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Structure of *N*-acetyl-L-glutamate synthase/kinase from *Maricaulis maris* with the allosteric inhibitor L-arginine bound

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ABSTRACT

Maricaulis maris *N*-acetylglutamate synthase/kinase (mmNAGS/K) catalyzes the first two steps in L-arginine biosynthesis and has a high degree of sequence and structural homology to human *N*-acetylglutamate synthase, a regulator of the urea cycle. The synthase activity of both mmNAGS/K and human NAGS are regulated by L-arginine, although L-arginine is an allosteric inhibitor of mmNAGS/K, but an activator of human NAGS. To investigate the mechanism of allosteric inhibition of mmNAGS/K by L-arginine, we have determined the structure of the mmNAGS/K complexed with L-arginine at 2.8 Å resolution. In contrast to the structure of mmNAGS/K in the absence of L-arginine where there are conformational differences between the four subunits in the asymmetric unit, all four subunits in the L-arginine liganded structure have very similar conformations. In this conformation, the AcCoA binding site in the *N*-acetyltransferase (NAT) domain is blocked by a loop from the amino acid kinase (AAK) domain, as a result of a domain rotation that occurs when L-arginine binds. This structural change provides an explanation for the allosteric inhibition of mmNAGS/K and related enzymes by L-arginine. The allosterically regulated mechanism for mmNAGS/K differs significantly from that for *Neisseria gonorrhoeae* NAGS (ngNAGS). To define the active site, several residues near the putative active site were mutated and their activities determined. These experiments identify roles for Lys356, Arg386, Asn391 and Tyr397 in the catalytic mechanism.

1. Introduction

A novel bifunctional enzyme that has both *N*-acetylglutamate synthase (NAGS, EC 2.3.1.1) and *N*-acetylglutamate kinase (NAGK, EC 2.7.2.8) activities and catalyzes the first two reactions in the L-arginine biosynthetic pathway was recently identified in some proteobacteria [1]. Interestingly, these bifunctional NAGS/K have higher protein sequence similarity to mammalian NAGS, a regulator of the urea cycle, than to the "classic" NAGS enzymes of most bacteria and plants. Bifunctional NAGS/K also exists in the same quaternary state and has a fold very similar to mammalian NAGS [2]. However, while both NAGS/K and mammalian NAGS are regulated by L-arginine, L-arginine is an allosteric inhibitor of NAGS/K but an allosteric activator of mammalian NAGS [3].

Structures of a "classic" NAGS from *Neisseria gonorrhoeae* (ngNAGS) with and without substrates and L-arginine bound indicate that this type of NAGS has a hexameric structure [4]. In contrast, *Maricaulis maris* NAGS/K, as well as NAGS/K from *Xanthomonas campestris* (xcNAGS/K), exists as tetramers [5]. Yeast NAGK (yNAGK) also forms tetramers, suggesting that tetrameric oligomerization is a common feature of non-classic NAGS and is evolutionarily conserved [6].

The catalytic and regulatory mechanisms of ngNAGS, are now well understood. However, no structures of a "non-classic" NAGS with L-arginine bound have been determined [5]. Although a

Abbreviations: AAK, amino acid kinase; GNAT, GCN5-related acetyltransferase; mmNAGS/K, *Maricaulis maris* *N*-acetyl-L-glutamate synthase/kinase; mmNAGS/K-Arg, mmNAGS/K bound with L-arginine; mmNAGS/K-CoA, mmNAGS/K bound with CoA; NAG, *N*-acetyl-L-glutamate; NAGK, *N*-acetyl-L-glutamate kinase; NAGS, *N*-acetyl-L-glutamate synthase; NAGS/K, *N*-acetyl-L-glutamate synthase/kinase; NAT, *N*-acetyltransferase; ngNAGS, *Neisseria gonorrhoeae* *N*-acetyl-L-glutamate synthase; RMSD, root mean standard deviation; tmNAGK, *Thermotoga maritima* *N*-acetyl-L-glutamate kinase; xcNAGS/K, *Xanthomonas campestris* *N*-acetyl-L-glutamate synthase/kinase; yNAGK, yeast *N*-acetyl-L-glutamate kinase.

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mechanism for L-arginine regulation was proposed based on the structure of mmNAGS/K with CoA bound at a non-functional site, the L-arginine binding site and its regulatory mechanism have not been experimentally established.

Here, we report the structure of mmNAGS/K with L-arginine bound (mmNAGS/K-Arg) and compare it with the previously determined structure of mmNAGS/K with CoA bound, but without L-arginine bound (mmNAGS/K-CoA). Site directed mutagenesis was also used to probe the function of several amino acid residues at or near the active site. These structures and mutagenesis experiments provide experimental proof of the mechanism of L-arginine inhibition previously proposed [5].

2. Materials and methods

2.1. Protein expression and purification

mmNAGS/K, xcNAGS/K and all mutants were expressed and purified as described previously [7]. Briefly, the proteins were expressed in *Escherichia coli* BL21(DE3) cells (Invitrogen) and purified with nickel affinity and DEAE columns (GE Healthcare). Protein purity was verified by SDS/PAGE gel and protein concentration was measured with a Nano-drop 1000 spectrophotometer (Thermo Scientific). The extinction coefficient obtained from the ExPASy web server (http://web.expasy.org/protparam/) was used to calculate protein concentrations. The protein was stored at 253 K in a buffer containing 50 mM Tris–HCl, pH 8.0, 50 mM NaCl, 10% glycerol, 5 mM β-mercaptoethanol and 1 mM EDTA.

2.2. Site-directed mutagenesis

Site-directed mutant genes of mmNAGS/K and xcNAGS/K were created using primers containing the desired mutations and the QuikChange Mutagenesis Kit according to the manufacturer's protocol (Strategene). The sequences of mutant DNA sequences were verified by DNA sequencing.

2.3. Activity assay

Enzymatic activity was determined by measuring *N*-acetyl-L-glutamate (NAG) production as described previously using stable isotope dilution and liquid chromatography mass spectrometry (LC–MS) [8]. The assay was performed in a solution containing 50 mM Tris, pH 8.5, 10 mM glutamate and 2.5 mM AcCoA in a 100 µl reaction volume. The reaction was initiated by the addition of enzyme, and the mixture was incubated at 303 K for 5 min, then quenched with 100 µl of 30% trichloroacetic acid containing 50 µg of *N*-acetyl-[$^{13}C_5$] glutamate (^{13}C-NAG) as an internal standard. Precipitated protein was removed by micro-centrifugation. The supernatant (10 µl) was submitted to LC–MS (Agilent) for separation and measurement. The mobile phase consisted of 93% solvent A (1 ml trifluoroacetic acid in 1 L water) and 7% solvent B (1 ml trifluoroacetic acid in 1 L of 1:9 water/acetonitrile) and the flow rate was 0.6 ml/min. L-glutamate, NAG and ^{13}C-NAG were detected and quantified by selected ion monitoring mass spectrometry.

2.4. Crystallization

Crystals of mmNAGS/K-Arg were grown in the presence of L-arginine by the sitting drop, vapor-diffusion method. Before crystallization, the purified protein (~10 mg/ml) was incubated with 1 mM L-arginine, and 10 mM NAG for 30 min. Screening for crystallization conditions was performed using sitting drop vapor diffusion in 96-well plates (Hampton Research) at 291 K by mixing 2 µl of the protein solution with 2 µl of the reagent solution from the sparse matrix Crystal Screens 1 and 2, and Index Screen (Hampton Research). The best crystals were grown from a reservoir solution containing 100 mM sodium cacodylate trihydrate, pH 6.2, 25% polypropylene glycol P400 and 200 mM magnesium chloride. Crystal were plate-shaped and reached a maximum length of 0.4 mm in 6–7 days.

2.5. Data collection and structure determination

Crystals were transferred from the crystallization plate to a well solution supplemented with 25% glycerol and then vitrified directly by liquid nitrogen. Diffraction data were collected at beamline 22-ID equipped with a MAR300 CCD at the Advanced Photon source (APS), Argonne National Laboratory, USA. All data were processed using the HKL2000 package [9]; statistics are summarized in Table 1. The structure was solved by molecular replacement using Phaser [10,11] and subunit X of the mmNAGS/K-CoA structure (PDB 3S6G) as the search model. After several cycles of refinements with Phenix [12] and model adjustments with Coot [13], L-arginine was visible in the electron density map and built into the model. Although NAG was included in the crystallization medium, it was not visible in the crystals at any stage of the refinement. In the last stage of refinement, the translation/liberation/screw (TLS) parameters were included and refined [14]. Five TLS groups per subunit were selected in the *N*-terminal segment, *N*-terminal lobe and the *C*-terminal lobe of the AAK domain and the *N*-terminal arm and the *C*-terminal arm of NAT domain, as defined previously [5]. The final R and R_{free} were 19.9% and 26.5%, respectively. Refinement statistics for the final refined model are given in Table 1. The final refined coordinates for mmNAGS/K-Arg have been deposited in the RCSB Protein Data Bank with the accession code 4KZT. All figures were drawn using program Pymol [15].

Table 1
Data collection and refinement statistics.

Data collection	
Bound ligands	L-arginine
Space group	*C*2
Wavelength (Å)	1.0
Resolution (Å)	40–2.80 (2.85–2.80)[a]
Unit-cell parameters (Å)	a = 165.8 Å b = 110.8 Å c = 117.2 Å β = 91.0°
Measurements	312,380
Unique reflections	47,676 (1431)
Redundancy	6.6 (2.9)
Completeness (%)	90.3 (54.1)
$\langle I/\sigma(I)\rangle$	37.0 (1.5)
R_{merg}(%)[b]	8.1 (65.6)
Refinement	
Resolution range (Å)	40–2.79 (2.86–2.79)
No. of protein atoms	13244
No. of water atoms	0
No. of hetero atoms	76
Rmsd of bond lengths (Å)	0.009
Rmsd of bond angle (°)	1.4
R_{work} (%)[c]	19.6 (37.8)
R_{free} (%)[d]	26.5 (48.2)
Ramachandran plot (%)	
Favored	83.3
Allowed	13.5
Generous	2.4
Disallowed	0.9

[a] Figures in brackets apply to the highest-resolution shell.

[b] $R_{merg} = \sum_h\sum_i|I(h,i)-<I(h)>|/\sum_h\sum_i I(h,i)$, where $I(h,i)$ is the intensity of the ith observation of reflection h, and $<I(h)>$ is the average intensity of redundant measurements of reflection h.

[c] $R_{work} = \sum_h||F_{obs}|-|F_{calc}||/\sum_h|F_{obs}|$.

[d] $R_{free} = \sum_h||F_{obs}|-|F_{calc}||/\sum_h|F_{obs}|$ for 5% of the reserved reflections.