Gabaculine-resistant Glutamate 1-Semialdehyde Aminotransferase of Synechococcus

DELETION OF A TRIPEPTIDE CLOSE TO THE NH2 TERMINUS AND INTERNAL AMINO ACID SUBSTITUTION

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Glutamate 1-semialdehyde aminotransferase (GSA-AT) is the last enzyme in the C5 pathway converting glutamate into the tetrapyrrole precursor δ-aminolevulinate in plants, algae, and several bacteria. Sequence analysis of the genes encoding GSA-AT in barley, Synechococcus, and Escherichia coli revealed 50–70% similarity in the primary structures of the proteins. The enzyme is inhibited rapidly by gabaculine when added in approximately stoichiometric amounts with the enzyme. A gabaculine-tolerant Synechococcus strain, GR6, was found to produce a GSA-AT less sensitive to the inhibitor. Accordingly, the mutant gene was isolated and sequenced. In comparison with the wild-type gene it contains a deletion of nine nucleotides (position 12–20) and a guanine to adenine substitution (position 743). This resulted in the loss of the amino acids serine, proline, and phenylalanine (position 5–7) close to the NH2 terminus of the enzyme and an exchange of Met-248 for isoleucine in the middle of the polypeptide chain. Wild-type and mutant GSA-AT were expressed in E. coli and purified close to homogeneity. Although the specific activity of the mutant GSA-AT was only one-fifth of the wild type, it displayed a 100-fold increased resistance to gabaculine. Peaks in the absorption spectrum of the purified recombinant GSA-ATs at 335 and 417 nm are typical of a transaminase containing a B6 cofactor. Incubation with substrate and with inhibitor induced spectral changes characteristic of other gabaculine-sensitive, B6-requiring enzymes.

Glutamate 1-semialdehyde aminotransferase (GSA-AT) catalyzes the net transfer of the amino group of GSA from C2 to C1 yielding δ-aminolevulinate (ALA), the universal precursor of tetrapyrroles. GSA-AT is the final enzyme in the tRNA-mediated C5 pathway that converts glutamate to ALA in three steps (for reviews, see Refs. 1–3). This pathway is present in algae, several bacteria, and plastids of higher plants. Other microorganisms like Rhodobacter and Rhizobium share with yeast and animals the single-enzyme pathway in which ALA is formed from succinyl-CoA and glycine (4).

GSA-AT was characterized first in barley. It is a dimeric enzyme consisting of two identical subunits of 46 kDa (5, 6). The complete nucleotide sequence of the gene encoding GSA-AT has been determined in barley (7), Synechococcus PCC6301, and Escherichia coli (8). The barley enzyme is synthesized in the cytoplasm as a 49.5-kDa precursor and posttranslationally imported into plastids. The molecular weights of GSA-AT deduced from the nucleotide sequences of single-copy Synechococcus and E. coli genes are 46,000 and 45,500, respectively. Comparative studies of the primary structure of GSA-AT of barley, Synechococcus, and E. coli indicate more than 48% sequence identity (8). Because congruence of secondary structure is also predicted, it is presumed that the three-dimensional structures of these GSA-ATs are similar.

In spite of these structural similarities, several contradictory observations concerning the involvement of the cofactor vitamin B6 have to be resolved. Synechococcus and cyanidium GSA-ATs require pyridoxamine phosphate (PMP) for the synthesis of ALA (9, 10) while the Chlorella enzyme shows enhanced activity with catalytic amounts of pyridoxal phosphate (PLP) (11). The activity of barley GSA-AT purified from soluble plastid extracts is not increased by supplementation with vitamin B6 (5). GSA-ATs from all organisms tested so far are very sensitive to the inhibitor gabaculine (3-amino-2,3-dihydrobenzoic acid) (12–14), and it has been assumed that the inhibition of tetrapyrrole synthesis by gabaculine is due to the inactivation of this enzyme. Gabaculine is also a potent inhibitor of γ-amino butyrate aminotransferase; it can be considered a rigid analog of γ-amino butyrate which forms m-carboxyphenylpyridoxamine phosphate with PLP tightly bound at the active site of the enzyme (15). It is an irreversible inhibitor of many other PLP-requiring aminotransferases. Soper and Manning (16) have suggested that the ability to carry out an exchange of β-protons of the natural substrate makes aminotransferases susceptible to gabaculine inactivation.

Addition of 5 μM gabaculine to a continuous culture of wild-type Synechococcus PCC 6301 resulted in rapid decline in culture density and in phycocyanin and chlorophyll content (17). However, the culture recovered within several hours.
indicating the selection of a gabaculine-tolerant spontaneous mutant. After successive exposure to 10 and 20 μM inhibitor, *Synechococcus* strain GR6 was isolated from the continuous culture and found to be unaffected by gabaculine concentrations up to 100 μM under photoautotrophic conditions in batch culture (17).

There are several possible explanations for the tolerance of *Synechococcus GR6* to gabaculine. The uptake of inhibitor may be prevented by an alteration of a transport process, gabaculine-catabolizing enzyme could be activated in the cyanobacterial cell, the gabaculine-sensitive enzyme in tetrapyrrole synthesis could be amplified, or the enzyme itself could have an altered amino acid sequence making it resistant to gabaculine.

This paper describes an investigation of the molecular basis of the gabaculine resistance of strain GR6. As the partially purified GSA-AT from this organism retains gabaculine tolerance, we have sequenced the GSA-AT gene of the mutant to identify the structural changes causing this effect. Both the *synechococcal* wild type (PCC6301) and mutant (GR6) genes have been expressed in *E. coli*. The two forms of recombinant GSA-AT have been purified and their properties compared.

**MATERIAL AND METHODS**

**Bacteria—Cyanobacterium Synechococcus PCC 6301** wild-type and mutant strain GR6 were grown photoautotrophically in liquid BG-11 at 30 °C and constant light (18). Media for GR6 were supplemented with gabaculine (5–100 μM). DH5α (F+ 4800lacZAM15 EndA1 recA1 hsdR17(km− mK+) supE44 thi1-1 λ– gyrA96 relA1 (lacZYA−argF)U169 (Bethesda Research Laboratory) was transformed with plasmid pGem7Zf(+). pGem7Zf(+) and pSAT GR6 described in this paper. LB media containing the pUHAl plasmid hosted the recombinant expression vector pDS 56/RBSII, SphI (23) was cleaved with BarnHI in the polycloning site of the expression vector. This resulted in the synechococcal wild type (PCC6301) and mutant (GR6) genes being expressed in *E. coli* cells transformed with recombinant expression vectors pSAT1.4 or pSAT GR6 carrying genes for wild-type and mutant GSA-AT, respectively, were used to inoculate 3 liters of fresh LB medium. At an OD = 600 nm of about 0.5, cells were induced with IPTG (1 mM), and incubation continued for 4 h. Cells were broken by lysozyme digestion (0.8 mg/mL, weight, *E. coli*) and subsequently treated with deoxycholate (4 mM) and DNase (2 μg/mL) as described (24). Soluble protein extracts obtained by centrifugation (1, 4 °C, 37,000 × g) were fractionated on a Sephacryl S-300 column in buffer (0.1 M Tricine, pH 7.9, 25 mM MgCl2, 0.1 mM diithiothreitol, 0.3 mM glycerol). Protein fractions containing GSA-AT activity and having highest concentration of 46-kDa protein determined by SDS-polyacrylamide gel electrophoresis were applied to a DEAE-cellulose column (Waters DE52). Protein was eluted with an exponential gradient (0.0–0.4 M NaCl) in the same buffer using a constant volume mixer (200 mL). A portion (30 mg) of the protein fraction with the highest GSA-AT activity was purified further by FPLC (Mono Q) in a buffer containing 20 mM Tricine, pH 7.9, 25 mM MgCl2, 0.1 mM dithiothreitol, and 0.3 mM glycerol. The eluting salt gradient was from 0.0 to 1.0 M NaCl. Molecular weights of active isolated GSA-ATs were determined on a calibrated Sephacryl S-200 column using molecular weight markers.

**Analysis of Recombinant Wild-type and Mutant GSA-AT**—Portions of protein fractions from each purification step were analyzed by electrophoresis in 12.5% SDS-polyacrylamide gels (26). These fractions were also assayed for GSA-AT activity by measuring ALA according to Mauzenn and Granick (27) with some modification; the assay mixture consisted of 0.1 M phosphate, pH 7.0, or 0.1 M Bistris buffer, pH 7.0, 120 μM GSA, and protein fraction. GSA was synthesized as described (28). Leucine (10 mM) was included until protein fractions were shown to be free of 5-ALA dehydratase. PMP and PLP were added as indicated. Assay mixtures were incubated routinely for 1 h at 30 °C, terminated by cooling in ice water and addition 100 μl of ethyl acetocetate, heated at 100 °C for 15 min, and finally mixed with 500 μl of Ehrlich reagent (27). ALA was determined spectrophotometrically at 553 nm using a molar extinction coefficient of 6.0 × 104 (27). Protein concentrations were determined by colorimetric techniques (29). Absorption spectra of purified GSA-AT were recorded on a Shimadzu spectrophotometer.

**RESULTS**

**Characterization of Gabaculine-resistant GSA-AT and Its Coding Nucleotide Sequence**—To test whether GSA-AT from gabaculine-tolerant *Synechococcus* strain GR6 is resistant to gabaculine inhibition, GSA-AT was partially purified using techniques described previously for the barley enzyme (20, 21). GSA-AT was present in the run-through fraction from the final affinity column, and the enzyme activity was resistant to gabaculine concentrations up to 1000 μM. These findings indicated that the enzyme in strain GR6 is rather insensitive to gabaculine. To identify the changes in enzyme structure associated with this resistance to inhibitor, the GSA-AT gene of GR6 was cloned and sequenced in the multicopy plasmid pGEM 7Zf(+). To exclude possible nucleotide errors due to incorrect synthesis by TAQ polymerase, the mutant gene encoding GSA-AT was sequenced independently from two different PCR amplifications. Furthermore, the wild-type gene was amplified and sequenced in parallel. Both strands of mutant and wild-type gene were sequenced and found to be complementary. Comparisons of wild-type and mutant genes revealed consistent changes at two different sites in the coding sequence. The first change involved a deletion of nine nucleotides from the wild-type gene at positions 12–20 in the coding sequence. This resulted in the elimination of amino acids 5-7 Ser-Pro-Phe (Fig. 1, top). The fused codon 4 obtained from this deletion resulted in
in the replacement of the cytosine at the third position by thymine. However, this base transversion is a neutral change and does not lead to an amino acid substitution. The second alteration affects a single nucleotide (position 743) in the wild-type gene. In the bottom panel the substitution of adenine for guanine at position 743 is presented. In both cases the 5' end of the nucleotide sequence is at the bottom.

Fig. 1. Nucleotide sequence comparison of the genes encoding wild-type and gabaculine-resistant Synechococcus glutamate 1-semialdehyde aminotransferase (GSA-AT). The top panel illustrates the deletion of nine nucleotides in the mutant gene (position 12-20 of the wild-type gene). In the bottom panel the substitution of adenine for guanine at position 743 is presented. In both cases the 5' end of the nucleotide sequence is at the bottom.

GSA-ATs was recovered in the 37,000 × g supernatant fraction and comprised 5-10% of the total soluble protein. Both recombinant enzymes migrated as a 46-kDa protein in SDS-polyacrylamide gels. Degradation products or truncated proteins due to a translation start at an internal ATG were not detectable. The solubility of a relatively large fraction of the total synchochoccal GSA-AT in E. coli is in marked contrast to the insolubility of the mature barley enzyme expressed in E. coli (7). In spite of 71% sequence identity between barley and Synechococcus GSA-AT, the barley protein was found largely in the pellet fraction of disrupted E. coli cells.

Soluble extracts from E. coli cells containing either wild-type or mutant Synechococcus GSA-AT formed 324 and 57 nmol of ALA/mg of protein/10 min, respectively, in 0.1 M phosphate buffer, pH 7.0, containing 10 μM each of PMP and PLP (Table I). On the other hand, the basal activity due to endogenous E. coli enzyme, of 0.018 nmol of ALA/mg of soluble protein/10 min (7) is less than 0.032% of the levels found in cells containing the Synechococcus genes. Mutant and wild-type aminotransferases were purified by gel filtration, ion exchange chromatography, and Mono Q FPLC. The elution profiles for the mutant enzyme at each purification step are shown in Fig. 2, and in each case activity coincided with the presence of a 46-kDa protein in fractions on SDS-polyacrylamide gels. The data for the wild-type enzyme are essentially the same. Approximately 90% of the activity recovered from the Sephacryl S-300 column was pooled and loaded on a DEAE-cellulose ion exchange column, and GSA-AT activity was eluted at approximately 0.15 M NaCl in 0.1 M Tricine buffer, pH 7.9. A portion containing 30 mg of protein of the most active fraction was purified further by FPLC on a Mono Q column; GSA-AT was eluted at a salt concentration of approximately 0.15 M. After these three purification steps the recombinant enzymes were essentially homogeneous as judged by SDS electrophoresis, even when using a 100-fold excess (cf. Fig. 2). Using a calibrated Sephacryl S-200 column GSA-ATs were found to have a molecular weight close to 1105 g/mol (data not shown). This is consistent with that expected for a dimer, as has been observed for many aminotransferases (30). Our gel filtration experiments suggest that the active form of the mutant enzyme is also a dimer in spite of the NH2-terminal deletion of 3 amino acid residues. The specific activities of the purified recombinant wild-type and mutant GSA-AT were 5.7 and 1.1 μmol of ALA/mg of protein/10 min, respectively, with recoveries of approximately 76% (Table I). The consistently lower activity of the mutant enzyme compared with the wild type at each stage of the purification can be attributed to differences in structure. Since this mutant was selected in cultures grown with stepwise increasing amounts of gabaculine, only mutations which improve the insensitivity to the inhibitor will be favored.

Cofactor Dependence of Wild-type and Mutant GSA-AT—In a previous investigation (6, 17), the activity of GSA-AT isolated from the wild-type Synechococcus was strictly dependent on PMP. After purification recombinant enzymes were active in the absence of added B6 cofactor (Table II). However, PMP and PLP increased the activity of each of the enzymes although the mutant was not affected to the same extent as the wild-type GSA-AT. This stimulating effect was higher in Bistris buffer than in phosphate (0.1 M, pH 7.0). In Bistris buffer the degree of stimulation with both enzymes was greater with PMP than with PLP.

The absorption spectra of the purified recombinant proteins from wild-type and mutant strains included two distinct peaks in addition to that due to protein (276 nm, at 335 nm (major) and 417 nm (minor) (Fig. 4A). The absorbance ratios for the
two enzymes were as follows: wild type 0.084 (335/276), 0.014 (417/276) and mutant 0.075 (335/276), 0.0096 (417/276). When purified recombinant wild-type or mutant enzyme was incubated with GSA (120 μM), there was a marked decrease in absorbance at 343 nm and a corresponding increase at 417 nm (Fig. 4B). During this incubation an additional transient peak was observed at λmax 485 nm. The absorption maxima of absorbing species of aspartate aminotransferase have been well established by correlation to model systems (31). By analogy the observed spectral changes upon GSA addition were assumed to be: internal and external aldimine (417 nm), PMP and ketamine (343 nm), quinonoid intermediate (485 nm).

**Effect of Gabaculine on the Recombinant Wild-type and Mutant Enzymes**—Gabaculine had a markedly different effect on the activity of the two recombinant GSA-ATs (Fig. 3). The wild-type enzyme was inhibited more than 50% by 3 μM gabaculine. In contrast, 100 times more inhibitor was needed to decrease the activity of the mutant enzyme to the same extent. Preincubation with 10 mM levulinate and/or gabaculine did not give increased inhibition. This suggests that reaction conditions for the conversion of GSA-AT to the pyridoxal form by GSA are sufficient for optimum gabaculine inhibition. Stepwise addition of 1-3 μM D/L gabaculine to the wild-type GSA-AT pretreated with GSA decreased absorbance at 417 nm and increased concomitantly absorbance at 343 nm (Fig. 4C). This is consistent with our assignment of the 417 nm peak to the internal (or external) aldimine and the spectral shift to the lower wavelength previously observed with aspartate aminotransferase (16). The *Synechococcus* wild-type enzyme was inactivated in a 1:1 molar ratio. The reaction of 25 μM gabaculine with the mutant enzyme (10 μM) was observed as a function of time (Fig. 4D). A 40-min incubation reduces the absorption band at 417 nm by half, indicating an approximate 50% inactivation of the enzyme. The partially purified barley GSA-AT was consistently found to be sensitive to gabaculine if it was preincubated with an amino acceptor. This suggests that this enzyme must be converted to the pyridoxal form, although characteristic spectral changes have not been observed in partially purified barley GSA-AT upon addition of levulinate (6).

**DISCUSSION**

The major factor contributing to gabaculine tolerance of *Synechococcus* strain GR6 is the insensitivity of glutamate semialdehyde aminotransferase (GSA-AT) to the inhibitor. At least 100 times more gabaculine is required to inhibit enzymatic ALA formation by purified mutant enzyme relative to its wild-type counterpart. This is clearly different from the mutants conferring gabaculine resistance in Chlamydomonas which contained gabaculine-sensitive GSA-AT in larger amounts (14). The basis of the insensitivity of the *synechococcal* GSA-AT to gabaculine has been investigated by sequencing gene copies produced by PCR amplification. These have been shown to code for gabaculine-sensitive and -resistant forms of the enzyme in an *E. coli* expression system.

Sequence comparisons of wild-type and mutant genes identified two specific differences: a deletion of 3 amino acid residues at positions 5–7 and the substitution of isoleucine for Met-248. These result not only in gabaculine resistance but also in a decrease in specific activity of GSA-AT in strain GR6. A comparison of the primary structure of GSA-AT with that of aspartate aminotransferase gives some indications why these changes lead to increased resistance and decreased catalytic activity. Aspartate aminotransferase is the most extensively analyzed Bs-requiring aminotransferase (for reviews see Refs. 32–34); it is a dimer of a 46-kDa polypeptide. Mehta et al. (35) have compared the 12 known amino acid sequences with four tyrosine and histidinol-phosphate aminotransferases and found that only 12 of 412 consensus amino acids are invariant among the 16 sequences. Most of the invariant residues apparently play a critical role in PLP or substrate binding. Although the overall sequence identity between *Synechococcus* GSA-AT and the aspartate aminotransferases is only 20% at best, 6 of these invariant residues can be identified tentatively in GSA-AT.

The sequence 222-D-X-A-Y-Q-G-F-228, which is conserved in all aspartate aminotransferases and partially in the other aminotransferases compared shows similarity to the sequence 245-D-E-V-M-T-G-F-251 in the GSA-AT. The NH$_2$ terminus of aspartate aminotransferase is responsible for the formation of a link between the two subunits of the catalytically active enzyme. Truncation of the first 9 amino acid residues from the NH$_2$ terminus of the native enzyme showed a decrease of activity and a corresponding increase in $K_m$ for the substrate (34). This indicates that the NH$_2$ terminus of aspartate aminotransferase is particularly important for the maintenance not only of the quaternary structure of the enzyme but also of the domain involved in substrate binding and catalysis. It is of interest that the deleted tripeptide -S-P-F- in GSA-AT corresponds in pig cytoplasmic aspartate aminotransferase and other aspartate aminotransferases to 4-S-X-F-6 (36).

Previous studies have shown that gabaculine affects Bs-dependent transaminases (15, 16). Based on the spectrum shown in Fig. 4A, purified wild-type GSA-AT should not be sensitive to gabaculine because the native enzyme is primarily in the pyridoxamine form. As shown in Fig. 4B, this form of the enzyme is converted rapidly to the putative pyridoxal upon addition of GSA. When gabaculine is added in stoichiometric amounts, relative to the enzyme concentration,
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TABLE II

Stimulation of activity of wild type and mutant enzyme by vitamin B6

The mutant enzyme was purified through the Mono Q column step. The assays were carried out for 10 min in 0.1 M phosphate or Bistris buffer, pH 7.0. The first column shows the amount of ALA synthesized. The second column indicates the percent increase of ALA formation in the presence of 10 μM PMP or PLP relative to the control without vitamin B6.

<table>
<thead>
<tr>
<th>Phosphatase buffer</th>
<th>Bistris buffer</th>
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<tr>
<td>μmol ALA/ mg protein/10 min</td>
<td>%</td>
</tr>
<tr>
<td>Wild type</td>
<td>2.13</td>
</tr>
<tr>
<td>PMP</td>
<td>3.14</td>
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<tr>
<td>PLP</td>
<td>3.47</td>
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<tr>
<td>Mutant</td>
<td>0.40</td>
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<tr>
<td>PMP</td>
<td>0.50</td>
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<td>PLP</td>
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Fig. 3. Gabaculine resistance of recombinant wild-type and mutant GSA-AT. GSA-AT purified by Sephacryl S-300, and DEAE-cellulose chromatography was assayed in 0.1 M phosphate buffer, pH 7.0, with 10 μM PMP and PLP in the presence of gabaculine (0-320 μM). The remaining activity of the two enzymes is plotted versus the gabaculine concentration.

near quantitative inhibition is obtained rapidly. This is consistent with the observation that preincubation with 10 mM levulinate did not give increased inhibition during subsequent reaction with GSA and gabaculine. Furthermore, spectral changes observed upon gabaculine addition (Fig. 4C) correspond directly to decreases observed in enzyme activity (data not shown). Spectral data for wild-type and mutant enzymes expressed in E. coli implicate vitamin B6 as a cofactor of GSA-AT. Peaks in the range 320-345 nm and at approximately 410-430 nm are typical of aminotransferases containing bound PMP and PLP, respectively (30). Although the activity of the natural enzyme isolated from Synechococcus 6301 was wholly dependent on the addition of PMP (6, 17), both purified recombinant enzymes retain appreciable activity without added cofactor. The reason for the difference in cofactor dependence of the natural and recombinant wild-type enzymes is not obvious. However, the activities of the wild-type and the mutant GSA-ATs increased when PMP or PLP was included in the assay. With aspartate aminotransferase, the cofactor is displaced from the enzyme by phosphate acryl S-300 column. Fractions 37–49 containing approximately 92% of GSA-AT activity were subjected to DEAE-cellulose column chromatography. Fraction 24 was purified further on a FPLC column (Mono Q). Purification of recombinant wild-type GSA-AT proceeded with essentially identical activity profiles and electrophoresis patterns.
at pH 5.0 (37); this has been attributed to competition between free phosphate and the 5'-phosphate group of PMP for a cationic binding site in the enzyme. The lower increase in GSA-AT activity with added cofactors in phosphate buffer compared with Bistris (Table II) and a 90% decrease in enzyme activity during incubation in phosphate buffer for 2 h (result not shown) would be consistent with a similar effect on cofactor binding by GSA-AT.

Since this paper was submitted, two articles on this enzyme have appeared. One reports the sequence of the hemL gene in *Salmonella typhimurium*, encoding GSA-AT, and discusses evolutionary relationships of this enzyme with other aminotransferases (38). The other shows that GSA-AT from *Chlamydomonas reinhardtii* is a membrane-associated 43-kDa monomer (39).

REFERENCES


2 μM gabaculine in 5-min intervals; D presents the time course of spectral changes of 10 μM mutant GSA-AT pretreated with 120 μM GSA after addition of 25 μM gabaculine. Spectra were recorded 1, 5, 10, 23, 30 and 40 min after the addition of gabaculine.

**Fig. 4.** Effect of cofactor, substrate, and inhibitor on the absorption spectra of recombinant wild-type and mutant GSA-AT in 0.1 M Bistris buffer, pH 7.0. A shows the spectrum of 10 μM wild-type GSA-AT purified by DEAE-cellulose chromatography; B is the spectra of mutant 10 μM GSA-AT after saturation with 12.5 μM PMP (stippled line) and after addition of 120 μM GSA (solid line); C shows spectral changes of 10 μM wild-type GSA-AT pretreated with 120 μM GSA after successive additions of 1, 3, 2, and
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