The ADP-glucose pyrophosphorylase from *Escherichia coli* comprises two tightly bound distinct domains

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Abstract Computational analysis of ADP-glucose pyrophosphorylases predicts a fold with two domains. Co-expression of two polypeptides comprising residues 1–323 and 328–431 from the *Escherichia coli* ADP-glucose pyrophosphorylase yielded an enzyme form as active as the wild type. The only difference from the wild type was a slightly modified affinity for allosteric effectors. The two polypeptides could not be separated by chromatographic procedures. Separate expression of these polypeptides produced inactive unstable forms. All these results indicated that the ADP-glucose pyrophosphorylase comprises two domains with a strong interaction between them. That interaction is important for allosteric properties and structural stability.

Keywords: Domain interaction; Glycogen synthesis; ADP-glucose pyrophosphorylase; Regulatory domain

1. Introduction

ADP-glucose pyrophosphorylase (ADP-Glc PPase[1], EC 2.7.7.27) catalyzes the formation of ADP-glucose (ADP-Glc) and release of PP_i from glucose 1-phosphate (Glc1P) and ATP. This is the first committed step in the synthesis of bacterial glycogen and starch in plants [1,2]. The ADP-Glc PPase is the key regulatory enzyme of the pathway and its activity is allosterically controlled by intermediates of the major carbon assimilatory pathway of the organism. For example, fructose 1,6-bisphosphate (FBP) activates the enzyme from *Escherichia coli*, whereas AMP is an inhibitor [1]. Other secondary activators of the *E. coli* enzyme are phosphoenolpyruvate (PEP), pyridoxal 5′-phosphate (PLP), and reduced nicotinamide adenine dinucleotides [3]. The reaction is reversible and can be assayed in both directions, synthesis (forward) and pyrophosphorolysis of ADP-Glc (reverse). In vivo, the reaction proceeds mainly in the synthesis direction [4].

The ADP-Glc PPases from enterobacteria are homotetramers (α_4) composed by subunits of ~50 kDa (~440 residues) encoded by the *glgC* gene [1]. The three dimensional structure of these enzymes is not available, but structural and computational analyses predict that the fold of ADP-Glc PPases has two domains [1,5]. Despite the low homology (~20% identity), the predicted structure of the central part of the ADP-Glc PPases (residues ~20–300) resembles other nucleotide-diphosphate-sugar pyrophosphorylases (NDP-sugar PPase) and it is postulated to be the catalytic core of the enzyme [1,5]. On the other hand, the C-terminus (~100 residues) was proposed to be important for regulation and specificity for activators [6]. A similar conclusion has been reached for plant ADP-Glc PPases [2]. Both domains are postulated to interact to regulate the activity because there are residues near the N-terminus that participate in the regulatory site [7–10]. In addition, the presence of an N-terminal tail (~10–20 residues) strongly determines the allosteric properties of the enzyme [11,12].

Partial proteolysis has been a classical procedure to probe domain boundaries [13–15]. In this paper, we used molecular biology techniques to test the 2-domain hypothesis. We divided the enzyme into two polypeptides corresponding to the putative domains, co-expressed them, purified the forms obtained, and characterized their properties.

2. Materials and methods

2.1. Materials

Synthesis and purification of oligonucleotides, and DNA sequencing were performed by the Genomics Technology Support Facility at Michigan State University. Perfect Protein™ marker was obtained form Novagen, Inc. The Mono-Q HR 10/10, 5/5 and Phenyl-Superose columns were purchased from Amersham Pharmacia Biotech. [^{32}P]PPi was purchased from Perkin–Elmer Life Sciences and [^{14}C]Glc1P from Amersham Pharmacia Biotech. All other reagents were purchased at the highest quality available.

2.2. Plasmids and expression vectors

The construction of pETEC, a pET24a derivative with the *E. coli* ADP-Glc PPase, has been previously described [6]. Plasmids pMAB5 and pMAB6 are modified versions of the respective compatible expression vectors pMON17335 and pMON17336 [16]. They have an *Nde*I rather than *Nco*I site for cloning.

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Abbreviations: ADP-Glc, ADP-glucose; ADP-Glc PPase, ADP-glucose pyrophosphorylase; FBP, fructose 1,6-bisphosphate; Glc1P, glucose 1-phosphate; NDP-sugar PPase, nucleotide-diphosphate-sugar pyrophosphorylase; PEP, phosphoenolpyruvate; PLP, pyridoxal 5′-phosphate

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2.3. Linker-scanning mutagenesis

Random introduction of a single 15-bp insertion per plasmid (pETEC) was performed by the technique of Hallet et al. [17] with the commercial GPS™ linker-scanning mutagenesis kit from New England Biolabs. From 130 insertions mutants obtained, 20 colonies had a single 15 bp inserted in the ADP-Glc PPase coding region. The plasmid with the insertion mutant 117 (pETEC-ins117) was selected for further studies and subcloned in pMAB5 (pMAB5-Ec-ins117) after digestion with *Nde*I–*Sac*I for expression in AC70R1-504 cells.

2.4. Truncated protein constructs

To obtain an enzyme with a C-terminal truncation of 108 residues ($Ec_{1–323}$), DNA from the 3′ coding region of the *E. coli* ADP-Glc PPase gene was removed as follows. A PCR fragment was amplified using pETEC-ins117 as a template, T7 promoter primer (5′-TAATACGACTCACTATAGGG-3′) and the oligonucleotide 5′-ACCGGAGAGCTCTGTTTAAACACG-3′, which introduced a *Sac*I site 3 bp after the previously inserted stop codon. The PCR fragment was subcloned into an *Nde*I–*Sac*I digested pMAB5 to obtain pMAB5-$Ec_{1–323}$.

2.5. Construction of a plasmid for expression of the C-terminus alone ($Ec_{328–431}$)

The DNA fragment encoding the C-terminal portion ($Ec_{328–431}$) was amplified from pETEC with the T7 terminator primer (5′-GCTAGTTATTGCTCAGCGG-3′) and the oligonucleotide 5′-GGTAGCCACCATATGACCCTTAACT-3′, which introduced an *Nde*I site. The fragment was subcloned into pMAB6 using the sites *Nde*I and *Sac*I to obtain pMAB6-$Ec_{328–431}$. The coding regions of all final products were verified by DNA sequencing. Fig. 1 summarizes the ADP-Glc PPase constructs used in this work.

2.6. Bacterial strains and expression of the recombinant enzymes

Escherichia coli AC70R1-504 cells without endogenous ADP-Glc PPase activity were used for expression of the enzymes subcloned in pMAB5 and pMAB6 [16]. The expression of pMAB5-Ec-ins117 and pMAB5-$Ec_{1–323}$ was performed as described previously for pML10 [18]. Expression of pMAB6-$Ec_{328–431}$ was performed as described previously for pMON17336 [16]. Co-expression of pMAB5-$Ec_{1–323}$ and pMAB6-$Ec_{328–431}$ was performed as was described previously for plasmids pML10 and pMON17336 [18].

2.7. Protein methods

Protein assay, electrophoresis (SDS–PAGE) and immunoblotting were performed as described previously [5]. N-terminal peptide sequence determination of blotted samples onto PVDF membranes (Bio-Rad) and soluble pure protein samples were performed at the Macromolecular Structure, Sequencing and Synthesis Facility at Michigan State University. Desalting was performed on Bio-Rad 10 DG chromatography columns. Samples were concentrated with Centricon-30 devices (Amicon Inc.).

2.8. Enzymatic assays

Assay A: Pyrophosphorolysis direction. Formation of [^{32}P]ATP from [^{32}P]PPi was determined by the method of Morell et al. [19]. The reaction was carried out for 10 min at 37 °C in a mixture that contained 50 mM HEPES (pH 8.0), 7 mM $MgCl_2$, 1.5 mM [^{32}P]PPi (1500–2500 cpm/nmol), 2 mM ADP-Glc, 4 mM NaF, and 0.05 mg/ml bovine serum albumin, plus enzyme in a total volume of 0.250 ml. Unless other activators were assayed or specifically stated, 2 mM FBP was added in the reaction mixture.

Assay B: Synthesis direction. Formation of ADP-[^{14}C]Glc from [^{14}C]Glc-1P was determined by the method of Yep et al. [20]. The reaction was carried out for 10 min at 37 °C in a mixture that contained 50 mM HEPES (pH 8.0), 7 mM $MgCl_2$, 0.5 mM [^{14}C]Glc-1P (~1000 dpm/nmol), 1.5 mM ATP, 1.5 units/ml pyrophosphatase, and 0.2 mg/ml bovine serum albumin, plus enzyme in a total volume of 0.20 ml, unless specifically stated. Unless other activators were tested, 2 mM FBP was added in the reaction mixture. One unit of enzymatic activity is one μmol of product, either [^{32}P]ATP or ADP-[^{14}C]Glc, formed per min at 37 °C.

2.9. Purification of Ec-ins117

After induction, 20 liters of AC70R1-504 cells transformed with pMAB5-Ec-ins117 was harvested and crude extracts were obtained as described previously [18]. The crude extract was precipitated with a 30–60% ammonium sulfate cut. After centrifugation, the precipitate was redissolved in *buffer A* (50 mM HEPES, pH 8.0, 5 mM $MgCl_2$, 0.1 mM EDTA and 10% sucrose) and desalted. The sample was applied onto a DEAE-Fractogel column (EMD Chemicals) and eluted with a linear NaCl gradient (0–0.5 M). The active fractions were pooled, desalted, applied to a Green A (Amicon Corp., Lexington, MA) affinity chromatography column (1 ml bed volume) and eluted with a 20 ml linear gradient of NaCl (0–2 M). Purest fractions were pooled, desalted, concentrated, and applied onto a Mono Q HR 5/5 (FPLC, Pharmacia) column equilibrated with *buffer A* and eluted with a linear NaCl gradient (0–0.5 M). The post-Mono Q fractions were pooled, concentrated, resuspended in *buffer B* (*buffer A* plus 1.2 M ammonium sulfate), and applied on a Phenyl-Superose (FPLC, Pharmacia) column equilibrated with *buffer B*. The sample was eluted with a decreasing linear gradient of ammonium sulfate (1.2–0.001 M). The purest fractions were pooled, concentrated, and applied to a Pharmacia Superdex 200 HR 10/30 column equilibrated with *buffer A*. The gel filtration chromatography was run at a flow rate of 0.25 ml/min and fractions with activity were pooled. After this step, Ec-ins117 was >95% pure, with a specific activity of 180 U/mg in the pyrophosphorolysis direction assay (Table 1).

2.10. Purification of $Ec_{1–323}$

The protein was monitored by immunoblot throughout the purification steps. AC70R1-504 cells harboring pMAB5-$Ec_{1–323}$ were grown and induced in 4 liters of LB medium. Crude extracts were precipitated with 0–30% ammonium sulfate and the pellet was resuspended in *buffer A* and desalted. Samples were further purified with a Mono Q HR 10/10 and a Green A affinity column as indicated above (Table 1). The fractions containing the enzyme, ~50–60% pure as detected by immunoblot analysis, were pooled, concentrated, and stored at −80 °C.

2.11. Purification of co-expressed $Ec_{1–323}$ + $Ec_{328–431}$

AC70R1-504 cells co-transformed with pMAB5-$Ec_{1–323}$ + pMAB6-$Ec_{328–431}$ were grown and induced in a 1-liter culture. The resulting crude extracts were applied onto a DEAE-Fractogel column (EMD Chemicals Inc.) and eluted with a linear NaCl gradient (0–0.5 M). The active fractions were pooled and precipitated with 30–60% ammonium sulfate. After centrifugation, the pellet was resuspended in *buffer A* and desalted. The sample was applied to a Mono Q HR 10/10 (FPLC,

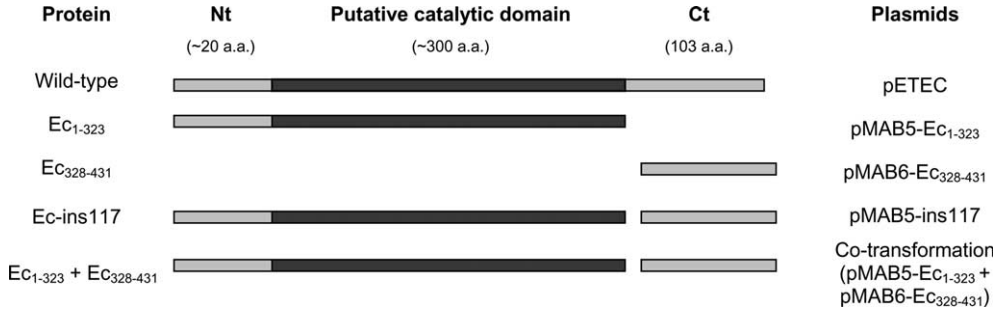

Fig. 1. *E. coli* ADP-Glc PPase constructs.

Table 1
Specific activities of mutant and wild-type *E. coli* ADP-Glc PPases

Sample	Specific activity		Purity[a] (%)
	Pyrophosphorolysis (U/mg)	Synthesis (U/mg)	
Wild-type	131 ± 3	54 ± 1	90
Ec-ins117	180 ± 9[b]	ND[c]	>95
Ec_{1-323}	< 0.001	<0.0001	50–60
$Ec_{1-323} + Ec_{328-341}$	132 ± 5	43 ± 1	>95

Concentration of FBP for the mutants was raised to 4 mM. For the sample $Ec_{1-323} + Ec_{328-341}$, concentration of ATP (synthesis direction) was raised to 5 mM.
[a] Purity of the samples was estimated from SDS–PAGE gels.
[b] Another independent purification produced a pure sample with an activity of 140 U/mg.
[c] Not determined.

Pharmacia) column equilibrated with *buffer A* and eluted with a linear NaCl gradient (0–0.5 M). Purest fractions, assessed by SDS–PAGE, were pooled, concentrated and applied to a Green A affinity chromatography column. The sample was eluted with a linear gradient of NaCl (0–2 M). After this step both co-expression products, $Ec_{1-323} + Ec_{328-431}$ accounted for >95% of the protein (Table 1).

2.12. Molecular mass determination

Purified wild-type *E. coli* ADP-Glc PPase and the mutants Ec-ins117, $Ec_{1-323} + Ec_{328-431}$, and Ec_{1-323} were applied to a column Pharmacia Superdex 200 HR 10/30 equilibrated with *buffer A* and run at 0.25 ml/min. Peaks were followed by absorbance at 280 nm, tested for enzymatic activity or confirmed by immunoblot (Ec_{1-323}). For calibration, ferritin (440 kDa), *E. coli* ADP-Glc PPase (200 kDa), aldolase (158 kDa), and hemoglobin (67 kDa) were used as standard proteins. The calibration line and the interval confidence (90%) were obtained after plotting the data as log (molecular mass) vs. elution volume in the program Origin® 5.0. Molecular mass was expressed with a confidence interval of 90%.

2.13. Kinetic characterization

Kinetic data were plotted as specific activity ($\mu mol\, min^{-1}\, mg^{-1}$) vs. substrate or effector concentration. Kinetic constants were acquired by fitting the data to the Hill equation with a non-linear least square formula using the program Origin™ 5.0. Hill plots were used to calculate the Hill coefficient n_H and the kinetic constants that correspond to the activator, substrate or inhibitor concentrations giving 50% of the maximal activation ($A_{0.5}$), velocity ($S_{0.5}$), and inhibition ($I_{0.5}$).

3. Results and discussion

Random insertions of a single 15-bp fragment into a recombinant *E. coli glg* C gene were produced to study structure–function relationships on the ADP-Glc PPase (unpublished results). One of the insertion mutants generated, Ec-ins117, had a stop codon after residue 323 in the translated polypeptide of the *E. coli glg*C gene. Surprisingly, the polypeptide still possessed ADP-Glc PPase activity although the stop codon would truncate 108 residues of the C-terminus. Colonies of BL21(DE3) cells expressing Ec-ins117 or the wild-type enzyme, but not a control plasmid (pET24a), stained brown in the presence of iodine vapors (unpublished results). This indicated high levels of glycogen in the cell and suggested the presence of ADP-Glc PPase activity [21]. For this reason, we characterized the properties of this putatively truncated form.

3.1. Purification and characterization of Ec-ins117

Despite the lower expression level of Ec-ins117 as observed by immunoblot and SDS–PAGE (not shown), the pyrophosphorolysis activity of this mutant was 0.31 U/mg in the crude extracts. That was two orders of magnitude lower than the overexpression of the wild-type ADP-Glc PPase but two orders of magnitude higher than pET24a, the plasmid control without insert (not shown). Attempts to isolate this ~37 kDa truncated protein resulted in the co-purification of a 12-kDa peptide even after five different chromatographic columns (Fig. 2). Despite the differences in molecular size, both polypeptides comigrated in a high-resolution gel filtration column (Superdex 200 HR). The N-terminal sequence of the 12-kDa band, obtained after SDS–PAGE and blotting, was MTLNSLVSGG, which indicated that the polypeptide corresponds to the C-terminus of the *E. coli* ADP-Glc PPase with residue 328 as the N-terminal methionine (103 amino acids). In addition, N-terminal sequencing of the purified Ec-ins117 in solution confirmed the presence of two polypeptides. There were both the C-terminal fragment together with the N-terminus of the enzyme (37 kDa band). It also indicated that the stoichiometry of the 37 and 12 kDa polypeptides was 1:1 (Fig. 3). Based on the sequence data, the polypeptide of 12 kDa appeared to be the product of an open reading frame that starts 24 bp downstream of the inserted stop codon, at Met^{328} (Fig. 4). The lower expression of this hetero-oligomer form can be explained by the absence of a proper ribosome-binding site before Met^{328}. Kinetic properties of the Ec-ins117 mutant purest sample were determined in the pyrophosphorolysis direction (Table 2). Apparent affinities for the substrates (ADP-Glc, PP_i), and cofactor (Mg^{2+}), were similar to those of the wild-type enzyme. The main difference was the apparent affinity for the activators. The $A_{0.5}$ for FBP was almost 6-fold higher for Ec-ins117 compared to the wild type.

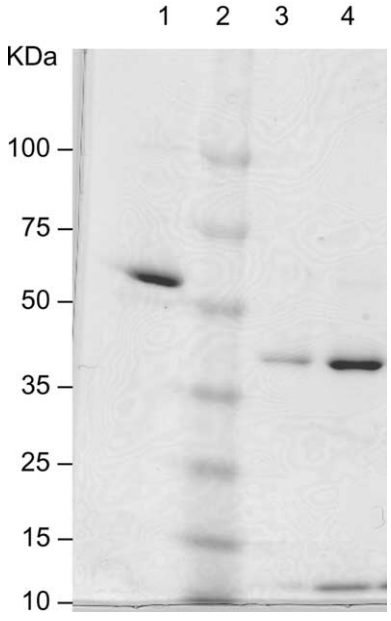

Fig. 2. SDS–PAGE of *E. coli* wild-type ADP-Glc PPase and Ec-ins117 mutant. Lane 1, *E. coli* wild-type ADP-Glc PPase; lane 2, pre-stained molecular mass markers; lane 3, Ec-ins117 mutant (0.5 μg); and lane 4, Ec-ins117 mutant (1.5 μg).

Amino Acid	Residue Number 1	2	3	4	5	6	7
				pmol			
D	3.11	2.35	2.27	2.88	2.21	2.66	**10.19**
N	0.72	0.51	0.87	**9.09**	1.44	**8.08**	2.78
S	3.24	**11.10**	2.04	1.96	**8.85**	1.53	2.32
T	1.41	**11.40**	0.49	0.57	0.35	0.22	0.77
E	0.49	0.50	0.63	**9.98**	2.59	1.33	1.24
M	**14.60**	0.33	0.20	0.23	0.27	0.36	0.43
V	**14.90**	1.89	1.03	1.03	1.21	1.78	**11.90**
K	0.49	0.47	ND	0.66	**13.39**	3.24	1.47
L	0.42	1.39	**29.18**	3.31	ND	**13.09**	2.98
Sequences deduced							
1	V	S	L	E	K	N	D
2	M	T	L	N	S	L	V

Fig. 3. N-terminal sequence analysis of the Ec-ins117 mutant Automated sequencing of the Ec-ins117 protein was performed directly from the purest fraction. The values are the picomoles of amino acid derivative found in each cycle of degradation. In each cycle of cleavage, major peaks are in bold. Residues Q, G, H, A, R, Y, P, W, F, I were negligible in all these cycles and were omitted for simplicity. ND: Not detectable.

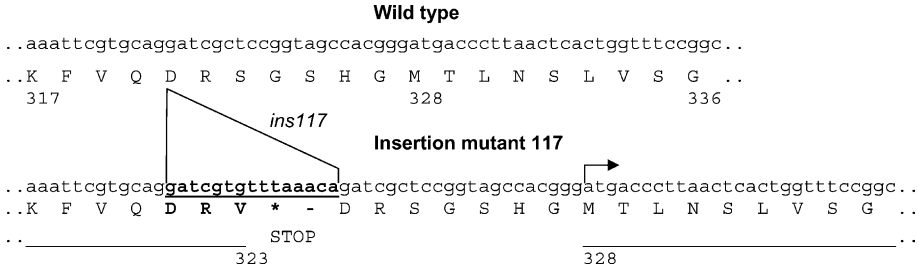

Fig. 4. Sequence of the insertion 117 in the *E. coli* ADP-Glc PPase. The upper sequence corresponds to the wild-type ADP-Glc PPase and the lower to the Ec-ins117, in which a 15-bp linker was inserted by linker-scanning mutagenesis as described in section 2. Underlined is the 15-bp insertion. A stop codon was introduced at residue 324, generating the transcription and translation of two polypeptides: the ~37 kDa (comprising residues 1-323), and the 12 kDa peptide starting at Met^{328}.

Table 2
Apparent affinity for the different effectors of the mutant Ec-ins117 and wild-type ADP-Glc PPase

Effectors	Enzyme		
	Wild type	Ec-ins117	Ratio[a]
Substrates	$S_{0.5}$ (μM)		
ADP-Glc	166 ± 26	241 ± 25	1.45
PP_i	81 ± 14	45 ± 13	0.55
Cofactor	$S_{0.5}$ (μM)		
Mg^{2+}	1420 ± 310	2147 ± 86	1.51
Activators	$A_{0.5}$ (μM)		
FBP	37.8 ± 5.2	214 ± 53	5.7
PLP	0.045 ± 0.004	0.156 ± 0.001	3.5
PEP	282 ± 15	>2000[b]	>7.1
Inhibitor	$I_{0.5}$ (μM)		
AMP	41 ± 4[c]	21 ± 8[d]	0.51

The assays were performed in the pyrophosphorolysis direction.
[a] This is the value of the kinetic constant of Ec-ins117 divided the wild type.
[b] Saturation was not reached.
[c] To observe AMP inhibition, activator should be added. In this case, FBP was 40 μM to avoid saturating concentrations of activator and allowed the competitive inhibition by AMP. That concentration is about the $A_{0.5}$ value.
[d] FBP was 300 μM, which is about the $A_{0.5}$ value.

3.2. Purification and characterization of C-terminal truncated forms

It was important to determine whether the truncated enzyme was active by itself or if the co-expression with the C-terminal 12 kDa polypeptide contributed to the reconstitution of the activity. To ensure the expression of a single polypeptide comprising residues 1–323 ($Ec_{1–323}$), a *Sac*I restriction site was introduced immediately downstream of the stop codon by site-directed mutagenesis. Only the $Ec_{1–323}$ coding fragment was subcloned in pMAB5, expressed, and purified as described under Section 2. With this approach, most of the expressed enzyme was insoluble, whereas the soluble fraction had a peptide of about 37 kDa and some lower bands recognized by immunoblotting, which indicated that it was highly proteolyzed (data not shown). $Ec_{1–323}$ (~37 kDa) was purified to ~50% purity where the main contaminants were lower bands still recognized by immunoblot. The activity was negligible (Table 1) and did not increase significantly with higher concentrations of FBP, up to 10 mM (data not shown). Gel filtration showed that the sample was highly aggregated (data not shown).

The total loss of activity after the removal of the C-terminus indicated that this domain is involved in more than regulation. In the inactive $Ec_{1–323}$ mutant, the C-terminal deletion yielded

Table 3
Kinetic parameters in the synthesis direction of *E. coli* ADP-Glc PPase wild type and $Ec_{1–323} + Ec_{328–431}$ form

Enzyme	ATP		Glc1P		FBP		AMP			
	$S_{0.5}$ (μM)	n_H	$S_{0.5}$ (μM)	n_H	$A_{0.5}$ (μM)	n_H	$I_{0.5}$[a] (μM)	n_H	$I_{0.5}$[b] (μM)	n_H
Wild type	301 ± 14	1.9	15.0 ± 1.3	1.0	92 ± 1	2.1	3.8 ± 0.4	1.1	92 ± 4	2.7
$Ec_{1–323} + Ec_{328–431}$	1410 ± 51	2.9	13.6 ± 1.2	1.2	327 ± 15	2.0	10 ± 2	1.2	41 ± 3	0.9

Except for the ATP curves, the concentration of ATP for the $Ec_{1–323} + Ec_{328–431}$ was 5 mM to ensure saturation. For the ATP and Glc1P curves, the concentration of FBP was 2 mM for the wild type and 4 mM for $Ec_{1–323} + Ec_{328–431}$.
[a] Inhibition curve performed in the presence of 30 μM FBP.
[b] Inhibition curve performed in the presence of 130 μM FBP.

a form highly susceptible to proteolysis. In an attempt to study which specific regions of this domain were important, deletions of 20–90 residues (every 10) from the C-terminus were evaluated. The truncated enzymes were expressed in small-scale systems and were visualized by SDS–PAGE and Western blot at their expected molecular sizes (data not shown). The recombinant products were found mainly in the insoluble fraction of the sample and the pyrophosphorolysis ADP-Glc PPase activity was negligible in all crude extracts (<0.0001 U/mg). These results suggest that most of, or maybe all of the ~100 residues of the C-terminus are necessary for maintaining a functional *E. coli* ADP-Glc PPase. It may be due to the role of specific residues or due to the overall structure of the domain that is necessary for the integrity of the enzyme.

3.3. Characterization of $Ec_{1–323} + Ec_{328–431}$

The results obtained with $Ec_{1–323}$ strongly suggested that the properties of Ec-ins117 are due to the presence of both the 37- and the 12-kDa polypeptides. To verify this possibility, the *E. coli glg*C fragment encoding the 103 C-terminal residues of ADP-Glc PPase ($Ec_{328–431}$) was subcloned into a compatible expression vector (pMAB6). Co-expression of $Ec_{1–323}$ and $Ec_{328–431}$ was performed in AC70R1-504 cells, yielding extracts with activity (39 U/mg) comparable to the wild type (24 U/mg) [6]. That level of expression was about 100-fold higher than the Ec-ins117. After the same purification procedure of the wild-type enzyme, $Ec_{1–323}$ and $Ec_{328–431}$ co-purified, indicating a very strong interaction. The 37-kDa fragment did not appear as proteolyzed as when it was expressed alone (data not shown). In addition, the specific activity values were comparable to that of the wild-type enzyme (Table 1). The molecular mass determined by gel filtration in native conditions of the co-expressed $Ec_{1–323} + Ec_{328–431}$ (227 ± 32 kDa) and Ec-ins117 mutant (213 ± 30 kDa) was not distinguishable from the wild-type enzyme (200 kDa). These results, together with the 1:1 stoichiometry (Fig. 3), indicate that the structures of both $Ec_{1–323} + Ec_{328–431}$ and Ec-ins117 are $\alpha'_4\omega_4$, rather than α_4 as the wild type (α' and ω being the polypeptides $Ec_{1–323}$ and $Ec_{328–431}$, respectively). The apparent affinity for the substrate ATP (4.7-fold higher $S_{0.5}$) and for the activator FBP (3.5-fold higher $A_{0.5}$ in the synthesis direction) was altered compared to those of the wild type (Table 3). The inhibition by AMP was not significantly changed and the apparent affinity for Glc1P was identical (Table 3).

4. Conclusions

Important structural information can be inferred from these results: (i) the stop codon inserted in the Ec-ins117 mutant, which generated a "nick" in the protein sequence, was in a non-critical position for the 3D structure of the protein. (ii) These data also support structure predictions and the hypothesis that the *E. coli* ADP-Glc PPase is organized in at least

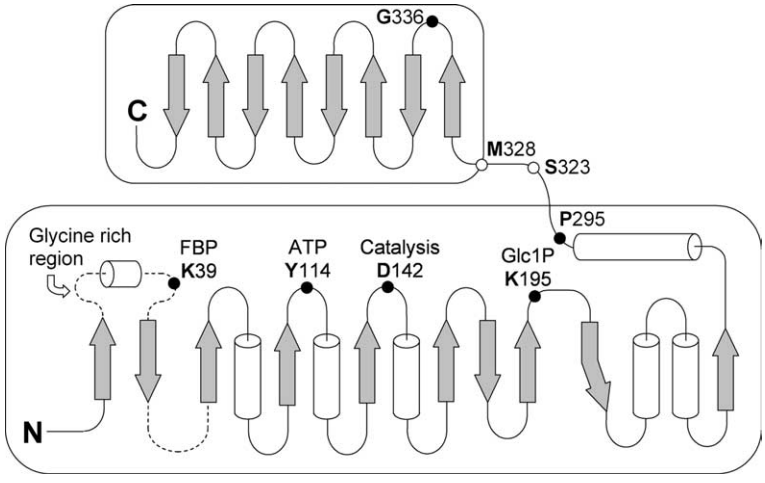

Fig. 5. Domain organization of the ADP-Glc PPase from *E. coli* The secondary structure is based on the prediction from [1,2]. Arrows and cylinders represent β-sheets and α-helices, respectively. Dark circles are important residues that interact with effectors or participate in catalysis. When Gly^{336} and Pro^{295} are replaced, allosteric mutants are generated [1]. After insertion of a stop codon, residues between Ser^{323} and Met^{328} were "nicked" (see Fig. 4).

two distinct domains [1]. According to the proposed model of domain organization [1,2,5], the stop codon was inserted in a loop that separates the two putative domains (Fig. 5). The data also agree with the hypothesis that the C-terminal domain is ~100 residues long and linked to the catalytic domain by a long loop (~30 residues) [1]. Removal of this domain yielded an inactive enzyme rather than a shorter unregulated version as other NDP-sugar PPases of 30–40 kDa [22–25]. Even the removal of 20 residues at the C-terminus rendered a form without activity. It is possible that the absence of even a small part of the C-terminal domain leaves the enzyme in a misfolded or insoluble form. On the other hand, a "nick" separating the C-terminus and the catalytic domain, obtained by the co-expression of both the N- and the C-terminal polypeptides, produced an enzyme with very similar properties to the wild type. Only the apparent affinity for the activator was slightly modified. Possibly, the lack of a covalent bond between the N- and C-domains yields a more relaxed enzyme that favors less the activated conformation. A slightly higher concentration of activator overcame this problem, 3.5- and 5.7-fold in the synthesis and pyrophosphorolysis direction, respectively. We cannot rule out the possibility that the residues removed in the "nick" ($S^{323}GSHG^{327}$) are the reason for the decrease in activator affinity. However, in other ADP-Glc PPases, those residues are not conserved or they are deleted, together with Met^{328} [1]. Most likely, they are part of an area that connects the two major domains and does not play a specific role (Fig. 5).

Partial proteolysis has been a classical procedure to detect domain boundaries [13–15]. In this paper, we used molecular biology techniques to probe whether the *E. coli* ADP-Glc PPase comprises two domains. After co-expression of the C- and the N-terminal domains, they remained non-covalently bound, with a tight interaction that cannot be disturbed even after passage through several chromatography columns. Presence of the C-terminal domain stabilizes the enzyme and prevents proteolytic degradation of the N-terminal domain. These results provide a structural insight for the differential roles of these regions of the ADP-Glc PPase. We propose that they are two strongly interacting domains that have to be expressed together to obtain a fully functional enzyme. According to previous analysis [1,5], the catalytic domain is located in the 37 kDa fragment (similar to most NDP-sugar PPases) and the responsibility for the regulation is shared by both the 12 and the 37 kDa fragments.

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