Dissection of *Escherichia coli* glutamate 5-kinase: Functional impact of the deletion of the PUA domain

Isabel Pérez-Arellano[a], Vicente Rubio[b], Javier Cervera[a,*]

[a] *Centro de Investigación Príncipe Felipe, Avda. Autopista del Saler, 16, Valencia 46013, Spain*
[b] *Instituto de Biomedicina de Valencia (CSIC), Jaime Roig 11, Valencia 46010, Spain*

Abstract **Glutamate 5-kinase (G5K) catalyzes the controlling first step of the synthesis of the osmoprotective amino acid proline, which feed-back inhibits G5K. Microbial G5K generally consists of one amino acid kinase (AAK) and one PUA (named after pseudo uridine synthases and archaeosine-specific transglycosylases) domain. To investigate the role of the PUA domain, we have deleted it from *Escherichia coli* G5K. We show that wild-type G5K requires free Mg for activity, it is tetrameric, and it aggregates to higher forms in a proline-dependent way. G5K lacking the PUA domain remains tetrameric, active, and proline-inhibitable, but the Mg requirement and the proline-triggered aggregation are greatly diminished and abolished, respectively, and more proline is needed for inhibition. We propose that the PUA domain modulates the function of the AAK domain, opening the way to potential PUA domain-mediated regulation of G5K; and that this domain moves, exposing new surfaces upon proline binding.**
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1. Introduction

Glutamate 5-kinase (G5K) catalyzes the first and controlling step of proline synthesis [1,2], a step that in animals is also the first of ornithine synthesis [3], and which is feed-back inhibited in microorganisms and plants by proline [1,4,5] and in animals by ornithine [6]. Since in plants and microorganisms proline plays a role in cell protection against osmotic stress, G5K is involved in osmoprotection [4,5,7]. In mammals G5K is crucial for the provision of ornithine for ammonia detoxification, as reflected in the clinical hyperammonia caused in humans by G5K deficiency [3,6].

Although plant and animal G5Ks are part of a bifunctional polypeptide composed of an N-terminal moiety corresponding to G5K and a C-terminal moiety corresponding to glutamyl 5-phosphate reductase (the enzyme catalyzing the second step of proline biosynthesis) [5–7], bacterial and yeast G5Ks are monofunctional single-polypeptide enzymes ([8,9] and Swiss-prot database, http://ca.expasy.org/sprot/). The majority of the microbial enzymes are ∼370-residue polypeptides composed, judged from homology searches, of (Fig. 1) a ∼260-residue N-terminal amino acid kinase (AAK) domain [10], and a ∼110-residue C-terminal region containing one PUA domain [11]. PUA domains were defined on the basis of sequence comparisons with pseudo uridine synthases and archaeosine-specific transglycosylases. They consist of ∼80 amino acids, and are folded as a six-strand β-sandwich and an α helix [12,13]. Although most bacterial G5Ks contain a C-terminal PUA domain, in some species such as *Streptococcus thermophilus* [9] the G5K polypeptide consists only of the ∼260-residue AAK domain, and yet, in addition to catalyzing the reaction, this small G5K is inhibited by proline [9].

Since the AAK domain appears sufficient for catalysis and for feed-back control, the functions of the PUA domain found in most microbial G5Ks are unclear. In other proteins, PUA domains are involved or are postulated to be involved in RNA binding [12,13], and, because of this, it has been speculated [11] that the PUA domain of G5K may be responsible, in as yet undetermined ways, for the reported involvement of G5K in gene expression regulation [14]. However, it is conceivable that the PUA domain may modify properties of the enzyme such as the stability, the substrate kinetics, the inhibition by proline or by ADP (another G5K inhibitor proposed to have a physiological role [1]), or the quaternary structure. To investigate these possibilities, we have deleted the C-terminal 167 residues (from residue 260 to the C-terminus) of *Escherichia coli* G5K, analyzing the consequences of this deletion. The results of these studies, reported here, reveal as important novel traits of *E. coli* G5K that the intact enzyme is tetrameric and proline-aggregable, and that its activity requires high concentrations of free Mg. Our results demonstrate that the PUA domain is not essential for either catalysis, proline inhibition or tetramer formation, and they show that the deletion of the PUA domain greatly reduces and increases, respectively, the concentrations needed of Mg for activation and of proline for inhibition of G5K. In addition, the truncation eliminates proline-triggered aggregation. On the bases of these findings we propose that the PUA domain might be a modulatory element of the function of G5K.

*Corresponding author. Fax: +34 96 328 97 01.
E-mail address: cervera@cipf.es (J. Cervera).

Fig. 1. Schematic representation of the domain organization in *E. coli* G5K. Figures denote residue numbers and the arrow signals the truncation site. The AAK and PUA domains are colored black and grey, respectively.

2. Materials and methods

2.1. Generation of truncated G5K

Cloning into and overexpression from the pET22b (Novagen, Madison, WI) derived plasmid pGKE of full-length *pro*B, the gene that encodes *E. coli* G5K, was previously reported [15]. The *pro*B gene was truncated immediately after an in-frame stop codon that replaced the normal codon 260, by PCR-amplification from plasmid pGKE, using the expand high fidelity PCR system (from Roche, Mannheim, Germany) and the primers 5′CAGAGACATATGAGTGACAGCC3′ and 5′GGTTTGGATCCGGTTACGCCTGGGCAT3′. The amplified DNA was introduced into pET22b exactly as described for wild-type G5K [15], yielding plasmid pGK260. All other steps for plasmid isolation, for overexpression of the protein in *E. coli* and for protein purification were previously reported [15] for the wild-type enzyme. The purified truncated protein was stored at −80 °C as a 35% saturation $(NH_4)_2SO_4$ precipitate.

2.2. Enzyme activity assays

Twenty five µl of appropriate dilutions of either the wild-type or the truncated enzyme in a solution of 1 mg ml^{-1} bovine serum albumin in 0.1 M imidazole, pH 7, containing 1 mM dithiothreitol, was added to 0.175 ml of a solution of the same buffer and thiol reagent, at 37 °C, containing sufficient amounts of the indicated compounds to give the following final concentrations: 20 mM ATP, 0.15 M Na glutamate, 80 mM $MgCl_2$, and, when used, the concentrations specified of L-proline or of ADP. When ATP was varied, the concentration of glutamate was 0.3 M and that of $MgCl_2$ always exceeded that of ATP by 60 mM. When glutamate was varied, ATP and $MgCl_2$ were present at concentrations of 20 and 80 mM, respectively. The reaction was terminated after 10 min with 0.2 ml of 10% (w/v) trichloroacetic acid, and ADP was measured spectrophotometrically at 340 nm in the neutralized supernatant [16]. When indicated, and particularly when the inhibition by ADP was studied, phosphate was determined at the end of the 10-min incubation using malacchite green [17]. When testing the formation of glutamyl 5-phosphate by conversion to γ-glutamyl hydroxamate (Table 1), 0.2 M hydroxylamine (neutralized) was included in the assay, and hydroxamate was determined colorimetrically [18] at the end of the 10 min incubation at 37 °C. Unless indicated, assay results are given as the means ± S.E. of at least three determinations. One enzyme unit produces 1 µmol of product min^{-1}. The program GraphPad Prism (GraphPad Software, San Diego, CA) was used for curve fitting of kinetic data, and to estimate the concentrations of free Mg (Mg_F) by interpolation in a plot constructed according to the expression:

$$Mg_T = Mg_F \times \{1 + [(K_1 \times ATP)/(1 + K_1 \times Mg_F)] + [(K_2 \times Glu)/(1 + K_2 \times Mg_F)]\},$$

where Mg_T, ATP and Glu are the total concentrations of these compounds, and K_1 and K_2 are the stability constants for the MgATP and MgGlu complexes (33 000 and 79 M^{-1}, respectively [19]). Protein was determined with the Bradford assay [20] using bovine serum albumin as standard. SDS–PAGE was performed according to Laemmli [21] using 12% gels.

2.3. Gel filtration chromatography

A Superdex 200 H/R 10/30 column fitted into a FPLC system (both from Amersham-Pharmacia, Freiburg, Germany) was used at a flow rate of 0.5 ml min^{-1}, at 23 °C, monitoring the A_{280} of the eluate. The solution used for dissolving the protein and for equilibrating and running the column was 50 mM Tris–HCl, pH 7.2, 1 mM dithiothreitol, 0.15 M NaCl, and, when used, the indicated concentrations of proline.

3. Results

3.1. Truncated G5K is catalytically active and reasonably stable

E. coli transformed with plasmid pGK260, which encodes the form of G5K that is truncated after residue 259, produced large amounts of the expected protein product [8], as
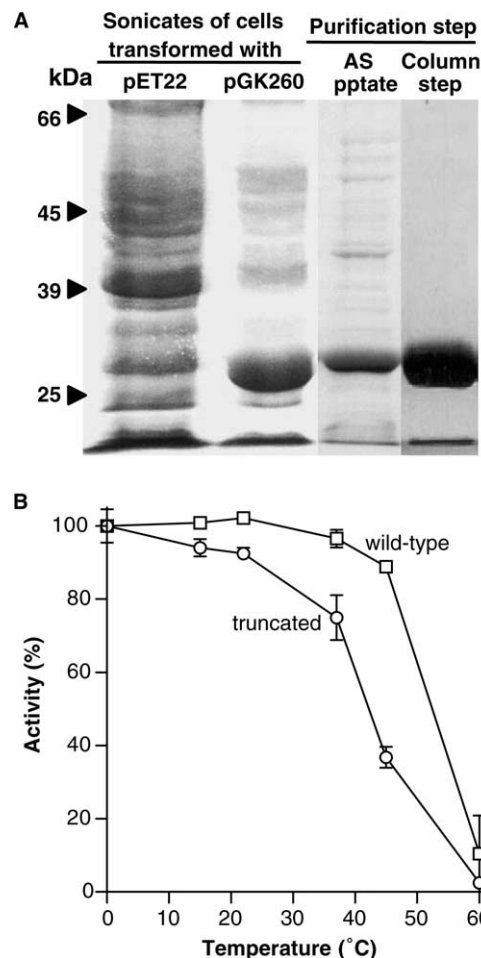

Fig. 2. Purification and thermal stability of truncated G5K. (A) SDS–PAGE analysis. Migration of protein standards is indicated with arrowpoints. AS, ammonium sulfate. (B) Truncated and wild-type G5K (0.2–0.3 mg ml^{-1}) were incubated 15 min at the indicated temperature in 50 mM Tris–HCl, pH 7.2, 1 mM dithiothreitol and 1 mg ml^{-1} bovine serum albumin, then chilled, and enzyme activity was assayed. The activity is given as a percentage of the activity before incubation.

Table 1
ADP, P_i and hydroxamate production catalyzed by wild-type and truncated G5K

Enzyme form	ADP	P_i (µmol min^{-1} mg^{-1})	Hydroxamate
Wild-type G5K	111 ± 5	125 ± 6	12 ± 1
Truncated G5K	51 ± 5	51 ± 1	5 ± 1

The standard assay (see Section 2) was used in the absence (ADP and P_i determination; amount of enzyme used, 0.05–0.28 µg) or in the presence of 0.2 M hydroxylamine (hydroxamate determination; 3–17 µg enzyme used).

revealed by the observation using SDS–PAGE of a large 27.4 kDa band (expected mass, 27265 Da) in the extracts of the pGK260-transformed cells but not in those of the pET22b-transformed cells (Fig. 2A, left two lanes). Truncated G5K was purified (Fig. 2A, right two lanes) to essential homogeneity in a yield of ∼20 mg pure protein L^{-1} of initial culture by the same simple two-step procedure (precipitation at 20%-saturation ammonium sulfate, and ion exchange chromatography) used earlier with wild-type G5K [15]. The polypeptide mass of the isolated protein, determined using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry, was 27118 Da, in agreement, within experimental error, with the mass of 27134 Da deduced from the sequence minus Met1.

Similarly to wild-type G5K, the truncated enzyme catalyzed the production of equimolar amounts of ADP and P$_i$ (P$_i$ is generated in the spontaneous cyclization of glutamyl 5-phosphate, see [22]) (Table 1), although at a rate representing, on a protein basis, ∼45% of the rate of the reaction with wild-type G5K (Table 1). When hydroxylamine was included into the assay, the wild-type and the truncated enzymes produced hydroxamate (demonstrated colorimetrically; Table 1), although this production, as previously reported [22] for purified *E. coli* G5K, exhibited with both enzyme forms ∼10% efficiency relative to ADP or P$_i$ production. The production of the hydroxamate proves that truncated G5K truly synthesizes glutamyl 5-phosphate. In addition, ADP release by the wild-type and the truncated enzyme forms proved to be strictly dependent on, and highly specific for, L-glutamate, with no ADP release (detection limit, 1% of the release with L-gluta-

mate) when 80 mM L-glutamate was replaced by the same concentration of either by L-aspartate, glutarate, γ-amino butyrate or L-norvaline (data not illustrated).

PUA domain deletion decreased modestly but significantly the thermal stability of G5K (Fig. 2B). The decrease in stability was most evident after 15-min incubation at 45 °C, since at this temperature >80% of the activity was retained by the wild-type enzyme but only ∼40% activity was retained by the truncated enzyme.

3.2. G5K requires free Mg for activity, and the truncation greatly decreases this requirement

Fig. 3A demonstrates that wild-type G5K requires for activity concentrations of free Mg which exceed amply those needed for formation of the MgATP complex [19]. The data allow estimation of a $K_a^{Mg} = 4.3 \pm 0.3$ mM, and are consistent with an inhibitory effect of high concentrations of Mg ($K_i \cong 15$ mM). The truncation greatly decreases the requirement of Mg, and thus, as soon as the concentration of Mg reaches that needed for converting virtually all ATP into the MgATP complex, the truncated enzyme exhibits its highest activity, which then falls, since the inhibition at high Mg is not abolished. In contrast with this dramatic effect of the truncation on the Mg-dependency of the activity, the truncation had no effect on the apparent K_m^{ATP} (1.6 ± 0.1 mM; $V^{[ATP]=\infty}$, 132 ± 2 and 75 ± 2 U mg^{-1} for wild-type and truncated G5K, respectively) and only changed modestly (<3-fold increase) the $S_{0.5}$ for glutamate (91 ± 4 and 227 ± 8 mM for wild-type and truncated G5K; the corresponding $V^{[Glu]=\infty}$ were 170 ± 2 and 108 ± 3 U mg^{-1}, respectively).
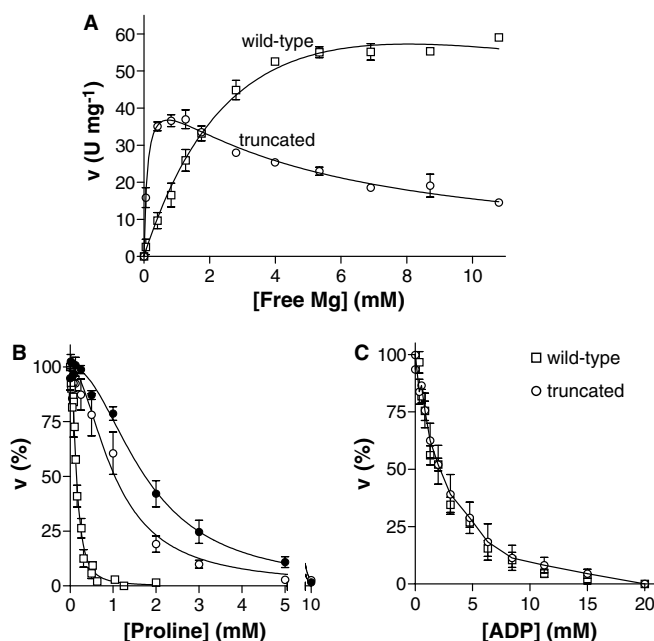

Fig. 3. Dependency of the activity of wild-type (□) and truncated (○, ●) G5K on free Mg^{2+}(A), proline (B) and ADP (C). The amount of enzyme used in each assay was in the 0.1–0.5 μg range. Assays contained 0.15 M glutamate, 2 mM (A and C) or 20 mM (B) ATP and the following MgCl$_2$ concentrations: (A) varied in the range 0–80 mM; (B, open symbols) 80 mM; (B, filled circles) 35 mM; (C) 62 mM. In (A) free Mg (Mg$_F$) was estimated as indicated in Section 2, and the lines fitted to the data conform to the equation for hyperbolic activation with inhibition at excess Mg [33] $v = V \times [Mg_F]/\{K_a + [Mg_F] + ([Mg_F]^2/K_i)\}$, for the following V, K_a and K_i values: 120 ± 4 U mg^{-1}, 4.3 ± 0.3 and 15 mM, respectively, for wild-type G5K; and 48 ± 2 U mg^{-1}, 110 ± 2 μM and 4.8 ± 0.5 mM, respectively, for the truncated enzyme. In (B) and (C) velocities are given as a percentage of the velocity of the same enzyme form in the absence of proline. In (B) data were fitted to sigmoidal inhibition kinetics, according to the expression, $v = 100 \times I_{0.5}^N/(I_{0.5}^N + [Pro]^N)$, for the following respective values for $I_{0.5}$ and N (the Hill coefficient): 0.15 ± 0.01 mM and 2.0 ± 0.1 for wild-type G5K; 1.04 ± 0.08 mM and 1.8 ± 0.2 for truncated G5K at 80 mM MgCl$_2$; and 1.8 ± 0.1 mM and 2.0 ± 0.2 for truncated G5K at 35 mM MgCl$_2$.

3.3. G5K truncation increases the concentrations of proline needed for inhibition

Fig. 3B demonstrates that G5K is completely and sigmoidally inhibited by proline with a half-inhibitory concentration ($I_{0.5}$) of 0.15 ± 0.01 mM and a Hill coefficient, $N = 2.0 \pm 0.1$ (the sigmoidicity is not patent in Fig. 3B for wild-type G5K because it occurs at too low proline concentrations relative to the scale used). Truncated G5K was also sigmoidally inhibited by proline

with a similar N value, but the proline concentrations needed for inhibition were nearly one order of magnitude higher ($I_{0.5} = 1.04 \pm 0.08$ mM) (Fig. 3B). The increase was even larger ($I_{0.5} = 1.8 \pm 0.1$ mM) when the activity of the truncated enzyme was assayed at optimal Mg concentration (Fig. 3B, filled circles). The effect of the truncation on proline inhibition appears highly specific, since ADP, another inhibitor believed to modulate G5K activity in vivo [1,22], inhibited identically the wild-type and the truncated enzyme forms (Fig. 3C).

3.4. Oligomeric state of G5K. Influence of proline and of the PUA domain deletion

Gel filtration assays showed that, in the absence of proline, G5K was eluted from a Superdex 200 column as a single peak at the position expected for a tetramer (Fig. 4A). The regular shape of the peak, the absence of other substantial peaks, and the independence of the elution position of the peak on protein concentration (range tested, 1–5 mg ml^{-1} in the sample applied to the column) indicate that the G5K tetramer is highly stable. The truncated enzyme was also eluted at the expected position for a tetramer, and thus, the PUA domain is not essential for tetramer formation. Addition of 10 mM proline had a dramatic effect on the position of elution of wild-type G5K (Fig. 4A) since the peak appeared much earlier, at a position fitting best the formation of an oligomer of ~10 subunits (Fig. 4B). The peak observed when proline was present (Fig. 4A) was wider, with a sharp front and a long tail, suggesting a rapid equilibrium between the tetramer and larger aggregates. As expected for a rapid-equilibrium self-aggregating system influenced by the concentration of proline, the position of the observed peak varied with proline concentration, giving an apparently hyperbolic dependency of the size of the protein (estimated from the elution position of the peak) on proline concentration, with an estimated K_D^{Pro} of 0.07 ± 0.01 mM, a value which is remarkably close to that of the $I_{0.5}^{Pro}$ for inhibition of G5K. The size of the enzyme oligomer extrapolated at infinite proline is 11.3 ± 0.5 subunits, and thus in the presence of proline the enzyme possibly forms dodecamers (trimers of tetramers). Enzyme truncation abolished this proline-triggered aggregation even when the concentration of proline was increased to 10 mM (Fig. 4), a value vastly exceeding the concentrations of proline causing complete inhibition of the truncated enzyme.
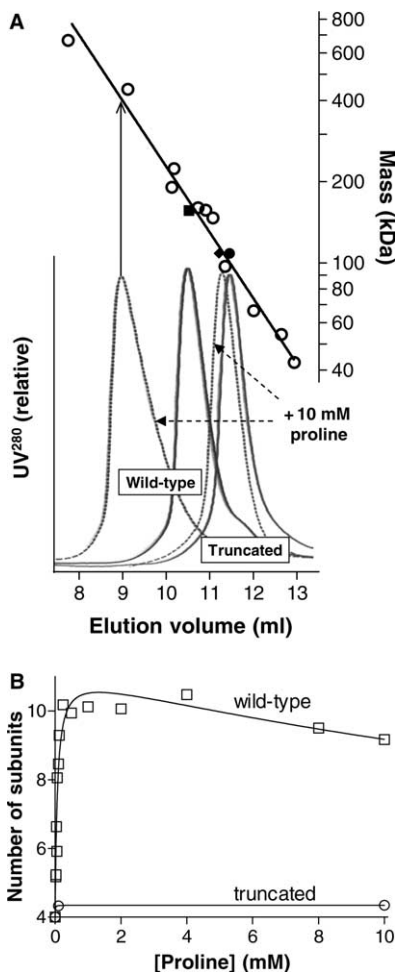
Fig. 4. Mass estimate of wild-type and truncated G5K by gel filtration, and the influence of proline. (A) Elution profiles of the wild-type and truncated enzymes (monitored as UV absorption) without (solid lines) or with 10 mM proline (dashed lines). A plot of molecular mass (in log scale) as a function of the elution volume is represented above the peaks. (○) Protein standards (masses are given in parentheses, in kDa): ovalbumin (42.7), *E. coli* acetylglutamate kinase (54.3), bovine serum albumin (66.4), *E. coli* aspartokinase III (97.1), yeast alcohol dehydrogenase (146.8), aldolase (156.8), γ-globulin (160), *Pseudomonas aeruginose* acetylglutamate kinase (190.5), β-amylase (223.8), ferritin (440), and bovine thyroglobulin (669). Truncated G5K in the absence (●) or in the presence (◆) of 10 mM proline, and (■) wild-type G5K in the absence of proline, fit well in the semilogarithmic plot assuming that they are tetramers (108.5 and 156.2 kDa, respectively). The position in the plot of wild-type G5K when eluted in the presence of 10 mM proline is indicated with a long arrow. (B) Aggregation triggered by proline of wild-type G5K and lack of aggregation of the truncated enzyme. Masses estimated by gel filtration have been converted into number of subunits in the oligomer. The curve fitted to the data for wild-type G5K corresponds to a hyperbolic change from 4 (no proline) to 11.3 ± 0.8 subunits (infinite proline) and a $K_a^{Pro} = 65 \pm 14$ μM, combined with inhibition of the aggregation at high proline ($K_i^{Pro} = 27 \pm 10$ mM).

4. Discussion

The present findings confirm that the AAK domain of *E. coli* G5K is responsible for catalyzing the reaction and for proline and ADP inhibition. A much smaller truncation of *E. coli* G5K, spanning only the C-terminal 35 residues, was already known not to render the enzyme inactive, since the mutant gene complemented proline auxotrophs lacking *proB* [23]. There is only a modest 55% decrease in the k_{cat} of the AAK domain when the PUA domain is deleted [estimated from the 45% decrease in specific activity at saturation of both substrates of the truncated (108 U mg^{-1}) versus the wild-type (170 U mg^{-1}) enzyme, and given the subunit masses of 27 and 39 kDa for each of the two enzyme forms].

Our results have revealed that *E. coli* G5K requires free Mg in excess of that needed for making the MgATP complex. This requirement is surprising, since in the G5K reaction there is no

activation of an acceptor hydroxyl group (an usual function of the second divalent cation in phosphoryl transferring enzymes [24,25]), and since the lysine residues used by AAK family enzymes [10,26,27] to catalyze phosphoryl group transfer are conserved in G5K (Lys10 and Lys217), excluding the need for replacing them by Mg as observed in *Pyrococcus furiosus* UMP kinase [28]. Whichever the mechanism, the high concentrations of free Mg needed may render the Mg requirement physiologically relevant, since in *E. coli* there is ~30 mM total Mg [29] of which the majority may be bound. Thus, changes in intracellular Mg concentration might influence G5K activity in vivo, and the PUA domain, by increasing the K_a for Mg relative to the isolated AAK domain of G5K, appears to act as a modulator of the Mg sensitivity of the enzyme, bringing it towards the expected in vivo range of cellular free Mg concentrations. Nevertheless, the AAK domain requires free Mg for activity, as made patent by the low activity exhibited by the truncated enzyme at the lowest free Mg concentration used (Fig. 3A), a concentration at which >90% of the ATP is as MgATP. Thus, Mg must bind in the AAK domain, and the much larger K_a for Mg exhibited by the wild-type enzyme possibly indicates that the PUA domain physically hampers Mg access to its binding site.

The large increase in the concentrations of proline needed for G5K inhibition when the PUA domain is deleted proves that the PUA domain, although not essential for proline inhibition, either alters the conformation of G5K to one having a higher affinity for proline, or it contributes some group to proline binding (although not many groups, since in this case the effect of the truncation on proline affinity would be larger [30]). In any case, the change in the apparent affinities for both proline and free Mg reveals an important potential of the PUA domain for modulating the catalytic and regulatory functions of the AAK domain of G5K, opening the way to the as yet unexplored possibility of PUA domain-mediated regulation. The alternative possibility that the observed changes in the affinity for proline or for Mg induced by the truncation were purely artifactual as a consequence of inspecific structural changes caused by the loss of the PUA domain appears highly unlikely, since it would be difficult to conceive that the loss of structural integrity actually increased the affinity for a ligand, as observed here for Mg, and also given the null or small effect of the truncation on other parameters such as the K_m for ATP, the $S_{0.5}$ for glutamate, the estimated k_{cat}, the thermal stability or the tetrameric quaternary structure of the enzyme.

Although the activity of the isolated AAK domain and the limited magnitude of the effect of the PUA domain deletion on the stability of G5K prove that these two domains are in G5K separate structural entities, the changes imposed by the PUA domain on stability, proline sensitivity, and Mg dependency, clearly point to the existence of interactions between these two domains. These interactions appear not to be extensive, for otherwise the truncation would have caused a larger effect on enzyme stability, and they seem to take place between the two domains of the same subunit, for otherwise PUA domain deletion should have triggered disaggregation, as observed previously with aspartokinase III (another AAK family enzyme having AAK and C-terminal domains) upon C-terminal domain deletion [31]. The lack of dissociation of truncated G5K and our preliminary crystallographic results [15] strongly suggest that *E. coli* G5K conforms to the canonical AAK family oligomerization pattern, in which the basic structure is a homodimer formed by the interactions between AAK domains, and in which higher oligomers result from the association of dimers, mediated also by AAK domain interactions [10,27,28]. Nevertheless, the tetrameric quaternary structure is a novel feature within the AAK family, since the structurally characterized members of this family are either dimers [10,27] or trimers of dimers ([28,32] and unpublished results of the laboratory of V. Rubio).

Our results show that the formation by G5K of aggregates larger than the tetramer requires both proline and the PUA domain, and thus, this domain must change its orientation when proline is bound, exposing new surfaces to allow its interaction with a surface of another subunit of a different tetramer. We presently ignore the functional significance of higher aggregate formation or of this PUA domain movement, but these might be related to the as yet poorly characterized but well established gene-regulatory functions of G5K [14], since these functions have been proposed (but not proven) to be mediated by the potentially RNA-binding PUA domain [11].

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