG GTA CCC TGG AAG CTT 3', to introduce a *KpnI* site; and 5' CGG GAA GAG CTC TGA ACA TAC ATG TA 3', to introduce a *SacI* site at the C-terminus. For the *A. tumefaciens* gene the primers were as follows: 5' ACA GAA CAT ATG TCG GAA AAA AGA G 3', to introduce an *NdeI* site at the N-terminus; 5' GAG ACG TCG GTA CCA TCG ATG 3', to introduce a *KpnI* site; and 5' AAA CAG GAG CTC GGC TGC AGC TA 3', to introduce a *SacI* site at the C-terminus. All of the mutations introduced in both wild-type genes were silent. The coding regions of the final products, pETEC, pETAT, pETEA, and pETAE, were sequenced to confirm that there were no unwanted mutations.

**Expression and Purification of the Wild-Type and Chimeric Enzymes.** *E. coli* BL21(DE3) cells were transformed with the plasmids pETEC, pETAT, pETAE, or pETEA to express the native ADPGlc PPases from *E. coli* and *A. tumefaciens* and the chimeric enzymes AE and EA, respectively. In all cases, cells were grown at 37 °C up to OD<sub>600</sub> ~0.6 and induced with 1 mM IPTG for 4 h at room temperature. 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 of cells/3 mL) and sonicated in the presence of 50 mM HEPES (pH 8.0), 5 mM MgCl<sub>2</sub>, 0.1 mM EDTA, and 10% sucrose (w/v) (buffer A). The sonicated suspensions were centrifuged at 12000g for 15 min, and the supernatants were retained. An ammonium sulfate cut (30–70%) of the supernatants was performed, followed by centrifugation of the samples for 20 min at 15000g. 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 4 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.

The post-Mono Q samples were concentrated using Centricon-30 ultrafiltration devices (Amicon, Inc.), then 10-fold diluted in buffer B (similar to buffer A except that sucrose was replaced by 1.2 M ammonium sulfate), and applied on a phenyl-Superose (FPLC, Pharmacia) column. After being washed with 5 column volumes of buffer B, the

samples were eluted with a gradient of buffer B with decreasing amounts of ammonium sulfate (30 bed volumes, from 1.2 to 0.001 M salt). The presence of ADPGlc PPases in the fractions of the phenyl-Superose chromatography was monitored by SDS-PAGE (27), as enzyme activity is substantially inhibited by the presence of sulfate (especially the enzyme from *A. tumefaciens*, as reported in ref 17). The fractions containing the 50 kDa subunits were pooled, concentrated, and brought to buffer A conditions using Centricon-30 ultrafiltration concentrators and assayed for activity in the pyrophosphorolysis direction (assay A). Concentrated, purified samples in buffer A were aliquoted and stored at -20 °C, conditions under which the wild-type and chimeric enzymes remained stable during for at least 3 months.

## RESULTS

**Expression and Purification of Chimeric ADPGlc PPases AE and EA.** We selected the *E. coli* and *A. tumefaciens* ADPGlc PPases (classes I and IV, respectively) to construct chimeric enzymes containing the N- or C-terminus from the other. The respective wild-type enzymes have been relatively well characterized and exhibit different specificity for allosteric regulators. The alignment between these two enzymes shows neither deletions nor insertions, and the enzyme from *E. coli* is only seven and four amino acids longer in the N- and C-terminus, respectively. The overall homology is relatively high (56% identity), and the identity of the fragments exchanged was 59% and 51% at the N- and C-terminus, respectively. Both chimeric proteins, AE and the reverse EA, were adequately expressed using the plasmids pETAE and pETEA in amounts sufficient for functional studies.

The specific activities of the crude extracts measured in the pyrophosphorolysis direction of the AE and EA chimeric enzymes were 1.2 and 1.3 units/mg, respectively, as compared to values of about 24 and 14 units/mg obtained for the wild-type enzymes from *E. coli* and *A. tumefaciens*, respectively. The wild-type and chimeric ADPGlc PPases remained stable during the purification, and the total yield was 50%, 45%, 25%, and 35% for the enzymes from *E. coli*, *A. tumefaciens*, AE, and EA, respectively. The enzymes were purified 6- (*E. coli*), 9- (*A. tumefaciens*), 22- (AE), and 29-fold (EA) and migrated as single protein bands of ~50 kDa in SDS-PAGE (data not shown). After the purification, the chimeric enzymes were shown to be relatively efficient compared to the wild types. The specific activities in the pyrophosphorolysis direction were 148 and 129 units/mg for the purified *E. coli* and *A. tumefaciens* wild types. The chimeric enzymes AE and EA had specific activities of 27 and 36 units/mg, respectively, which are between 20% and 30% of the value of the wild-type enzymes.



Table 1: Kinetic Parameters for Substrates of the Different Wild-Type and Chimeric ADPGlc PPases in the Synthesis Direction<sup>a</sup>

enzyme	$S_{0.5}$ ( $\mu$ M)						specific activity (units/mg)	
	Glc-1-P	$n_H$	ATP	$n_H$	$Mg^{2+}$	$n_H$	(+) activator	(-) activator
<i>E. coli</i>	45 $\pm$ 3	1.0	300 $\pm$ 20	2.2	2400 $\pm$ 100	3.5	56 $\pm$ 3	3.5 $\pm$ 0.2
<i>A. tumefaciens</i>	60 $\pm$ 10	1.2	86 $\pm$ 8	1.5	2100 $\pm$ 150	2.6	62 $\pm$ 2	10.0 $\pm$ 0.5
AE	92 $\pm$ 8	1.4	162 $\pm$ 20	1.4	1510 $\pm$ 200	1.9	18 $\pm$ 2	3.2 $\pm$ 0.3
EA	39 $\pm$ 5	1.5	155 $\pm$ 10	2.0	1720 $\pm$ 90	2.2	15 $\pm$ 1	6.8 $\pm$ 0.3

<sup>a</sup> The kinetic constants of the enzyme from *E. coli* and AE were determined as described in Experimental Procedures in the presence of saturating concentrations of the activator FBP (1 mM). The activators used for enzymes from *A. tumefaciens* and EA were F6P and pyruvate, respectively. The deviations of the different  $S_{0.5}$  were calculated as described in Experimental Procedures. The deviation of the specific activity is the difference between two independent duplicates.

**Thermal Stability of the Chimeric ADPGlc PPases.** To test the stability of the chimeric enzymes, a heat treatment was performed as described previously (10). After incubation at 60, 55, and 50 °C for 5 min, the remaining activity of the chimeric enzyme AE was 37%, 65%, and 87%, respectively, whereas the chimeric enzyme EA retained 45%, 70%, and 75% of the initial activity, respectively. These results indicate that the stability of the folding of the chimeric enzymes AE and EA was not seriously altered since the wild-type enzymes generally retain between 70% and 90% of the activity at 60 °C (10, 25).

**Substrate Kinetics.** As shown in Table 1, the apparent  $S_{0.5}$  values for Glc-1-P, ATP, and  $Mg^{2+}$  of both chimeric enzymes, AE and EA, are similar to those exhibited by the enzymes from *E. coli* or *A. tumefaciens*. In addition, Table 1 shows that the AE and EA chimeric proteins are relatively efficient enzymes in the synthesis direction, reaching  $V_{max}$  values of 18 and 15 units/mg, respectively. This is between 30% and 40% of the value of the wild-type enzymes when assayed at optimal levels of the corresponding activator. In the absence of activators, the chimeric enzymes showed specific activities of the same magnitude as the wild types (Table 1).

**Activator Specificity of Chimeric ADPGlc PPases.** The main kinetic differences found in the characterization of the chimeric ADPGlc PPases were with respect to the regulation. Each chimeric ADPGlc PPase exhibited distinctive regulatory properties as illustrated in Figure 2. The chimeric enzyme containing the N-terminus from the *A. tumefaciens* enzyme and the C-terminus of the *E. coli* ADPGlc PPase (AE) was activated 3.0-, 5.7-, and 2.2-fold by FBP, F6P, and pyruvate, respectively, with different apparent affinities for the respective effector (Figure 2a). On the other hand, Figure 2b shows that the reverse chimeric enzyme, EA, was only activated by pyruvate (2.5-fold).

The kinetic constants describing the regulatory properties of chimeric ADPGlc PPases and those exhibited by the wild-type enzymes are shown in Table 2. The native enzyme from *E. coli*, as a class I enzyme, was mainly activated by FBP (16-fold activation,  $A_{0.5}$ , 30  $\mu$ M), and F6P had no effect. This is in good agreement with previously published data (11, 28, 29). Pyruvate activated only at very high concentrations with an  $A_{0.5}$  of approximately 14000  $\mu$ M (>6-fold activation). The class IV ADPGlc PPase from *A. tumefaciens* showed a higher apparent affinity for F6P and pyruvate than for FBP (Table 2), with these compounds increasing the enzyme activity by 6-, 4.5-, and 3.6-fold, respectively. These results were consistent with data from the literature (25). Remarkably, Table 2 shows that the chimeric enzyme AE showed relatively high apparent affinities for FBP (nearly 20-fold

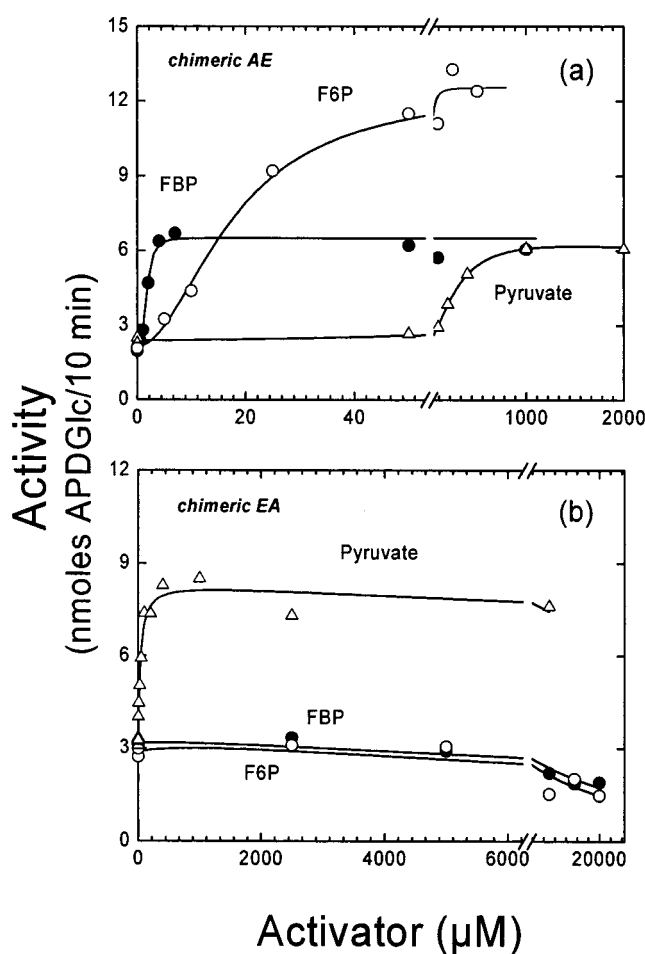


FIGURE 2: Activation of the chimeric ADPGlc PPases AE (a) and EA (b). The enzymes were assayed in the ADPGlc synthesis direction, as described under Experimental Procedures, with the addition of specified varying concentrations of FBP (filled circles), F6P (empty circles), or pyruvate (triangles).

higher than the *E. coli* wild-type enzyme) and also for F6P (about 5-fold higher than the *A. tumefaciens* wild-type enzyme). With respect to pyruvate, the  $A_{0.5}$  value for the chimeric enzyme AE was 3-fold higher than for the *A. tumefaciens* enzyme but 47-fold lower than that corresponding to the enzyme from *E. coli* (Table 2). On the other hand, pyruvate was a very specific activator of the chimeric enzyme EA since F6P and FBP showed no activation up to 20 mM, and the  $A_{0.5}$  value for pyruvate was about 3-fold lower than for the wild-type enzyme from *A. tumefaciens* (Table 2).

Table 2 shows the ratios of the  $A_{0.5}$  values of F6P/FPB, pyruvate/FPB, and pyruvate/F6P for the ADPGlc PPases from *E. coli*, *A. tumefaciens*, and the chimeric enzyme AE.

Table 2: Activation Properties of the Different Wild-Type and Chimeric ADPGlc PPases

enzyme	$A_{0.5}$ ( $\mu\text{M}$ )						activator affinity ratio		
	FBP	$n_H$	F6P	$n_H$	pyruvate	$n_H$	F6P/FBP	Pyr/FBP	Pyr/F6P
<i>E. coli</i>	$30 \pm 4$	2.0	na <sup>a</sup>		$14000 \pm 3000^b$	1.1		470	
<i>A. tumefaciens</i>	$900 \pm 100$	1.3	$88 \pm 23$	1.4	$100 \pm 15$	1.2	0.10	0.11	1.10
AE	$1.8 \pm 0.3$	2.5	$18 \pm 3$	2.0	$300 \pm 25$	2.1	10	166	17
EA	na		na		$36 \pm 3$	1.3			

<sup>a</sup> na: no activation was observed when assayed up to 20 mM concentration. <sup>b</sup> Kinetics were performed up to 20 mM concentration, conditions under which complete saturation was not reached. The activity at the highest concentration was 60% of the calculated  $V_{\max}$ . The deviations of the different kinetic constants were calculated as described in Experimental Procedures.

The *E. coli* enzyme has a pyruvate/FBP ratio of about 500, as the enzyme is largely specific for FBP activation. Pyruvate is a very poor regulator, and F6P exerts practically no effect. The *A. tumefaciens* enzyme shows values of about 0.1 for the F6P/FBP and pyruvate/FBP apparent affinity ratios, indicating that the enzyme has 10-fold higher apparent affinity for F6P or pyruvate than for FBP. The pyruvate/F6P ratio is near 1, which indicates that both activators have a similar apparent affinity. The analysis of the three activator ratios for the chimeric enzyme AE in Table 2 indicates that although the chimeric ADPGlc PPase is activated by FBP, F6P, and pyruvate, its apparent affinity for FBP is 10- and 170-fold higher than for F6P and pyruvate, respectively. Moreover, the chimeric enzyme AE is 100- or 1700-fold more specific for FBP with respect to F6P or pyruvate than the *A. tumefaciens* ADPGlc PPase, and AE exhibits 17-fold higher apparent affinity for F6P than for pyruvate. A very significant aspect is that the chimeric enzyme AE has greater apparent affinity for FBP than the *E. coli* enzyme and higher apparent affinity for F6P than either the *E. coli* or *A. tumefaciens* enzymes. On the other hand, the chimeric enzyme EA has the highest apparent affinity for pyruvate ( $A_{0.5}$ , 35  $\mu\text{M}$ ).

**Inhibition of Chimeric ADPGlc PPases by AMP.** AMP is a high-affinity allosteric inhibitor of the class I ADPGlc PPase from *E. coli*, and the inhibitory effect is observed only in the presence of the activator (30). Figure 3 illustrates the effect of AMP on the activity of the different wild-type and chimeric enzymes, each one assayed in the presence of the respective activator. The *E. coli* ADPGlc PPase is highly sensitive to AMP, as it is inhibited with an  $I_{0.5}$  of 7  $\mu\text{M}$  in the presence of 0.1 mM FBP (Figure 3). The *A. tumefaciens* enzyme assayed in the presence of 0.3 mM F6P was poorly inhibited by AMP at concentrations higher than 1 mM with an  $I_{0.5}$  of about 3 mM (Figure 3). Both of the chimeric ADPGlc PPases exhibited a very low sensitivity to AMP. Figure 3 also shows that the AE enzyme (assayed at 6  $\mu\text{M}$  FBP) was slightly activated (up to 1.4-fold) by AMP in the range of 0.1–2 mM and inhibited only at very high concentrations with an  $I_{0.5}$  of 9 mM. On the other hand, the chimeric enzyme EA activated by 0.1 mM pyruvate was relatively insensitive to AMP (Figure 3).

## DISCUSSION

ADPGlc PPases from different sources seem to evolve from a common ancestor and probably share a similar 3D structure (6, 10). The central region of the protein has been identified as a substrate binding and catalytic domain by secondary structure prediction, alignment with other sugar nucleotide pyrophosphorylase enzymes, and further site-

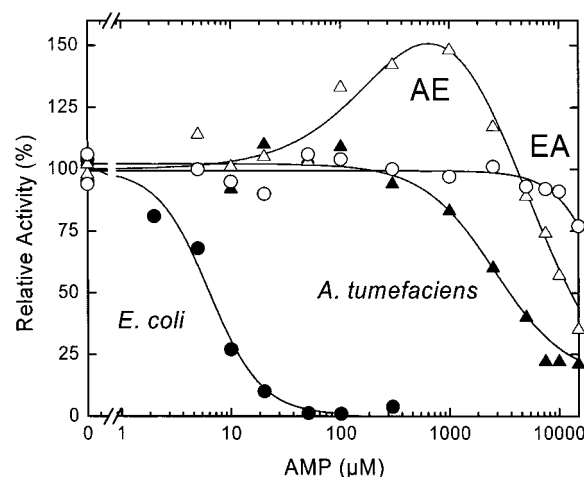


FIGURE 3: Effect of AMP on the wild-type and the chimeric ADPGlc PPases. Each enzyme was assayed in the ADPGlc synthesis direction at the specified varying concentrations of AMP and in the presence of the specific activator (at a concentration about 3-fold the  $A_{0.5}$  value) as follows: *E. coli* (filled circles, at 100  $\mu\text{M}$  FBP); *A. tumefaciens* (filled triangles, at 250  $\mu\text{M}$  F6P); AE (open triangles, at 6  $\mu\text{M}$  FBP); EA (open circles, at 100  $\mu\text{M}$  pyruvate).

directed mutagenesis (10). It is expected that the N- and C-termini account for the distinctive regulatory properties displayed by the different classes of ADPGlc PPases.

The C-terminus of the ADPGlc PPases from oxygenic photosynthetic organisms (class VII) has been clearly recognized as involved in the binding and allosteric regulation by 3PGA/ $P_i$  (18, 19, 31–36). On the other hand, residues that are critical for the binding of the activators have been found only on the N-terminus of enzymes from heterotrophic bacteria that correspond to classes I and IV (13–15, 17). No residues have been identified on the C-terminus of the latter enzymes to be involved in the binding of regulators, and the regulatory residues identified in class VII oxygenic photosynthetic ADPGlc PPases are missing. Moreover, the homology of this region is very low between the enzymes from class VII and the rest. Only two allosteric mutants (P295S and G336D) were found and characterized in the C-terminus of the *E. coli* enzyme, but they had higher rather than decreased apparent affinities for the activator (28, 37–39).

Construction of hybrid polypeptides (chimeric proteins) has been utilized previously as a useful tool to study structure–function relationships of different enzymes (40–42). Since the function of the C-terminus of the ADPGlc PPases from heterotrophic organisms is unknown, we constructed chimeric proteins exchanging 153 amino acids of the C-terminus of the enzyme from *E. coli* and *A.*

*tumefaciens* and studied their altered properties. Interestingly, the chimeric enzymes showed distinctive apparent affinities for the activators, which allowed us to evaluate the role of the C-terminus in the specificity for them. Moreover, the chimeric ADPGlc PPases were not significantly different in their apparent affinities for substrates than the wild-type enzymes, which agrees with the model where the central region of the protein contains the substrate binding and catalytic domain (10).

The chimeric ADPGlc PPase that has the N-terminus from the *A. tumefaciens* and the C-terminus from the *E. coli* enzymes (AE) was activated by FBP, F6P, and pyruvate. The most outstanding property of the AE enzyme was the higher apparent affinity toward FBP. In fact, the  $A_{0.5}$  value for FBP activation of the chimeric enzyme AE was 1 order of magnitude lower than the one that corresponds to the class I (*E. coli*) enzyme and 500-fold lower with respect to the class IV (*A. tumefaciens*) enzyme. In addition, the chimeric enzyme AE showed a 10-fold preference to be activated by FBP rather than by F6P, a value that is the inverse of that exhibited by the *A. tumefaciens* enzyme. All of these results strongly support the fact that the C-terminus of the ADPGlc PPase from *E. coli* largely contributes to determine the selectivity for the activator FBP. The higher apparent affinity of the chimeric enzyme AE for FBP when compared to the *E. coli* enzyme may be explained by the slightly shorter (seven amino acid) N-terminus of the former. It has been shown that a shorter N-terminus could decrease the  $A_{0.5}$  for the activator in ADPGlc PPases (16, 43, 44). It has been proposed that the N-terminal region of the ADPGlc PPase may play a role as an "allosteric switch" to regulate enzyme activity (16, 45). This is predicted as a loop (10) that possibly interferes with the transition between two different conformations of the enzyme (activated and nonactivated). The shorter N-terminus in the chimeric enzyme AE may cause the activated form to be favored, facilitating the activation by FBP.

The reverse chimeric enzyme (EA) was only activated by pyruvate with a higher apparent affinity (3-fold) than the class IV enzyme from *A. tumefaciens*. These data suggest that the C-terminus of the *A. tumefaciens* enzyme determines the high apparent affinity for the activator pyruvate, but the residues critical for F6P selectivity lie outside this region. The latter indicates that the binding of F6P and pyruvate in class IV ADPGlc PPases occurs at different sites as has been recently proposed by site-directed mutagenesis studies (17). Since it has been found previously that Lys-39 of the class I *E. coli* enzyme interacts with the allosteric activator (13–15), it would have been expected that the FBP activated the chimeric enzyme EA. However, the absence of this allosteric effect suggests that the regulation by FBP is critically determined by a combined arrangement between the N- and C-terminus in the 3D structure of the protein.

The class I *E. coli* ADPGlc PPase binds AMP with high affinity, but none of the chimeric enzymes were efficiently inhibited by AMP, indicating that the inhibitory site is apparently disrupted. One explanation is that the region that determines the interaction with the inhibitor must have critical residues from both the N- and the C-terminus of the protein. Another possibility to explain the insensitivity toward AMP is that the switch is located in the middle of the inhibitory site and it could have been deleteriously modified

in the chimeric constructs. In agreement with this, Arg-294, which has been demonstrated to be involved in the P<sub>i</sub> inhibition of an enzyme from class VII (35, 36), is about 25 amino acids nearer the homologous area where the fragments were switched to construct the chimeric enzymes. It is not clear why the chimeric enzyme AE is slightly activated by AMP at ~1 mM in the presence of 6  $\mu$ M FBP. However, it is not surprising since many allosteric inhibitors behave similarly. It is possible that the AMP at such high concentrations competes with the FBP site.

Differences in selectivity for the regulators of the ADPGlc PPases play a key metabolic role in the organisms that use ADPGlc for synthesis of polysaccharides as a carbon and energy storage. It is possible that a common enzyme ancestor evolved to other forms having different regulatory properties according to their metabolic environment and developed in at least seven characterized classes of ADPGlc PPases (1, 8). It is not known whether the regulatory sites are located in the same or distinct domains in the protein structure of these classes. Some of these enzymes are relatively nonspecific regarding the selectivity for allosteric regulators, which would indicate a certain flexibility to undergo evolutionary changes. Unfortunately, the structure-to-function relationships of the regulatory site(s) in heterotrophic bacteria are far from clear. A more comprehensive characterization of the structure of the allosteric sites will greatly help to understand the evolutionary mechanism. In this work we show that a single "crossover" in the gene renders two ADPGlc PPases that would belong to different classes than their parents. From enzymes of class I (*E. coli*) and class IV (*A. tumefaciens*) were obtained two ADPGlc PPases that could be included as class V (chimeric enzyme AE) and class VI (chimeric enzyme EA).

This is the first time that it is shown that the C-terminus of ADPGlc PPases from heterotrophic bacteria plays a relevant role for the activator selectivity and that a simple exchange of polypeptide fragments could generate an enzyme from a different metabolic class. It has been previously reported that a single mutation (K419Q) in the activator site of the ADPGlc PPase from *Anabaena* yields an enzyme with altered activator specificity. The activation fold by 3PGA reduced from 10.5- to 3.8-fold and the activation fold by FBP increased from 1.7- to 6.3-fold. However, the apparent affinity for FBP decreased 30-fold, and the mutation lowered the  $V_{max}$  to only 2% of the activity seen for the wild-type enzyme (32). Conversely, in this work, the selectivity for activators in the chimeric enzymes was completely reverted with apparent affinities that were even higher than the respective wild-type enzymes. It is not known what part of the C-terminus could be responsible for the change in selectivity. To further explore this problem, construction of truncated and more chimeric enzymes is in progress. Ultimately, it will be very important to determine the 3D structure of crystallized ADPGlc PPases from different sources as the regulatory site could be compared to understand the mechanisms of the evolution of the regulatory site. Efforts on this direction are currently underway (46).

## REFERENCES

1. Preiss, J. (1984) *Annu. Rev. Microbiology* 38, 419–458.
2. Preiss, J. (1991) in *Oxford Surveys of Plant Molecular and Cell Biology* (Miflin, B., Ed.) pp 59–114, Oxford University Press, Oxford.



3. Preiss, J., and Sivak, M. N. (1998) in *Comprehensive Natural Products Chemistry* (Pinto, B. M., Ed.) pp 441–495, Pergamon Press, Oxford.
4. Preiss, J., and Romeo, T. (1994) *Prog. Nucleic Acid Res. Mol. Biol.* 47, 299–329.
5. Sivak, M. N., and Preiss, J. (1998) in *Advances in Food and Nutrition Research* (Taylor, S. L., Ed.) pp 1–199, Academic Press, San Diego.
6. Smith-White, B. J., and Preiss, J. (1992) *J. Mol. Evol.* 34, 449–464.
7. Krishnan, H. B., Reeves, C. D., and Okita, T. W. (1986) *Plant Physiol.* 81, 642–645.
8. Iglesias, A. A., and Preiss, J. (1992) *Biochem. Educ.* 20, 196–203.
9. Takata, H., Takaha, T., Okada, S., Takagi, M., and Imanaka, T. (1997) *J. Bacteriol.* 179, 4689–4698.
10. Frueauf, J. B., Ballicora, M. A., and Preiss, J. (2001) *J. Biol. Chem.* 276, 46319–46325.
11. Kumar, A., Tanaka, T., Lee, Y. M., and Preiss, J. (1988) *J. Biol. Chem.* 263, 14634–14639.
12. Hill, M. A., Kaufmann, K., Otero, J., and Preiss, J. (1991) *J. Biol. Chem.* 266, 12455–12460.
13. Gardiol, A., and Preiss, J. (1990) *Arch. Biochem. Biophys.* 280, 175–180.
14. Parsons, T. F., and Preiss, J. (1978) *J. Biol. Chem.* 253, 6197–6202.
15. Parsons, T. F., and Preiss, J. (1978) *J. Biol. Chem.* 253, 7638–7645.
16. Wu, M. X., and Preiss, J. (1998) *Arch. Biochem. Biophys.* 358, 182–188.
17. Gomez-Casati, D. F., Igarashi, R. Y., Berger, C. N., Brandt, M. E., Iglesias, A. A., and Meyer, C. R. (2001) *Biochemistry* 40, 10169–10178.
18. Ball, K., and Preiss, J. (1994) *J. Biol. Chem.* 269, 24706–24711.
19. Charng, Y. Y., Iglesias, A. A., and Preiss, J. (1994) *J. Biol. Chem.* 269, 24107–24113.
20. Morell, M., Bloom, M., and Preiss, J. (1988) *J. Biol. Chem.* 263, 633–637.
21. Shen, L., and Preiss, J. (1964) *Biochem. Biophys. Res. Commun.* 17, 424–429.
22. Preiss, J., Shen, L., Greenberg, E., and Gentner, N. (1966) *Biochemistry* 5, 1833–1845.
23. Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J., and Klenk, D. C. (1985) *Anal. Biochem.* 150, 76–85.
24. Okita, T. W., Rodriguez, R. L., and Preiss, J. (1981) *J. Biol. Chem.* 256, 6944–6952.
25. Uttaro, A. D., Ugalde, R. A., Preiss, J., and Iglesias, A. A. (1998) *Arch. Biochem. Biophys.* 357, 13–21.
26. Bi, W., and Stambrook, P. J. (1998) *Anal. Biochem.* 256, 137–140.
27. Laemmli, U. K. (1970) *Nature* 227, 680–685.
28. Kumar, A., Ghosh, P., Lee, Y. M., Hill, M. A., and Preiss, J. (1989) *J. Biol. Chem.* 264, 10464–10471.
29. Meyer, C. R., Ghosh, P., Nadler, S., and Preiss, J. (1993) *Arch. Biochem. Biophys.* 302, 64–71.
30. Gentner, N., and Preiss, J. (1967) *Biochem. Biophys. Res. Commun.* 27, 417–423.
31. Sheng, J., Charng, Y. Y., and Preiss, J. (1996) *Biochemistry* 35, 3115–3121.
32. Charng, Y. Y., Sheng, J., and Preiss, J. (1995) *Arch. Biochem. Biophys.* 318, 476–480.
33. Ballicora, M. A., Fu, Y., Nesbitt, N. M., and Preiss, J. (1998) *Plant Physiol.* 118, 265–274.
34. Greene, T. W., Woodbury, R. L., and Okita, T. W. (1996) *Plant Physiol.* 112, 1315–1320.
35. Sheng, J., and Preiss, J. (1997) *Biochemistry* 36, 13077–13084.
36. Frueauf, J. B., Ballicora, M. A., and Preiss, J. (2002) *Arch. Biochem. Biophys.* 400, 208–214.
37. Meyer, C. R., Yirsa, J., Gott, B., and Preiss, J. (1998) *Arch. Biochem. Biophys.* 352, 247–254.
38. Meyer, C. R., Bork, J. A., Nadler, S., Yirsa, J., and Preiss, J. (1998) *Arch. Biochem. Biophys.* 353, 152–159.
39. Ghosh, P., Meyer, C., Remy, E., Peterson, D., and Preiss, J. (1992) *Arch. Biochem. Biophys.* 296, 122–128.
40. Wales, M. E., and Wild, J. R. (1991) *Methods Enzymol.* 202, 687–706.
41. Hong, S., and Preiss, J. (2000) *Arch. Biochem. Biophys.* 378, 349–355.
42. Kuriki, T., Stewart, D. C., and Preiss, J. (1997) *J. Biol. Chem.* 272, 28999–29004.
43. Iglesias, A. A., Barry, G. F., Meyer, C., Bloksberg, L., Nakata, P. A., Greene, T., Laughlin, M. J., Okita, T. W., Kishore, G. M., and Preiss, J. (1993) *J. Biol. Chem.* 268, 1081–1086.
44. Ballicora, M. A., Laughlin, M. J., Fu, Y., Okita, T. W., Barry, G. F., and Preiss, J. (1995) *Plant Physiol.* 109, 245–251.
45. Wu, M. X., and Preiss, J. (2001) *Arch. Biochem. Biophys.* 389, 159–165.
46. Binderup, K., Watanabe, L., Polikarpov, I., Preiss, J., and Arni, R. K. (2000) *Acta Crystallogr., Sect. D: Biol. Crystallogr.* 56, 192–194.

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