BIOCHEMISTRY
including biophysical chemistry & molecular biology

Rapid Report

pubs.acs.org/biochemistry

Structure and Activity of PA5508, a Hexameric Glutamine Synthetase Homologue

Jane E. Ladner,[†,‡] Vesna Atanasova,[†] Zuzana Dolezelova,[†] and James F. Parsons*[,†]

[†]Institute for Bioscience and Biotechnology Research, University of Maryland, and [‡]National Institute of Standards and Technology, 9600 Gudelsky Drive, Rockville, Maryland 20850, United States

Supporting Information

ABSTRACT: The structure of PA5508 from *Pseudomonas aeruginosa*, a glutamine synthetase (GS) homologue, has been determined at 2.5 Å. Surprisingly, PA5508 forms single hexameric rings rather than the stacked double rings that are characteristic of GS. The C-terminal helical thong motif that links GS rings is present in PA5508; however, it is folded back toward the core of its own polypeptide, preventing it from interacting with a second ring. Interestingly, PA5508 displays a clear preference for aromatic amine substrates. Unique aspects of the structure illustrate how the enzyme is able to catalyze reactions involving bulky amines rather than ammonia.

Pseudomonas aeruginosa is an ever-present, opportunistic pathogen and a constant threat to hospitalized persons, those with immune disorders, and cystic fibrosis patients.[1] The ubiquitous nature of *P. aeruginosa* stems from its ability to thrive on an unusually wide variety of carbon and nitrogen sources. Among these are alkyl polyamines, which are found in all organisms and perform a variety of functions in vivo.[2] When present in the environment, bacteria can use polyamines such as spermidine and putrescine as sole sources of carbon and nitrogen. *P. aeruginosa* has a particularly refined ability to grow on polyamines. The first catabolic step in polyamine utilization is γ-glutamylation by a glutamine synthetase (GS) homologue. *P. aeruginosa* encodes seven GS homologues that have overlapping specificity toward spermidine, putrescine, and other biogenic polyamines. Six of the seven are induced in the presence of either spermidine or putrescine.[2] The seventh, PA5508, is not, and its cellular function is unknown. The genetic context of PA5508 additionally suggests that it may have a unique function. PA5506 is an RpiR-type transcriptional regulator that controls the expression of the *pa5506−5509* operon. Sequence analysis identifies PA5507 as a hydrolase, possibly with specificity toward amides, while PA5509 is annotated as a formylglutamate lyase. Typically, γ-glutamylated polyamines are further catabolized by a combination of oxidation and dehydrogenation steps that appear to be inconsistent with the products of the genes cotranscribed with PA5508.

As part of an effort to understand the role of the PA5508 and to assess how GS homologues with alternative functions differ from authentic GS, we conducted a structural and biochemical evaluation of PA5508. Here we show that PA5508 is a previously uncharacterized GS-like protein that exhibits significant γ-glutamyl aromatic monoamine ligase activity. Despite being considerably similar to GS, PA5508 displays no ability to produce glutamine. Several of the biogenic polyamines appear to be poor substrates for PA5508. Surprisingly, tryptamine, serotonin, dopamine, and other biogenic amines are readily converted by PA5508 to the corresponding γ-glutamyl derivatives. The amino acids tyrosine, tryptophan, and lysine are not substrates.

The structural data shed light, for the first time, on the large family of bacterial GS-like enzymes with alternative functions. PA5508 and similar proteins are significantly more similar to GS than are members of the γ-glutamyl cysteine ligase (γ-GCL) family. PA5508 homologues are widespread, yet little biochemical or structural data are available. GS is a critical enzyme in the biosphere because of its indispensable role in nitrogen metabolism. Accordingly, it has been the focus of numerous revealing crystallographic and mechanistic studies.[3−5] Three classes are recognized: GS-I, -II, and -III, which are distinguished by chain length, oligomeric arrangement, and regulatory features. GS-I and GS-III enzymes form dodecamers of two six-membered rings, while GS-II enzymes form a sandwich of five-membered rings. GS-I enzymes also feature a conserved tyrosine that is subject to adenylation, a scheme that regulates the activity of the enzyme. PA5508 is most similar to the bacterial GS-I enzymes, but it forms only single rings and is apparently not regulated by adenylation. In addition, two key acidic residues involved in catalysis in GS are not conserved in PA5508. Instead, two nonpolar residues, Trp296 and Val42, occupy analogous positions, suggesting that PA5508 may bind and sequester larger substrates via nonpolar interactions.

The 2.5 Å resolution structure of PA5508 was determined by molecular replacement (Figure 1). One polypeptide chain was present in the crystallographic asymmetric unit. Experimental details of the protein purification, crystallization, data collection, and refinement procedures and statistics are provided in the Supporting Information. The final model includes 426 of the 443 residues, one glutamate, one Mg^{2+} ion, and one Ca^{2+} ion. One disordered segment (residues 365−381) corresponds to the adenylation loop of GS-I located on the outside edge of the GS ring. PA5508 has no tyrosine residue in this region, suggesting that it is not subject to regulatory control by adenylation. Glutamate and Mg^{2+} are bound in the PA5508 active site in the same orientation that has been observed in

Received: November 1, 2012
Revised: December 7, 2012
Published: December 12, 2012

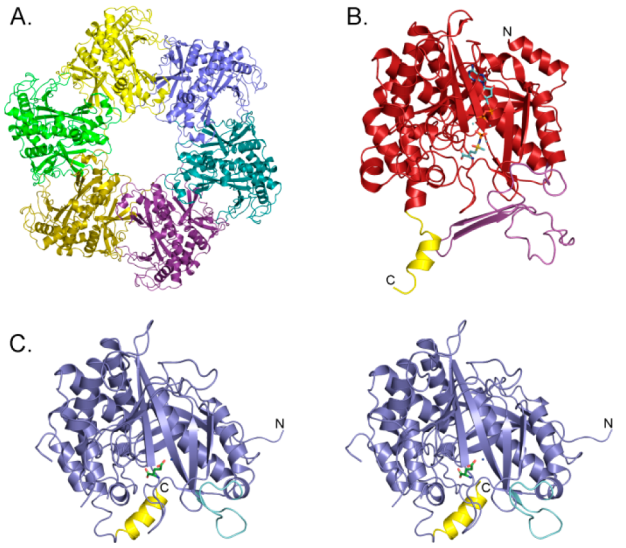

Figure 1. Structural basis for the single-ring structure of PA5508. Unlike GS-I, which forms dodecamers of stacked six-membered rings, PA5508 forms only single rings. (A) PA5508 hexamer colored by chain. (B) Structure of GS-I (PDB entry 2BVC) illustrating the extended C-terminal helical thong motif (yellow) that anchors hexameric GS-I rings together. Also colored magenta is the two-stranded β-sheet, absent from PA5508, that may prevent the C-terminal helix of GS from folding back and adopting the orientation seen in PA5508. (C) Stereoview of the PA5508 monomer illustrating the orientation of its C-terminal helix (yellow). Colored cyan is the shorter loop analogous to the two-stranded β-sheet region of GS. Also shown are the bound ligands, glutamate and Mg^{2+}.

GS-I (Figure S1 of the Supporting Information). Mg^{2+} occupies a site analogous to the n1 site of GS.[5] The Ca^{2+} ion, likely present because of the crystallization cocktail, was found at the subunit interface and near Glu441.

The structure of PA5508 can be superimposed on that of either *Mycobacterium tuberculosis* GS [MtGS, Protein Data Bank (PDB) entry 2BVC] or *Salmonella typhimurium* GS (StGS, PDB entry 1FPY) with a root-mean-square deviation (rmsd) of 2.3 Å over ~380 residues. The level of sequence identity of the superimposed residues is 26% in each case. PA5508 is more distantly related to γ-GCL and related enzymes.[6] These enzymes feature active sites similar to those of GS and PA5508; however, they do not form oligomeric rings, and the monomers superimpose with rmsd values of >3.5 Å over only ~240 residues.

The most striking difference between PA5508 and GS-I enzymes is that PA5508 forms only single hexameric rings (Figure 1 and Figures S2 and S3 of the Supporting Information). In GS-I enzymes, the C-terminal helix of the polypeptide, known as the "helical thong", extends away from the rest of the subunit (Figure 1B) and is anchored in a hydrophobic cavity on a second hexameric ring.[4] The head-to-head arrangement of the rings stems from 12 embedded helices tethering the two rings together. In PA5508, however, the C-terminal helix is oriented very differently. Instead of extending away from the subunit, it is bent back toward the core of its own chain, preventing it from interacting with another ring (Figure 1C). The orientation of the C-terminal helix in PA5508 may be a result of a large sequence deletion in PA5508 relative to GS. The ~55-residue region after Asp140 of MtGS is only ~12 residues long in PA5508. Most notably, a two-stranded β-sheet seen in GS is not present in PA5508 (Figure 1B, C). This sheet may prevent the C-terminal helix of GS from folding backward.

The C-terminal helix of PA5508 also engages in an interaction not seen in GS. Tyr439 interacts with Asn51 from the important Val42 loop, which may aid in positioning Val53 and Ile54 along one wall of the active site (Figure S3 of the Supporting Information). These residues along with Val42 and Phe148 form a hydrophobic patch that could favorably interact with nonpolar substrates. The corresponding Asp54 loop of MtGS is 10 residues shorter than the Val42 loop and does not interact with the MtGS helical thong motif.

The mechanism of GS involves the ordered sequential binding of ATP followed by glutamate and ammonia.[7] Interestingly, no nucleotide is present in the structure of PA5508 even though glutamate was found. Crystals were obtained only using glutamate and either ADP or ATP in the crystallization mixture, but we have not observed density consistent with a bound nucleotide. The result is puzzling as a comparison to the MtGS nucleotide binding site shows that virtually all residues involved in nucleotide binding are conserved in PA5508. The one notable exception is Lys215 of MtGS that directly interacts with the α-phosphate of ADP. Met176 is the corresponding residue in PA5508. The glutamate binding site of PA5508 also closely resembles that of MtGS. In both enzymes, the n1 metal coordination involves the glutamyl substrate and three active site glutamate residues.

An essential element of GS catalysis is the closing of the flap containing Glu335 of MtGS (Glu327 of StGS[5,7]). The active site is located between two subunits, and the flap is latched in place by the interaction of Glu335 with Asp54 from an adjacent subunit within the same ring. This closure sequesters the glutamyl phosphate (Glu-P) intermediate and prevents its premature hydrolysis. It has been proposed that ammonium then enters the latched active site. Asp54 then aids in orienting and deprotonating ammonium for attack on Glu-P.[4] In PA5508, a different scheme is likely necessary because a bulky substrate would be unable to enter the closed active site. Additionally, in PA5508, hydrophobic residues occupy the positions corresponding to Glu335, and Asp54 of MtGS (Figure 2). One possibility, given that we observed glutamate bound, but not ADP, is that that binding follows a different order such that the Glu-P intermediate is not formed until the amine substrate is already bound.

GS structures have been described as existing in either a "taut" (active) or a "relaxed" (inactive) state.[5,8] The relaxed form is metal-free, while the taut form has two or three occupied metal ion sites. Relaxed forms of GS also feature an active site β-strand that is out of register relative to the taut conformation. It is not clear whether PA5508 can adopt two distinct conformations; however, the model presented here is consistent with a taut form, though only one Mg^{2+} ion is seen. When compared to the taut form of MtGS, the β-strand starting with Glu175 is fully in register with the corresponding strand of taut MtGS (Figure S1 of the Supporting Information).

To assess the function of PA5508 and rapidly screen potential PA5508 substrates, we developed a liquid chromatography–mass spectrometry-based assay designed to detect γ-glutamyl products formed by PA5508. Initial results indicated that PA5508 was unable to synthesize glutamine when incubated with glutamate, ammonia, Mg^{2+}, and ATP. We then examined whether several alkyl polyamines known to be substrates for bacterial homologues of PA5508 were also

dx.doi.org/10.1021/bi3014856 | *Biochemistry* 2012, 51, 10121–10123

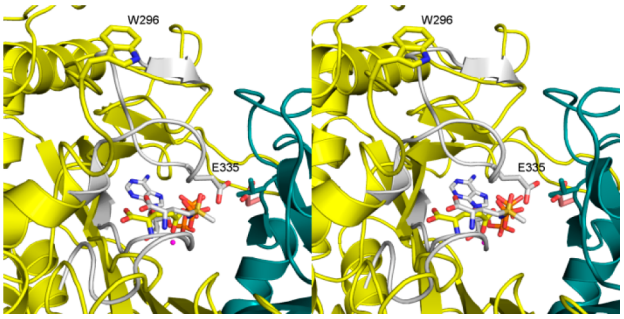

Figure 2. Examination of the active site of PA5508 suggests that it may be optimized to handle bulky and hydrophobic amine substrates. Stereoview of the active site of PA5508 (yellow and green cartoon) and comparison with key elements of GS (MtGS; gray cartoon; active site ligands ADP and methionine sulfoximine also shown). A key interaction between Glu335 and Asp54 (gray) of an adjacent subunit closes off the active site of GS. Asp54 is also essential in binding and activating ammonia for nucleophilic attack on the glutamyl phosphate intermediate. In PA5508, the active site is occupied only by glutamate and the corresponding loop, with Trp296 in place of Glu335, has not closed over the active site. Val42 occupies the position of Asp54, further suggesting that hydrophobic interactions may be important in substrate recognition and active site closure prior to catalysis.

PA5508 substrates. Results indicated that the common polyamines putrescine, spermidine, and spermine could be converted to the γ-glutamyl conjugates, but the reactions were very slow. An expanded screen, however, revealed that several aromatic and nonaromatic cyclic amines were excellent substrates for PA5508. Notably, several biogenic amines were readily converted to the corresponding γ-glutamyl derivatives. These included tryptamine, tyramine, dopamine, serotonin, octopamine, and norepinephrine. Interestingly, the amino acids tyrosine and tryptophan were nonsubstrates. To further characterize the activity and to provide a benchmark for a detailed evaluation of possible PA5508 substrates, we examined the activity toward tryptamine in greater detail. The data reveal a k_{cat} of 0.59 s^{-1} and a K_m of 8 mM (Figure S4 of the Supporting Information). This compares favorably with the performance of γ-glutamylputrescine synthetase, PuuA, from *Escherichia coli*, which exhibits a k_{cat} of 5.9 s^{-1} and a K_m for putrescine of 44.6 mM.[9] PuuA, however, also differs from PA5508 in key respects. It is reported to be a GS-like dodecamer in solution, and the key GS residue, Glu335, is conserved in PuuA. These results suggest that legitimate polyamine utilization enzymes may be more similar to GS than PA5508. To further evaluate the substrate preference of PA5508, we compared tryptamine and three alkyl polyamines as substrates using a glutamate depletion assay. The results indicate that tryptamine is a substantially better PA5508 substrate than any of the three polyamines (Figure S5 of the Supporting Information). A coupled spectroscopic assay following ATP hydrolysis was also used and indicated that dopamine was the best of the cyclic amine substrates evaluated to date, exhibiting a specific activity more than twice that observed with tryptamine (Table S2 of the Supporting Information). While the identity of the actual PA5508 substrate is not yet known, because of its spectral properties, tryptamine may prove to be a convenient alternative substrate for assays of γ-glutamyl ligase activity in PA5508 homologues.

In summary, we have determined the structure of the novel GS homologue, PA5508. It is, to the best of our knowledge, the closest GS homologue with an alternative function to be determined. The surprising observation that PA5508 does not form GS-like dodecamers is explained by a key sequence deletion and the resulting alternative orientation of the GS helical thong motif. Substitutions at key active site residues also suggest how PA5508 may cope with bulkier amine substrates. We initially anticipated that PA5508 was a γ-glutamyl polyamine ligase, but the data suggest that it may instead act on aromatic monoamines. It is not yet clear if the observed activity is serendipitous, evidence of a catabolic pathway, or if there is a host-to-cell signaling element involved. Biogenic amines have previously been implicated in host-to-cell signaling and virulence in several other pathogens, including enterohemorrhagic *E. coli* and *S. typhimurium*.[10,11]

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Detailed experimental procedures, crystallographic processing and refinement information, five figures, and two tables listing experimental results. This material is available free of charge via the Internet at http://pubs.acs.org.

Accession Codes

PDB entry 4HPP.

■ AUTHOR INFORMATION

Corresponding Author

*Telephone: (240) 314-6158. E-mail: parsonsj@umd.edu.

Notes

The authors declare no competing financial interests.

■ ACKNOWLEDGMENTS

We thank Dr. Andrey Galkin and Dr. Everett Pesci for providing reagents and other assistance.

■ REFERENCES

(1) Wagner, V. E., and Iglewski, B. H. (2008) *Clin. Rev. Allergy Immunol. 35*, 124−134.
(2) Yao, X., He, W., and Lu, C.-D. (2011) *J. Bacteriol. 193*, 3923−3930.
(3) van Rooyen, J. M., Abratt, V. R., Belrhali, H., and Sewell, T. (2011) *Structure 19*, 471−483.
(4) Eisenberg, D., Gill, H. S., Pfluegl, G. M., and Rotstein, S. H. (2000) *Biochim. Biophys. Acta 1477*, 122−145.
(5) Krajewski, W. W., Jones, T. A., and Mowbray, S. L. (2005) *Proc. Natl. Acad. Sci. U.S.A. 102*, 10499−10504.
(6) Hibi, T., Nii, H., Nakatsu, T., Kimura, A., Kato, H., Hiratake, J., and Oda, J. (2004) *Proc. Natl. Acad. Sci. U.S.A. 101*, 15052−15057.
(7) Gill, H. S., and Eisenberg, D. (2001) *Biochemistry 40*, 1903−1912.
(8) Gill, H. S., Pfluegl, G. M. U., and Eisenberg, D. (2002) *Biochemistry 41*, 9863−9872.
(9) Kurihara, S., Oda, S., Tsuboi, Y., Kim, H. G., Oshida, M., Kumagai, H., and Suzuki, H. (2008) *J. Biol. Chem. 283*, 19981−19990.
(10) Lesouhaitier, O., Veron, W., Chapalain, A., Madi, A., Blier, A.-S., Dagorn, A., Connil, N., Chevalier, S., Orange, N., and Feuilloley, M. (2009) *Sensors 9*, 6967−6990.
(11) Sperandio, V., Torres, A. G., Jarvis, B., Nataro, J. P., and Kaper, J. B. (2003) *Proc. Natl. Acad. Sci. U.S.A. 100*, 8951−8956.