Salt-Tolerant and Thermostable Glutaminases of Cryptococcus Species Form a New Glutaminase Family

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Genes encoding salt-tolerant and thermostable glutaminases were isolated from Cryptococcus species. The glutaminase gene, CngahA, from C. nodaensis NISL-3771 was 2,052 bp in length and encoded a 684-amino acid protein. The gene, CagahA, from C. albidus ATCC20293 was 2,100 bp in length and encoded a 700-amino acid protein. These glutaminases showed 44% identity. By searches on public databases, we found that these glutaminases are not similar to any other characterized glutaminases, but are similar to certain hypothetical proteins. On searching the conserved domain with the basic local alignment search tool (BLAST), it was found that they have the amidase domain and are members of the amidase signature superfamily. They were expressed in Saccharomyces cerevisiae, and their activity was detected on the cell surface. This study revealed that they are a new type of glutaminase with the amidase signature sequence, and that they form a new glutaminase family.

Key words: Cryptococcus; salt-tolerant; glutaminase; cloning; amidase signature sequence

Glutaminase (Glutamine amidohydrolase, EC 3.5.1.2) catalyzes the hydrolytic deamidation of L-glutamine, resulting in the production of L-glutamate and ammonia. L-Glutamate is the most important umami factor in the yeast microbes for salt-tolerant glutaminases, and found one that they form a new glutaminase family.

Materials and Methods

Strains and media. Purified glutaminase proteins and their genes were obtained from Cryptococcus nodaensis NISL-3771 and Cryptococcus albidus ATCC20293. Several glutaminase genes have been cloned and expressing them in Saccharomyces cerevisiae species and expressing them in Saccharomyces cerevisiae. Sequence analysis revealed that they were new, previously unidentified glutaminases with the amidase signature (AS) sequence and that they formed a new glutaminase family.

Enzyme purification. The glutaminases from C. nodaensis were purified as previously described. The glutaminase enzyme isolated CaGahA (Cryptococcus albidus glutamine amidohydrolase), was purified and characterized. Enzymatic characterization showed that it was suitable for manufacturing soy sauce. It was found that the glutamate content of soy sauce increased with the use of CaGahA in laboratory-scale soy sauce production.

A salt-tolerant glutamimase was also found in Cryptococcus nodaensis. This glutaminase, CnGahA, was more salt-tolerant and thermostable than CaGahA. CnGahA was found to hydrolyze not only glutamine but also asparagine, so it was defined as a glutaminase-asparaginase.

Several glutaminase genes have been cloned and characterized from bacteria, fungi, and mammals. In this study, we focused on cloning salt-tolerant and thermostable glutaminase genes from Cryptococcus species and expressing them in Saccharomyces cerevisiae. Sequence analysis revealed that they were new, previously unidentified glutaminases with the amidase signature (AS) sequence and that they formed a new glutaminase family.

Abbreviations: Gah, glutamine amidohydrolase (glutaminase); RACE, rapid amplification of cDNA ends; PAGE, polyacrylamide gel electrophoresis; BLAST, basic local alignment search tool; DIG, digoxigenin

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30 min. The supernatant contained the crude enzyme. The crude enzyme solution was heated at 60°C for 1 h. After heating, its pH was adjusted to 7.0 with 0.2 M Na₂HPO₄. The preparation was heated at 60°C for 1 h, then immediately cooled on ice, and then centrifuged at 7,000 rpm for 30 min to remove the precipitate. Solid ammonium sulfate was added to the cooled supernatant to a final concentration of 1.2 M, and the precipitate was removed after centrifugation at 10,000 rpm for 30 min. The supernatant was loaded onto the TSKgel Phenyl-5PW column (Tosoh, Tokyo), equilibrated with 0.1 M phosphate buffer (pH 7.0) containing 1.2 M ammonium sulfate. The bound proteins were eluted with a linear gradient of ammonium sulfate from 1.2 M to 0 M and with ethylene glycol from 0% to 20% for 60 min at a flow rate of 5.0 mL/min. The active fractions were collected, and the buffer was replaced with 10 mM phosphate buffer (pH 7.0) by using Centriprep YM-10 (Millipore, Billerica, MA) and then loaded onto the TSKgel G3000SW column (Tosoh) equilibrated with 0.1 M phosphate buffer. The active fractions were collected and concentrated using Centriprep YM-10. The concentrated enzyme solution was loaded onto the TSKgel G3000SW column (Tosoh) equilibrated with the same buffer. The bound proteins were eluted with a linear gradient of NaCl from 0 M to 1.0 M for 60 min at a flow rate of 1.0 mL/min. The active fractions were collected and concentrated using Centriprep YM-10. The concentrated enzyme solution was loaded onto the TSKgel G3000SW column (Tosoh) equilibrated with 0.1 M phosphate buffer containing 0.2 M NaCl (pH 7.0), and eluted at a flow rate of 0.7 mL/min. This enzyme preparation was used for peptide sequencing.

**Determination of the molecular weights of the glutaminase proteins.**

The apparent molecular mass of the native enzyme was determined by gel filtration using TSKgel G3000SW. Glutamate dehydrogenase (MW 290,000), lactate dehydrogenase (MW 142,000), enolase (MW 67,000), adenylate kinase (MW 32,000), and cyctochrome c (MW 12,400) were used as molecular weight markers. The molecular mass of the denatured enzyme was determined by SDS-PAGE. A gradient gel (10–20%) was used for SDS-PAGE (Cosmo Bio, Tokyo).

**Determination of the N-terminal and internal amino acid sequences of the purified glutaminases.**

The N-terminal amino acid sequence of CnGahA was determined using a 213 Protein Sequencer (Applied Biosystems, Foster City, CA). Analysis of the internal amino acid sequence of CnGahA was performed as described below. The purified glutaminase was boiled in 50 mM Tris–HCl buffer (pH 9.0) containing 1% SDS for 10 min, and then digested with lysylendopeptidase at 37°C for 2 h. The peptide fragments were separated by reverse-phase HPLC using an Asahipack ODP-4E column (Showa Denko, Tokyo) with a linear gradient of acetonitrile (5–95%). The peptide sequences were determined with a 492 Protein Sequencer (Applied Biosystems). Analysis of the internal amino acid sequence of CaGahA was performed as described below. Three separate polypeptides on SDS–PAGE were transferred to an Immuno-Blot PVDF Membrane (Bio-Rad, Hercules, CA) by the semi-dry blotting method.19) The transferred membrane was stained with Coomassi Brilliant Blue using Quick CBB staining solution A (Wako, Osaka, Japan). The stained bands of the proteins were cut out and analyzed with a 492 Protein Sequencer (Applied Biosystems).

**DNA and RNA manipulations.** General DNA and RNA manipulations were performed as described by Sambrook et al.20) C. albidus and C. nodensis were grown in YM medium at 30°C until OD₆₀₀ reached 3.0, and were harvested by centrifugation at 3,000 rpm for 10 min. The wet cells were quickly chilled with liquid nitrogen and disrupted by mortar and pestle. Chromosomal DNA was extracted using SepaGene (Sanko Junyaku, Tokyo), and total RNA was extracted from the disrupted cells using RNAeasy (Qiagen, Hilden, Germany), following the manufacturer’s instructions.

**Cloning of the chromosomal gene and cDNA-encoding glutaminase from C. albidus.** Cloning of the glutaminase gene from C. albidus was performed by the procedure described above. The oligonucleotide sequences of the glutaminase from C. albidus were obtained by reverse translation of the peptide sequences, indicated by underlined letters. For peptide sequence TVSAKTVPGISIAYASYNHASD, the forward primer Peptide I-s was used. For peptide sequence YIASYNHA, the reverse primer Peptide F-s was used. For peptide sequence LYGTDYAEOQND, the reverse primer peptide G-as was used. Standard PCR was performed to amplify the glutaminase gene fragments using equal amounts of forward and reverse primers and chromosomal DNA from C. nodensis as template. The reaction program was as follows: 3 min hold at 94°C, followed by 45 cycles for 30 s at 94°C, 30 s at 52°C, and 3 min at 72°C. The amplified PCR fragment was cloned into the pcR2.1/TOPO vector (Invitrogen) and sequenced. The PCR product was used as a probe in Southern and Northern blotting.

**Inverse PCR was performed to amplify the flanking regions of the glutaminase gene.** Chromosomal DNA was completely digested with EcoRV or Sall, and separated by agarose gel electrophoresis on a 1% agarose gel. The gel around the 1.5- to 3.0-kbp region was cut out to extract the digested chromosomal DNA. This was subsequently ligated and used as the template for inverse PCR. To obtain the 5’ flanking region, CnInv5'-s and CnInv5'-as were used as PCR primers. To obtain the 3’ flanking region, CnInv3'-s and CnInv3'-as were used as PCR primers. 5’-RACE was performed using a First Choice RLM-RACE Kit (Ambion, Austin, TX), and 3’-RACE was performed using a 3’-Full RACE Core Set (Takara, Kyoto, Japan) following the manufacturers’ instructions. CnRace5'-as and CnRace3'-as were used as PCR primers for 5’ RACE and 3’ RACE respectively. The entire glutaminase gene was amplified using the first-strand cDNA generated by 3’ RACE, using primers Cn-s and Cn-as. All PCR reactions were performed with Ex-taq polymerase (Takara). Nucleotide sequences were determined with a Thermo Sequenase Cycle Sequencing Kit (USB, Cleveland, OH) and DNA sequence LIC-4200L(S)-2 (LI-COR, Lincoln, NE).

**Cloning of the chromosomal gene and cDNA-encoding glutaminase from C. albidus.** Cloning of the glutaminase gene from C. albidus was performed by the procedure described above. The oligonucleotide sequences of the glutaminase from C. albidus were obtained by reverse translation of the peptide sequences, indicated by underlined letters. For peptide sequence TVSAKTVPGISIAYASYNHASD, the forward primer Peptide I-s was used. For peptide sequence YIASYNHA, the reverse primer Peptide F-s was used. For peptide sequence LYGTDYAEOQND, the reverse primer peptide G-as was used. Standard PCR was performed to amplify the glutaminase gene fragments using equal amounts of forward and reverse primers and chromosomal DNA from C. nodensis as template. The reaction program was as follows: 3 min hold at 94°C, followed by 45 cycles for 30 s at 94°C, 30 s at 52°C, and 3 min at 72°C. The amplified PCR fragment was cloned into the pcR2.1/TOPO vector (Invitrogen) and sequenced. The PCR product was used as a probe in Southern and Northern blotting.

**Sequence analysis of the glutaminases.** The nucleotide and protein sequences of the AS enzymes were obtained from the Uniprot database (http://www.uniprot.org/). A homology search was performed against

### Table 1. Oligonucleotides Used in This Study

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Sequence (5’–3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cloning primer for CnGahA</td>
<td><strong>TTTTGTYCICRCARTCYCRTA</strong></td>
</tr>
<tr>
<td>peptide-s</td>
<td><strong>GGIGTIAICWSIGTGYCTTCYATCCARCC</strong></td>
</tr>
<tr>
<td>peptide-as</td>
<td><strong>GAAGTTATGACTTTACCCAGG</strong></td>
</tr>
<tr>
<td>CnInv5'-s</td>
<td><strong>ATCGTATGGTCGAGCCAGTCGTATGCATGCTGC</strong></td>
</tr>
<tr>
<td>CnInv5'-as</td>
<td><strong>CATCCGTGGGCGACAACGACAGTTTG</strong></td>
</tr>
<tr>
<td>CnRace3'-s</td>
<td><strong>ccakcgtggggcgaacagcagtatttg</strong></td>
</tr>
<tr>
<td>Cn-as</td>
<td><strong>ATTTAAGTCTTACACCCAGCTGGTACCGC</strong></td>
</tr>
</tbody>
</table>

Restriction enzyme sites used in cloning is in italics.
Heterologous expression of glutaminase in S. cerevisiae. The entire amplified gene was digested with 
KpnI and EcoRI and cloned into pYES2 (Invitrogen). The plasmids in which the Cn
gahA and CgahA genes were cloned into pYES2 were named pYESTKF and pYESCKF
respectively. These were introduced into S. cerevisiae INVSc (Invitrogen). Yeasts were transformed by the lithium acetate method.22) The recombinant protein was expressed following the manufacturer’s instructions for the pYES2 vector.

**Results**

**Purification of C. nodaensis glutaminase**

The purified CnGahA was obtained as a single peak by gel filtration, and was observed as a single band on SDS–PAGE (Fig. 1). The molecular weight of the purified CnGahA was estimated to be about 270 kDa by gel filtration TSK-gel G3000SW, and to be 78 kDa by SDS–PAGE analysis. These results suggest that native CnGahA was probably present as a trimer or tetramer. The mobility of CnGahA on SDS–PAGE was found to change under PNGaseF treatment. Hence we think that CnGahA is a glycoprotein (Fig. 1).

**Cloning and sequencing analysis of CngahA**

The N-terminal sequence of the purified CnGahA revealed that at least three polypeptides were present. Considering the signal intensities of the various amino acids, three N-terminal sequences were identified, as follows: peptide A, GVTSLGAFQPASV; peptide B, KVASVSPQGAQYL; and peptide C, SGSIPOQQYLAH. The yields of the latter two peptides were much less than that of peptide A. Furthermore, peptide B and peptide C were found to have similar sequences (the same amino acids are indicated in bold). Hence we concluded that peptide B and peptide C were derived from the same polypeptide.

The purified CnGahA was digested with lysylendopeptidase and subjected to chromatography by reverse-phase HPLC. Four internal peptides fragments were isolated and their N-terminal amino acid sequences were determined, as follows: peptide D, TSQFA; peptide E, EFIDTEVL; peptide F, GVTSLGAFQPASV; and peptide G, LWYEDYAEQND[.]

A combination of forward primer peptide F-s and reverse primer peptide G-as (Table 1) was used to amplify a 1.6-kbp chromosomal DNA fragment of *C. nodaensis* NISL–3771. The peptide sequence of peptide D was found in the internal region of the amino acid sequence as deduced from the amplified fragment.

Since it was expected that the amplified fragment contained introns, both chromosomal DNA and cDNA were analyzed. Finally, a 5.7-kb fragment containing the glutaminase gene was isolated from chromosomal DNA by inverse PCR, and a 2.5-kb cDNA fragments were isolated by 5’ and 3’ RACE. Sequence analysis of the chromosomal DNA and the cDNA fragment revealed that the open reading frame (ORF) was 2,052 bp long and encoded a 684-amino acid protein with a molecular mass of 73,801, containing six introns (Fig. 2, accession no. A610785). All N-terminal peptide sequences and internal peptide sequences isolated from digested CnGahA were found in the deduced amino acid sequence (Fig. 2). These results indicate that the three N-terminal sequences determined from the purified CnGahA were derived by N-terminal truncation of CnGahA. The signal sequence in the region from Met1 to Ser23, as determined by SIGNALP analysis, suggested that CnGahA is a secreted protein. Southern blotting revealed that a single band of a different size was present after EcoRI, Smal, or Xbal digestion, indicating that the CngahA gene was present as a single copy in the chromosomal DNA of *C. nodaensis* NISL–3771 (data not shown). The purified CnGahA showed a single band on SDS–PAGE, and the N-terminal sequence of it was derived from the N-terminal truncated form of CnGahA. Furthermore, the CnGahA gene was present as a single copy in the chromosomal DNA. These results suggest that CnGahA is a homotrimer or homotetramer.

Public DNA and protein databases were searched for similar glutaminases, but none were found. The highest overall homology was found with hypothetical proteins from *Penicillium chrysogenum* Wisconsin 54–1255.
Sclerotinia sclerotiorum (38% identity; E value, 8e^-102),
Aspergillus oryzae RIB40 (38% identity; E value, 6e^-102),
Aspergillus flavus (38% identity; E value, 7e^-101),
Nectria haematococca (39% identity; E value, 1e^-98), and
Saccharomyces cerevisiae strain CBS4411 (37% identity; E value, 1e^-93). None of these proteins, however, have been biochemically characterized.

23) Glutamyl-tRNA Gln amidotransferase and putative amidase had low identity to CnGahA. BLAST searches revealed that CnGahA had a conserved amidase domain (AS), a CCGT superfamily domain, and a Asp-tRNAAsn/Glu-tRNAGln amidotransferase A subunit-related amidase domain. These results indicate that CnGahA is a new type of glutaminase and a member of the AS superfamily.

24) The catalytic triad, Lys_265, Ser_345, and Ser_380 of CnGahA, found exclusively in the AS sequences,25–28 was absolutely conserved in CnGahA.

Gene expression in yeast

The cloned CnGahA gene was expressed in S. cerevisiae. Its glutaminase activity was not detected in the extracellular fraction but on the cell surface, as observed for native CnGahA. The glutaminase activity of the transformant carrying the pYESTKF-containing CnGahA gene increased gradually with growth under inducing conditions (Fig. 3).

Thermostability and salt tolerance of recombinant CnGahA

To determine the thermostability and salt tolerance of recombinant CnGahA, we examined the glutaminase on the cell surface of recombinant yeast. Residual activities after heat treatment at 60°C and 70°C for 30 min were 100% and 70% respectively. Furthermore, the recombinant CnGahA in the reaction mixture containing 20% NaCl exhibited approximately 70% activity as compared to that in the reaction with 0% NaCl. These results indicate that the CnGahA expressed in S. cerevisiae was...
salt-tolerant and thermostable, similarly to native CnGahA.15

Molecular cloning and expression of the glutaminase gene from C. albidus

Purified CaGahA was obtained as a single peak by gel filtration, but was observed as a smear band on SDS–PAGE (data not shown). Because the purified CaGahA was separated into three polypeptides by treatment with PNGaseF on SDS–PAGE, we concluded that CnGahA, CaGahA is a truncated glycoprotein. The N-terminal amino acids of the three polypeptides separated on SDS–PAGE were determined as follows: peptide H, LHEISAVMPMGVFEGTQQL; peptide I, TVSAXTVPSGPYIASYNHASD; and peptide J, NVAVPRALEGVRVAKDLXYDTAGL.

The sequences of the latter two peptides were similar to those of the CnGahA-derived peptides. A combination of oligonucleotide primers Peptide I-s and Peptide J-as was subjected to PCR for amplification of a 120-bp chromosomal DNA fragment of the CagahA gene. Because the amino acid sequence deduced from the amplified fragment was similar to the sequence of the internal region of CnGahA, we concluded that the amplified fragment was part of the glutaminase gene of C. albidus.

The chromosomal DNA and cDNA of CagahA were obtained by the same method used for CnGahA gene cloning. The ORF was 2,100 bp long, and encoded a 700-amino acid protein with a calculated molecular mass of 76,474 (Accession no. A610786). The chromosomal DNA was interrupted by seven introns. Three separated polypeptide sequences isolated from the purified CaGahA were found in the internal region of the deduced amino acid sequence translated from the ORF. SIGNALP analysis revealed the signal sequence in the region from Met1 to Ser18 of CaGahA, and indicated that CaGahA is also a secreted protein. The overall amino acid sequence of CaGahA showed 44% identity to CnGahA, whereas the region around the AS sequence comprised residues 268–391 of CaGahA showed 67% identity to the corresponding region of CnGahA.

The cloned gene was expressed in S. cerevisiae, and its glutaminase activity was detected on the cell surface, similarly to the observations for CnGahA. The transformants carrying pYESCKF containing CagahA or pYES2 as control were cultured for 30h under the inducing condition, and the glutaminase activity of the transformant carrying pYESCKF was found to be 11 times higher than that of the transformant carrying pYES2.

Discussion

In this study, we cloned two salt-tolerant, thermostable glutaminase genes from Cryptococcus species. Sequence analysis revealed that these glutaminases constitute a new family of glutaminases that have an AS sequence. Many proteins with the AS sequence have been found in various organisms. It is difficult to predict the physiological functions of the members of the AS superfamily found in the sequence database, because the identified physiological functions and substrate specificities of enzymes in the AS family vary widely. Recently, Ko et al. reported that AS enzymes are to be classified into six subfamilies.29 Although CnGahA and CaGahA were predicted to contain a Glu-tRNAGln amidotransferase A subunit (GatA) that shows glutaminase activity,30 these proteins were not classified in the GATA subfamily, but in a different subfamily altogether (Fig. 4). Our study has established a new functional classification for the AS superfamily. Although the glutaminase reaction is a kind of amidase reaction in that it involves hydrolysis of the amide bond (CO–NH), GatA is the only known enzyme with an AS sequence having glutaminase activity. The results of this study suggest the diversity of glutaminases, and lay a ground work for research into the physiological role of these enzymes.

The cryptococcal glutaminases described in this report are located on the cell surface. It is not likely that these glutaminases attach to the cell surface via transmembrane binding, because they have no transmembrane domain according to a prediction made by the SOSUI program (http://bp.nuap.nagoya-u.ac.jp/sosui/). These glutaminases were solubilized from the cell surface with a lytic mixture consisting of Cellulase ONOZUKA R-10, which is used for the degradation of the plant cell wall. Hence, we concluded that these glutaminases are bound to the cell wall. There are two main classes of covalently linked wall proteins, GPI wall proteins and mild alkali-soluble proteins in fungi.31 The former form the largest class, and the latter is less numerous, but these glutaminases have no GPI anchor attachment site according to GPI modification site prediction (http://mendel.imp.ac.at/gpi/fungi_server.html). PIR-proteins (proteins with an internal repeat) are representative of mild alkali-soluble proteins. PIR-proteins contain variable numbers of glutamine-containing internal repeats (core sequence, DGQXQ) and a four cystein domain in the C-terminal region. These typical features were not confirmed for CnGahA and CaGahA, but several proteins contain neither a consensus sequence for GPI modification nor PIR-specific repeats as found in mild alkali-soluble proteins.32 The common feature of these proteins is the presence of a Kex2p substrate site (KK/KR) in the N-terminal region, and this site is preceded by at least three additional positively charged residues (including at least two histidines). Both CnGahA and CaGahA were present
in the Kex2p substrate site of the N-terminal region, but additional positively charged residues (including at least two histidines) were not confirmed. The mechanism of attachment to the cell surface is unclear.

We found many hypothetical proteins similar to CnGahA and CaGahA by BLAST search. Multiple alignments are shown in Fig. 5. The Lys-cis-Ser-Ser catalytic scissors of GatA were absolutely conserved in GaHAs. Substitution of these amino acid residues significantly decreased the glutaminase activity of GatA. Substitution of these amino acid residues was not confirmed. The mechanism of glutamine is different from that of GatAs. This suggests that the N-terminal regions of these GaHAs play roles in localization. N-Terminal sequence analysis revealed that the purified CnGahA contained three different N-terminals (Peptides A, B, and C), but all these peptides were derived from CnGahA. We concluded that CnGahA is truncated, and that the majority of the purified CnGahA had the peptide A sequence at the N-terminal, according to the signal intensity of the peptide sequence, but the cleavage site did not correspond to the predicted signal peptide cleavage site. There are two possible explanations for this inconsistency. One is that these glutaminases are degraded by a contaminating protease during purification, and the other, that they are cleaved by proteolytic processing. The purified CnGahA was observed as a single band on SDS–PAGE (Fig. 1), and the potential Kex-2p cleavage site (KR) was present just before the N-terminal sequence of the purified CnGahA (corresponding to the peptide A sequence). Hence, we assumed that CnGahA is cleaved by proteolytic processing, and that part of the CnGahA was subsequently degraded by a contaminating protease during purifica-
tion. These differences in localization and proteolytic processing might reflect of the different physiological roles played by them.

Many hypothetical proteins similar to CnGahA and CaGahA were found by BLAST search, almost all of them in Ascomycetes. It would be interesting to investigate the physiological roles of these glutaminases in these organisms.

Acknowledgments

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References


Fig. 5. Multiple Alignments of Glutaminases.

Glutaminase of Cryptococcus nodaeensis (CnGahA), glutaminase of Cryptococcus albidaus (CaGahA), the hypothetical protein from Saccharomyces cerevisiae (Q06733 YEAST), Penicillium chrysogenum (B6HDX2 PENCW), Magnaporthe grisea (Q2KH33 9PEZI), Aspergillus flavus (BSNCR0 ASPFN), and Glu-tRNAGln amidotransferase subunit A from Staphylococcus aureus (GATA STAAM) are compared. The region after the amidase signature sequence (252–665 of CnGahA and 267–671 of CaGahA) was predicted to be a Glu-tRNAGln amidotransferase A subunit domain. Conserved amino acid residues are highlighted. Asterisks indicate the three invariant residues of the catalytic triad, and solid circles indicate the residues involved in the recognition of glutamine in GatA.

A New Glutaminase Family


