Mechanism of arginine regulation of acetylglutamate synthase, the first enzyme of arginine synthesis

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N-acetyl-l-glutamate synthase (NAGS), the first enzyme of arginine biosynthesis in bacteria/plants and an essential urea cycle activator in animals, is, respectively, arginine-inhibited and activated. Arginine binds to the hexameric ring-forming amino acid kinase (AAK) domain of NAGS. We show that arginine inhibits Pseudomonas aeruginosa NAGS by altering the functions of the distant, substrate binding/catalytic GCN5-related N-acetyltransferase (GNAT) domain, increasing \( K_{\text{cat}} \) and decreasing \( V_{\text{max}} \) and triggering substrate inhibition by AcCoA. These effects involve centrally the interdomain linker, since we show that linker elongation or two-residue linker shortening hampers and mimics, respectively, arginine inhibition. We propose a regulatory mechanism in which arginine triggers the expansion of the hexameric NAGS ring, altering AAK–GNAT domain interactions, and the modulation by these interactions of GNAT domain functions, explaining arginine regulation.

1. Introduction

In microorganisms and plants, N-acetyl-l-glutamate synthase (NAGS) catalyzes the first arginine biosynthesis step and is feed-back inhibited by arginine [1]. In contrast, in mammals, NAGS is activated by arginine [2,3] and its product, N-acetyl-l-glutamate (NAG), is the essential activator of the first urea cycle enzyme, carbamoyl phosphate synthetase I (CPSI). Thus, NAGS deficiency is an inborn error causing clinical hyperammonaemia [4].

Bacterial NAGS gene sequences (Fig. 1A; http://www.experimental.org) revealed a two-domain organization of the NAGS polypeptide, with \( \sim 280- \) and \( \sim 150\)-residue N- and C-terminal domains belonging, respectively, to the amino acid kinase (AAK) and GCN5-related N-acetyltransferase (GNAT)-acetyltransferase families. Since the AAK domain particularly resembles the hexameric, arginine-inhibitable acetylglutamate kinase (NAGK) [5], and since Escherichia coli NAGS was proven hexameric [6], bacterial NAGS was proposed [5] to be, as NAGK, a hexameric ring of AAK domains, formed by linking three AAK dimers through their inter-domain linker (Fig. 1E), since we show that linker elongation or two-residue linker shortening hampers and mimics, respectively, arginine inhibition. We propose a regulatory mechanism in which arginine triggers the expansion of the hexameric NAGS ring, altering AAK–GNAT domain interactions, and the modulation by these interactions of GNAT domain functions, explaining arginine regulation.

Using sequence comparisons and site-directed mutagenesis we localized Pseudomonas aeruginosa NAGS (PaNAGS) the arginine site in the AAK domain, in the same place as in NAGK, next to the interdimeric boundary and the N-terminal \( \alpha \)-helix (Fig. 1B) [8]. We clarify here, using mutant forms of PaNAGS in which the interdomain linker (Fig. 1E) was elongated or shortened, how the binding of the effector at its remote site (Fig. 1B) can influence the activity of the GNAT domain. Our data support the same basic mode of regulation by arginine as in NAGK [5]: the triggering by the effector of hexameric ring widening and flattening (Fig. 1D). This widening would be expected to displace the GNAT domains from their normal resting places, because of pulling mediated by the interdomain linker (Fig. 1C and D). In agreement with this expectation, we find that linker elongation hampers arginine inhibition and that two-residue linker shortening mimics the effect of arginine, which we also characterize here. These studies provide further [8] support for a key role of the AAK domain in modulating GNAT domain functions. Since we find that one-residue linker shortening can increase NAGS activity and that one-residue linker shortening can increase NAGS activity and that one-residue linker...
lengthening can render arginine an activator, a similar mechanism may underlie arginine activation of mammalian NAGS [2,3].

2. Materials and methods

PaNAGS containing the GSLEH6 C-terminal tail was purified as described [8]. PaNAGS with the mutant linker sequences 282QEAQA-FEQ, 282QEFQ and 282QEQ (Fig. 1E; abbreviated +A, +2A, −Q and −EQ, respectively) were prepared by the overlapping extension method using a commercial kit (Quickchange, from Stratagene), the pET-22b plasmid carrying the wild-type (WT) gene as template, and appropriate mutagenic forward and reverse oligonucleotides (primer sequences will be provided on request). Mutant purifications were as for WT enzyme [8], using the storage and dilution solutions reported in [6].

NAGS activity was assayed colorimetrically with Ellman’s reagent, as CoA release, as previously reported [8], except for the use of 30 mM glutamate and 4 mM AcCoA and for the omission of MgCl2. When the concentration of AcCoA was varied, assay volume was increased as required, from the standard 0.02 ml, up to 0.3 ml, for higher sensitivity. Blanks without enzyme were run in parallel and were subtracted. Color production was linear in all assays for at least 10 min. One enzyme unit produces 1 μmol CoA min−1. Results of at least duplicate assays were fitted with GraphPad Prism (GraphPad Software, San Diego) to either hyperbolic kinetics or to substrate inhibition kinetics [10] \( (v = V \times [S]/(K_{m} + [S]) + [S]/K_{i}); \) S, substrate, v and V, activities at a given [S] and at [S] = ∞; \( K_{m} \), apparent \( K_{m} \) for S; \( K_{i} \), substrate inhibition constant). Protein was determined by the Bradford assay [11] using bovine serum albumin as standard.

Fig. 1. Bacterial NAGS and its engineered forms used here. (A) Domain composition and (B) schematic architecture of bacterial NAGS showing the gross locations of the arginine site [8] and of G146 (an AAK domain residue that, when mutated, affected \( K_{m} \) and \( V_{max} \) [8]) to highlight its relative locations with respect to the GNAT domain. (C) Detail of the NgNAGS structure [7] showing the GNAT–AAK domain relations. Domains from different subunits are in different colors. Residue G148 (corresponding to G146 of PaNAGS), is highlighted with a sphere and labelled. (D) Proposed ring expansion upon arginine binding. The arrows indicate the movements leading to ring enlargement and flattening. Note the corresponding displacement of the GNAT domains. For clarity only the three GNAT domains that are closer to the viewer are represented. (E) Interdomain linker identification in PaNAGS from its alignment with the NgNAGS linker, and mutations engineered into the PaNAGS linker. (F) Coomassie-stained SDS–PAGE of the WT and mutant forms (St, molecular weight standards), and, at the bottom, activity of each enzyme form in the standard assay (mean ± S.D.).
3. Results

3.1. Preparation and activity of the mutants

We identified from the sequence (Fig. 1E) the PaNAGS interdomain linker, and we prepared mutants (see sequences in Fig. 1E) with 1 and 2 extra alanines (+A, +2A) or lacking one (−Q) or two (−EQ) residues. These mutants were easily purified to homogeneity from *E. coli* BL21 (DE3) cells (Fig. 1F) and were active and stable, although their specific activities were 38%, 45% and 70% lower for +A, +2A and −EQ, respectively and 45% higher for −Q, relative to WT NAGS.

3.2. Linker elongation mutations decrease \( V_{\text{max}} \) and hamper arginine inhibition

The lower activity of the +A and +2A mutants resulted from \( V_{\text{max}} \) decrease (Table 1), but in all other respects these mutants presented similar kinetics as WT NAGS, with hyperbolic plots for AcCoA and with substrate inhibition at high glutamate concentrations (Fig. 2A and B and Table 1).

As expected for arginine inhibition resulting from hexameric ring widening (Fig. 1D), linker lengthening dramatically hampered arginine inhibition. Whereas WT NAGS was virtually completely inhibited by 2 mM arginine, none of these mutants

<table>
<thead>
<tr>
<th>NAGS form</th>
<th>[Arg] (mM)</th>
<th>Kinetic parameters for AcCoA</th>
<th>Kinetic parameters for glutamate</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>( K_{\text{App}} ) (mM)</td>
<td>( V_{\text{AcCoA}} = (U \text{ mg}^{-1}) )</td>
</tr>
<tr>
<td>WT</td>
<td>−</td>
<td>0.09 ± 0.01</td>
<td>80 ± 1</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.05 ± 0.02</td>
<td>15 ± 2</td>
</tr>
<tr>
<td>+A</td>
<td>−</td>
<td>0.10 ± 0.02</td>
<td>45 ± 1</td>
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<tr>
<td></td>
<td>2</td>
<td>0.14 ± 0.02</td>
<td>58 ± 1</td>
</tr>
<tr>
<td>+2A</td>
<td>−</td>
<td>0.07 ± 0.02</td>
<td>42 ± 1</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.13 ± 0.03</td>
<td>50 ± 3</td>
</tr>
<tr>
<td>−Q</td>
<td>−</td>
<td>0.24 ± 0.04</td>
<td>175 ± 11</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.15 ± 0.04</td>
<td>13 ± 2</td>
</tr>
<tr>
<td>−EQ</td>
<td>−</td>
<td>0.26 ± 0.11</td>
<td>56 ± 10</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.22 ± 0.05</td>
<td>18.2 ± 1.7</td>
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Kinetic parameters were determined as indicated in Section 2, and correspond to the data in Fig. 2. \( K_{\text{App}} \), apparent \( K_m \) values (30 mM fixed glutamate; 4 mM fixed AcCoA). \( K_i \), inhibition constant in the substrate inhibition equation. Standard errors are given.

Fig. 2. Dependency of enzyme activity on the concentrations of the substrates for the different enzyme forms in the absence (A, B and inset in B) and in the presence (C, D) of 2 mM arginine. When fixed, AcCoA and glutamate were at respective concentrations of 4 mM and 30 mM. The lines correspond either to hyperbolic kinetics or to hyperbolic kinetics with substrate inhibition (see Table 1 for the values of the individual constants). Enzyme concentrations were, for variable glutamate, in the respective absence and presence of arginine, 0.35–1.6 and 1.1–5.5 \( \mu \text{g} \text{l}^{-1} \) (the concentrations differed for different enzyme forms); and, for varied AcCoA, depending in addition on the concentration of this substrate, in the respective absence and presence of arginine, 0.04–0.61 and 0.14–1.2 \( \mu \text{g} \text{l}^{-1} \). For all enzyme forms the specific activity of the enzyme was independent of protein concentration (tested in the 0.04–0.61 \( \mu \text{g} \text{l}^{-1} \) range, at 1 mM AcCoA and 30 mM glutamate, in the absence of arginine).
was inhibited substantially by concentrations of arginine below 5 mM (Fig. 3).

Interestingly, low arginine concentrations activated, although little, mutant +A (Fig. 3; significant differences with respect to absence of arginine are illustrated with asterisks). An activation too small to be statistically significant was also observed with the +2A mutant. The activation reflects the binding to these mutants of arginine, as expected given the intactness of the arginine site. The arginine activation appears to result from a decrease in substrate inhibition by glutamate triggered by 2 mM arginine (Fig. 2C and D; Table 1); otherwise, 2 mM arginine affected little the substrate kinetics of these two mutants, triggering only on the +2A mutant some substrate inhibition by AcCoA (an inhibitory effect that was counterbalanced by the decreased substrate inhibition by glutamate). In contrast, 2 mM arginine had dramatic effects on WT NAGS (Fig. 2C and D; Table 1), increasing ~16-fold apparent $K_{m}$, causing substrate inhibition by AcCoA ($K_{i} = 2.4$ mM) and reducing ~60% $V_{\text{max}}$ (estimated by calculating the expected $V_{\text{max}}$ if arginine changed only the $K_{m}$ and $K_{i}$ values, Table 1).

3.3. Effects of linker shortening

As expected for the hexameric ring expansion model of arginine inhibition, the −EQ mutant caused on the enzyme some effects that were similar to those caused by arginine (Fig. 2A and B; Table 1): it decreased enzyme activity, increasing the $K_{m}$ and inducing substrate inhibition by AcCoA. Even 1-residue shortening (−Q mutant) increased $K_{m}$ and triggered AcCoA inhibition, (Fig. 2A and B; Table 1). However, the −Q mutant was more active in the standard assay (Fig. 1F) and exhibited a higher $V_{\text{max}}$ than WT NAGS (Fig. 2A and B; Table 1).

Both deletion mutants were sensitive to arginine (Fig. 3) at similar concentration ranges than the WT enzyme. The arginine inhibition of the −EQ mutant appeared incomplete, but this was a relative effect of the poor activity of this mutant, since its absolute activity at infinite arginine concentration was estimated to be only marginally higher (~2-fold higher) than for WT NAGS. The kinetic factors responsible for arginine inhibition with these two mutants were grossly similar as for WT NAGS (Fig. 2C and D; Table 1).

4. Discussion

The high similarity in architecture [5,7] and arginine site [5,8] between NAGS and NAGK strongly suggest that, as with NAGK [5], arginine triggers the expansion and flattening of the hexameric enzyme ring (Fig. 1D). Since in NAGS the substrates bind and catalysis proceeds in a separately folded GNAT domain [7,8], arginine-triggered overall ring changes must affect GNAT domain functions by altering the interactions of this domain with the AAK domain. These interactions were previously proven to be important [8]. Ring expansion should drag the GNAT domain out of its normal position on the AAK domain (Fig. 1D). Therefore, linker lengthening should alleviate this drag and should hamper arginine inhibition, whereas linker shortening should mimic arginine in dragging out of position the GNAT domain. These two predictions have been fulfilled in our experiments, which thus provide support for this mechanism of regulation, in addition to revealing a key role of the interdomain linker in the intramolecular transduction of the arginine signal.

Arginine is found here to alter NAGS activity by a multiplicity of effects. It increases $K_{c_{\text{AcCoA}}}$, it elicits and prevents substrate inhibition by, respectively, AcCoA and by glutamate, and it decreases $V_{\text{max}}$. The NgNAGS structure ([7] and Fig. 1C) provides clues on how small changes in GNAT–AAK domain interactions can trigger these effects. This is particularly clear concerning glutamate, since its γ-COOH is bound to Arg425 and its binding pocket wall includes residues 390–392, two elements that are adjacent to residues (Thr393, Asn394, Asn426 and His428) which anchor the GNAT domain on the AAK domain. Similarly, it is conceivable that arginine, by altering the position of the GNAT domain, might make extra room between the surfaces of the AAK and GNAT domains that sandwich the phosphoadenosine moiety of the AcCoA molecule [7], possibly allowing aberrant AcCoA conformations that result in non-productive complexes and in substrate inhibition by AccOAc. Finally, the influence of arginine on the $V_{\text{max}}$ can be explained if $K_{\text{cat}}$ is determined by conformational changes associated with the opening and closing of the active centre (a likely possibility, in view of the deepness of the acetyl group transfer site [7]). Given the intimate contact between both domains, it is conceivable that small changes in the interactions may hamper or facilitate active site opening/closing in the GNAT domain.

Our demonstration that the $V_{\text{max}}$ can be augmented by one-residue shortening (−Q mutant), most likely because of altered interdomain interactions, and our observation that arginine can become an activator (although modest) by simply adding one residue (+A) to the linker, may be relevant for understanding arginine activation of NAGS in land vertebrates [2,3,12]. In fact, even in the case of mammalian NAGS, arginine has a dual role, being an activator at low concentration, and inhibiting the enzyme at higher concentration [2]. Our results suggest that not much change appears necessary in NAGS for shifting the effects of arginine from inhibition to activation, as observed in the transition from marine life forms to land tetrapods.

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References


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