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Primary structure of a key enzyme in plant tetrapyrrole synthesis: Glutamate 1-semialdehyde aminotransferase

(barley/chlorophyll/δ-aminolevulinate/cDNA/expression in *Escherichia coli*)

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ABSTRACT **The formation of δ-aminolevulinate from glutamate 1-semialdehyde (GSA) is catalyzed by glutamate 1-semialdehyde aminotransferase (EC 5.4.3.8). The active form of the barley enzyme appears to be a dimer of identical subunits with a molecular mass of 46 kDa. From the purified enzyme, amino acid sequences of the N-terminal ends of the mature protein as well as an internal peptide were determined. DNA primers deduced from these peptide sequences were used to amplify with the polymerase chain reaction a cDNA sequence encoding part of the enzyme. Screening a cDNA library with this DNA fragment identified a full-length clone encoding the 49,540-Da precursor of the GSA aminotransferase. The transit peptide for chloroplast import consists of 34 amino acids. GSA aminotransferase and a precursor form were expressed on a multicopy plasmid in *Escherichia coli*. Both recombinant gene products reacted with an antibody against the barley GSA aminotransferase. Active barley GSA aminotransferase expressed in *E. coli* was shown to be active in assays of bacterial cell extracts. As a gene symbol for barley GSA aminotransferase, *Gsa* is proposed.**

δ-Aminolevulinate acid (ALA) is the universal precursor for the biosynthesis of tetrapyrroles, which include many oxygen- and electron-carrying porphyrins such as chlorophyll and heme as well as linear molecules such as the prosthetic group of phytochrome. Two different pathways have evolved for the formation of ALA from amino acids. In mammals, yeast, and some bacteria, glycine is condensed with succinyl-CoA to form ALA in a single step catalyzed by δ-aminolevulinate synthetase (EC 2.3.1.37) (1). In higher plants, algae and—as it turned out recently—in many bacteria, ALA is synthesized from glutamate by three enzymatic reactions (2). In the first step, glutamate is esterified to $tRNA^{Glu}$ by glutamic acid–tRNA ligase, which also provides glutamyl–tRNA for protein synthesis (3–5). Glutamyl–tRNA is reduced in an NADPH-requiring reaction to glutamate 1-semialdehyde (GSA) (6–9). Finally, ALA is formed from GSA by an isomerization (10, 11), which is catalyzed by an aminotransferase, GSA aminotransferase [glutamate-1-semialdehyde 2,1-aminomutase; (*S*)-4-amino-5-oxopentanoate 4,5-aminomutase, EC 5.4.3.8]. In higher plants, ALA is synthesized in the chloroplast stroma by cooperation of the nuclear and chloroplast genome. The $tRNA^{Glu}$ used to activate the glutamic acid is encoded in chloroplast DNA, while the ligase, dehydrogenase, and GSA aminotransferase are encoded by nuclear genes and are synthesized as precursors in the cytoplasm (2).

As reported in a previous paper (12), GSA aminotransferase has been purified from greening barley seedlings and the cyanobacterium *Synechococcus* strain PCC 6301. The aminotransferase from barley probably consists of two identical subunits with a molecular mass of 46 kDa, while the subunit of the bacterial enzyme has the same molecular mass but may function as a monomer.

Very similar amino acid compositions and 69% homology of the N-terminal sequences characterize the two enzymes. An interesting difference was discovered when the cofactor requirements were compared: The monomeric cyanobacterial enzyme needs pyridoxamine phosphate for activity, while such a requirement could not be demonstrated for the dimeric barley enzyme. Concentration-dependent GSA hydrate dimerization has been observed and such dimers are likely intermediates in the chemical conversion of GSA to ALA (9). Possibly, the dimeric barley enzyme uses as substrate two head-to-tail arranged GSA molecules and effects an exchange of amino groups between them (13). This would make an amino group donor in the form of pyridoxamine phosphate superfluous.

To determine the primary structure of the barley GSA aminotransferase, a cDNA clone was obtained with synthetic oligonucleotides encoding the N-terminal and an internal peptide established by amino acid sequence analysis. In the present paper, the nucleotide sequence encoding the precursor of the barley GSA aminotransferase is presented.* The cDNA gene was expressed in *Escherichia coli* and the resulting protein was shown to catalyze the conversion of GSA into ALA. Availability of the clone makes it possible to test whether this enzyme or its gene is involved in the regulation of chlorophyll biosynthesis. Heterologous expression of the clone provides a basis to analyze the mechanism of ALA formation from GSA. As a gene symbol for GSA aminotransferase, *Gsa* is proposed.

MATERIALS AND METHODS

Bacteria and Vectors. *E. coli* strain λ 1090 [Δ*lacU169 proA*$^+$ Δ*lon araD139 strA supF*(*trpC22*::Tn*10*)] was used as pMC9 (14) host for λgt11 (15). DH5α [F$^-$ φ80d/*lacZ*ΔM15 *endA1 recA1 hsdR17*($r_K^- m_K^+$)*supE44 thi-1* λ$^-$ *gyrA96 relA1* (*lacZYA-argF*)*U169*)] (Bethesda Research Laboratories) was transformed with the different cDNA subclones in pGEM-7Zf(+) (Promega) and grown on LB medium (16) with ampicillin (100 μg/ml). M15 (ΔM15 *CI*$^+$ *kan*^{r} *lacI*^{q}) (17) containing the pDM1 plasmid or SG 13009 (F$^-$ *his pyrD* Δ *lon-100 rpsL*) (18) with the pUHA1 plasmid hosted the expression vectors pATB9 and pATE19 and were grown on medium containing ampicillin (50 μg/ml) and kanamycin (25 μg/ml). M15 and SG 13009 were obtained from D. Stüber (Hoffmann–La Roche). Standard transduction and transformation procedures for *E. coli* (16) were used throughout.

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Abbreviations: ALA, δ-aminolevulinate; GSA, glutamate 1-semialdehyde; IPTG, isopropyl β-D-thiogalactopyranoside; PCR, polymerase chain reaction.

*The sequence reported in this paper has been deposited in the GenBank data base (accession no. M31545).

Cloning Strategy. Poly(A) RNA for the cDNA synthesis was isolated from 5-day-old dark grown barley seedlings cv. Bonus after 5 hr of illumination. cDNA was prepared and inserted into the vector λgt11 (*lac5 cI857 nin5* S100) by Lisbeth Skou Hønberg (Carlsberg Laboratory, Copenhagen) according to ref. 15. A 400-μl aliquot of the cDNA library (10^9 plaque-forming units per μl) was extracted with phenol and the DNA was used for the polymerase chain reaction (PCR). Oligonucleotides derived from sequences of two peptides from the GSA aminotransferase (12) were used as primers (see Fig. 1). They comprised 768 and 384 different combinations of oligonucleotides. The sense primer was synthesized with an added *Hin*dIII linker at the 5′ end and the antisense primer was synthesized with an *Eco*RI linker. Twenty-five cycles of PCR were carried out with 1 unit of *Thermus aquaticus* DNA polymerase (*Taq* polymerase) with a temperature program of 1 min of denaturation at 95°C, 2 min of annealing at 45°C, and 3 min of chain extension at 72°C (19) (Perkin–Elmer/Cetus). An aliquot of the amplified cDNA was again subjected to 25 cycles of amplification but with an annealing temperature of 55°C. The PCR product was separated on a 1% agarose gel, eluted, digested with *Eco*RI and *Hin*dIII, and subcloned into the *Eco*RI/*Hin*dIII sites of pGEM-7Zf(+). It was subsequently discovered that the DNA sequence spanning from the sense to the antisense primer contained a *Hin*dIII restriction site whereby the desired PCR product was cut into a 380-base-pair (bp) *Hin*dIII/*Hin*dIII fragment and a 64-bp *Hin*dIII/*Eco*RI fragment. Two plasmids were selected by nucleotide sequencing for further work, one containing the 64-bp *Hin*dIII/*Eco*RI fragment and the other with an insert of the 380-bp *Hin*dIII/*Hin*dIII fragment ligated with an unrelated *Hin*dIII/*Eco*RI sequence. The two relevant DNA inserts were ^{32}P-labeled by nick-translation and used to screen the λgt11 library by *in situ* plaque hybridization (20). cDNA inserts were subcloned into the *Eco*RI site of pGEM-7Zf(+).

Other DNA and RNA Methods. Small and large scale preparation of plasmid and phage DNA were carried out as recommended (16). Poly(A) RNA for Northern blots was isolated according to ref. 21. RNA was separated on a 1.2% formaldehyde/agarose gel and blotted onto a nitrocellulose filter (Schleicher & Schuell). Nucleic acid bound to nitrocellulose filters was prehybridized in 6× SSC (1× SSC = 0.15 M NaCl/0.015 M sodium citrate)/0.02% Ficoll/0.02% polyvinylpyrrolidone/0.1% SDS/50 μg of salmon sperm DNA per ml at 65°C for 1 hr. Hybridization was performed with nick-translated DNA in 3× SSC/0.02% Ficoll/0.02% polyvinylpyrrolidone at 65°C overnight. Nucleotide sequencing was carried out by the dideoxynucleotide chain-termination method (22) with a Sequenase kit (United States Biochemical).

Construction and Performance of the Expression Vectors. The cDNA encoding the GSA aminotransferase was fused to the initiation codon and ribosome binding site of the expression vectors pDS56/RBSII, -1 or pDS56/RBSII, *Sph* I (23). Toward this end, the cDNA insert was cut at the 3′ end with *Eco*RI and shortened at the 5′ end with the restriction endonuclease *Bgl* I, which removes 40 bases, and with *Ecl*XI, which removes 119 bases from the clone. The vector was cleaved with *Bam*HI, the cDNA and vector overhangs were filled with T4 polymerase, and the ends were treated with calf intestinal phosphatase. After ligation, the plasmids were transformed into *E. coli*.

Plasmids with the desired orientation of the cDNA inserts were identified by restriction fragment analysis of minipreparations and subsequently tested for expression of the GSA aminotransferase.

Expression of the barley GSA aminotransferase and its precursor in *E. coli* strain SG 13009 was analyzed immunologically with an antibody against the barley enzyme (12), which does not cross-react with the enzyme from *E. coli*. The cells were grown overnight at 37°C. Fresh medium was inoculated with 1/400th vol of the culture. After reaching a cell density of 0.6 at A_{600}, the cells were induced with isopropyl β-D-thiogalactopyranoside (IPTG) at a final concentration of 1 mM and incubation was continued for up to 12 hr. For analysis of the total cell content, the cells of 1-ml aliquots were sedimented and resuspended in gel loading buffer (56 mM dithioerythritol/56 mM Na_2CO_3/2% SDS/12% sucrose/0.04% bromophenol blue), subjected to SDS/PAGE, and stained with Coomassie blue. To separate soluble from insoluble proteins of the *E. coli* cell, 500-ml cultures were harvested and the cells were washed once in buffer (0.1 M Tricine/NaOH, pH 7.9/0.3 M glycerol/25 mM $MgCl_2$/1 mM dithiothreitol). After resuspension in 2 ml of the same buffer and disruption of the cells by sonication three times for 30 sec, pellets and supernatants were obtained by centrifugation at 13,000 × *g* for 15 min. The two fractions were analyzed after SDS/PAGE by staining with Coomassie blue and Western blotting as described (12).

Since no enzymatic activity or GSA aminotransferase protein was detectable in the supernatant and activity in the pellet was very limited, the following alternative procedure for detecting barley enzyme activity was used. SG 13009 cells containing the pATE19 plasmid were grown in 1-liter cultures for 12 hr with or without IPTG, lysed with lysozyme (0.8 mg per g wet weight), and treated with sodium deoxycholate (4 mg per g wet weight) and DNase (24). The lysate was centrifuged at 12,000 × *g* for 15 min and the supernatant was desalted by passing through a Sephadex G50 column. The pellet was washed twice in 0.5% (vol/vol) Triton X-100 and both fractions were analyzed for GSA aminotransferase activity in 1-ml aliquots as described (8). An assay without cell extract was performed as reference and nonenzymatically formed ALA was subtracted. Absorption spectra were taken from 450 nm to 650 nm of the pyrrole formed with ethyl acetoacetate. The amounts of ALA were determined by using the molar extinction coefficient at 553 nm as 7.2×10^4 for a light path of 1 cm (25), characteristic for pyrrole treated with Ehrlich's reagent.

RESULTS

Isolation of a cDNA Clone Encoding GSA Aminotransferase. GSA aminotransferase is located in the stroma of higher plant chloroplasts and is purified in the active form by serial affinity

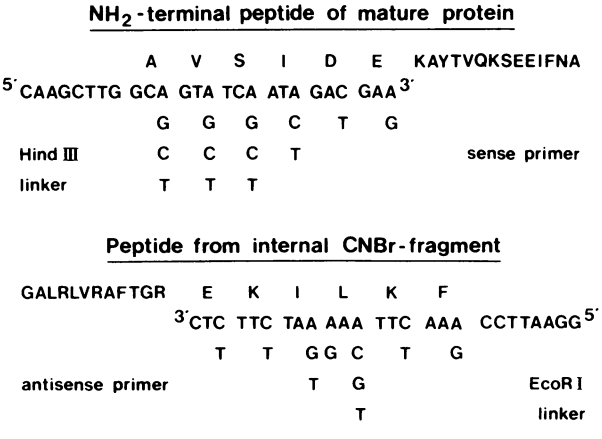
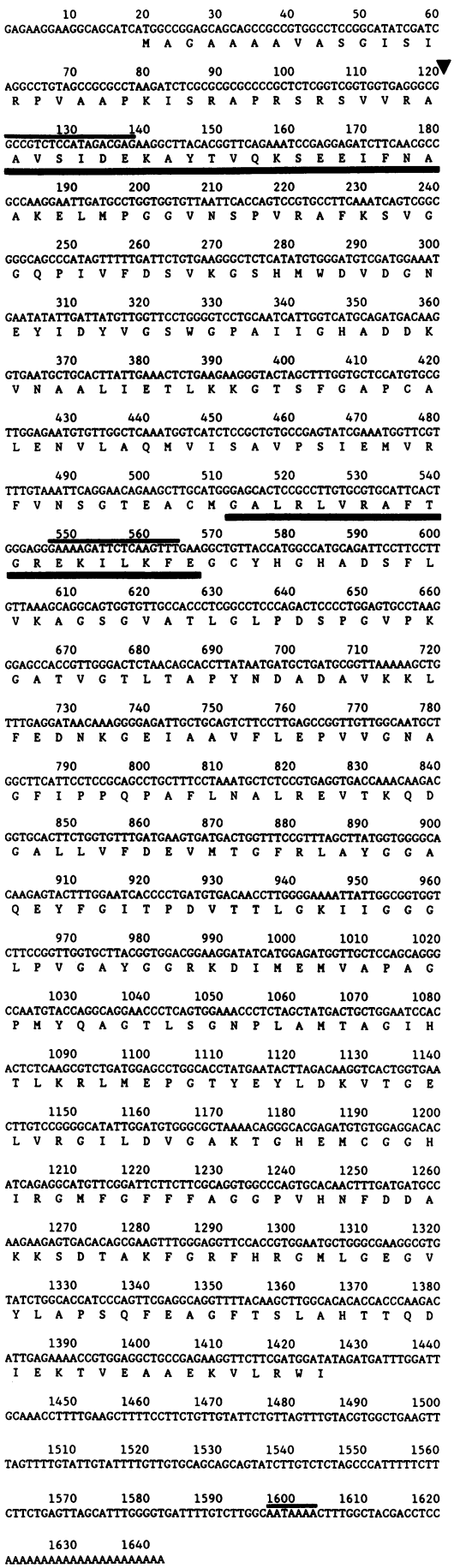


FIG. 1. Amino acid sequences (single-letter code) of the N-terminal peptide of the mature GSA aminotransferase and an internal peptide obtained by cyanogen bromide cleavage. Mixed oligonucleotides with 768-fold redundancy encoding the six N-terminal amino acids were synthesized and used as a sense primer in the PCR. For the antisense primer, mixed oligonucleotides with 384-fold redundancy encoding six amino acids of a 17-kDa internal peptide were synthesized. A *Hin*dIII linker was added to the sense primer and an *Eco*RI linker was added to the antisense primer.



chromatography and preparative nondenaturing gel electrophoresis (12). By electroblotting onto polyvinylidine difluoride membranes and microsequencing, the N-terminal amino acid sequence given in Fig. 1 was obtained. Cyanogen bromide cleavage yielded the largest internal fragment with a molecular mass of ≈17 kDa, which after blotting gave a sequence of 19 amino acid residues (Fig. 1). Appropriate mixtures of oligonucleotide sense and antisense primers corresponding to 6 amino acids in the sequenced peptides were synthesized as shown in Fig. 1 and used to amplify from a barley leaf cDNA library the appropriate 444-bp fragment. As described above, the PCR product happened to contain a *Hin*dIII restriction site and therefore had to be subcloned into two different plasmids. Both inserts detected a 1.6-kilobase cDNA clone in the λgt11 library.

The Primary Structure of the GSA Aminotransferase. The nucleotide sequence of the clone comprising 1642 bp is presented together with the deduced amino acid sequence in Fig. 2. A long open reading frame encoding 469 amino acids is present. The molecular mass of this protein calculates to 49,501 Da. Since the N-terminal amino acid of the mature enzyme is the alanine in codon position 121, a 34-residue-long transit peptide for chloroplast import can be deduced. The mature enzyme has a molecular mass of 46,172 Da, which agrees closely with the molecular mass of 46 kDa determined by SDS/PAGE.

A data bank search has so far revealed one striking similarity of the determined sequence: the 83 C-terminal amino acids have 48% identity and 64% similarity to the C-terminal part of a protein encoded by the DNA sequence adjacent to and diverging from the *fhuB* gene of *E. coli* (26). Genetic mapping has identified the *popC* gene in this region of the *E. coli* chromosome (27). Mutations in this gene lead to auxotrophy for ALA. Since *E. coli* synthesizes the porphyrin precursor ALA from glutamate (28), the *popC* gene product is a good candidate for the GSA aminotransferase.

A hydropathy plot of the sequence reveals the characteristics of a hydrophilic protein with a few hydrophobic domains. The 34-amino acid transit peptide for import of the GSA aminotransferase precursor into etioplasts and developing chloroplasts shares with other nuclear-encoded plastid proteins a high number of nonpolar and positively charged amino acid residues. Preceding the AUG start codon in the mRNA is the sequence GCAUCAUGG, which is similar to the eukaryotic consensus initiation site sequence CC (A/G) CCAUGG (29). In the 3′ 191-bp-long noncoding region of the mRNA is a polyadenylylation signal at position 1597, which is 23 bp upstream of the poly(A) site.

Expression of the Barley GSA Aminotransferase in *E. coli*. To obtain larger amounts of purified GSA aminotransferase for functional studies of the enzyme, the cDNA sequence encoding the mature protein was inserted into the cloning site (*Bam*HI) of the pDS56/RBSII, *Sph* I vector (Fig. 3). The coding sequence was obtained by cutting the recombinant pGEM-7Zf(+) plasmid at the 3′ end of the insert with *Eco*RI and at the 5′ end with *Ecl*XI, which cleaves one nucleotide in front of the transit peptide cleavage site. The cloning site is located downstream of the coliphage T5 promoter P_{N25} fused to the *lac* operator and a ribosome binding site. Insertion into this cloning site provides the open reading frame for the mature GSA aminotransferase with a methionine start codon and three additional codons (Fig. 3). In an analogous way, a

FIG. 2. Nucleotide and deduced amino acid sequence (single-letter code) of the cDNA encoding GSA aminotransferase of barley. Underlined amino acid sequences were determined by peptide sequencing. Lines above the nucleotide sequence delineate the primers used in the PCR. The polyadenylylation signal is marked at nucleotide position 1597. Arrowhead indicates the transit peptide cleavage site.

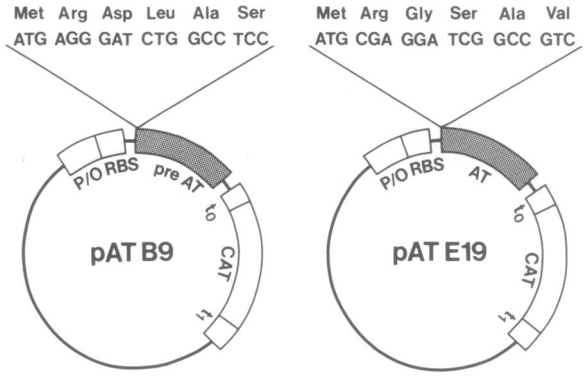


FIG. 3. Expression plasmids pATB9 containing the coding region for pre-GSA aminotransferase (pre AT) and pATE19 containing the coding sequence for mature GSA aminotransferase (AT). P/O, T5 promoter and *lac* operator (21); RBS, ribosome binding site; t_0, t_1, terminator; CAT, chloramphenicol acetyltransferase gene; above the vectors are shown the transitions between the plasmid and the cDNA sequences. In pATB9, the cDNA sequence starts with the codon for the ninth amino acid of the transit peptide. In pATE19, the cDNA sequence begins with the codon for the N-terminal alanine of the mature protein.

DNA sequence encoding the precursor protein with a deletion of the nine N-terminal transit peptide residues was inserted into the polylinker of plasmid pDS56/RBSII, -1.

Transformants of *E. coli* SG 13009 harboring one or the other plasmid were grown for 2, 3, and 6 hr in medium containing 1 mM IPTG as inducer. Increasing amounts of the mature (45 kDa) and precursor (50 kDa) GSA aminotransferase were identified (Fig. 4 *Right*).

In this figure is also presented an experiment in which an induced 12-hr culture was compared to an uninduced one. Upon harvest and lysis of the cells by sonication, the sedimentable internal proteins (pellets) were separated from the soluble ones. As shown in Fig. 4 (*Center*), the recombinant gene products are present in high amounts in pellets of IPTG-induced cells. This is confirmed in Western blots with an antibody against barley GSA aminotransferase (*Left*). Minor bands of smaller polypeptides identified by the antibody are considered to be degradation fragments. There is a thin band from the pellets of uninduced cells reacting with the aminotransferase antibody. Since the latter does not recognize the *E. coli* enzyme, it signifies a basic level of transcription of the gene from the vector.

Concentrated cell extracts of IPTG-induced and uninduced cultures of *E. coli* with the recombinant plasmid pATE19 were tested for GSA aminotransferase activity. The reaction mixture contained between 1 and 15 mg of protein, 500 μM GSA, and 10 mM levulinate in 0.1 M Tricine, pH 7.9/0.3 M glycerol/25 mM $MgCl_2$/1 mM dithiothreitol. Routinely, ALA was purified by ion-exchange chromatography (Dowex 50WX8) before it was condensed with ethyl acetoacetate. The amount of ALA was determined colorimetrically with Ehrlich's reagent. The rate of spontaneous conversion of GSA to ALA served as reference. GSA aminotransferase assays of sonicated total extracts from IPTG-induced M15 cells containing the pATE19 plasmid yielded 1.3 nmol of ALA per mg of protein per hr. The uninduced cell extract synthesized 0.11 nmol of ALA per mg of protein per hr. Assays for GSA aminotransferase of soluble extracts from IPTG-induced SG 13009 cells with the pATE19 plasmid synthesized 7.3 nmol of ALA per mg of protein per hr, which compares to a yield of 0.18 nmol of ALA per mg of protein per hr in uninduced cells. Fig. 5 shows the characteristic maximal absorption at 553 nm in the spectra of the ALA pyrroles treated with Ehrlich's reagent from the latter experiment. It is concluded that active barley GSA aminotransferase is synthesized by the *E. coli* cells containing the plasmid pATE19.

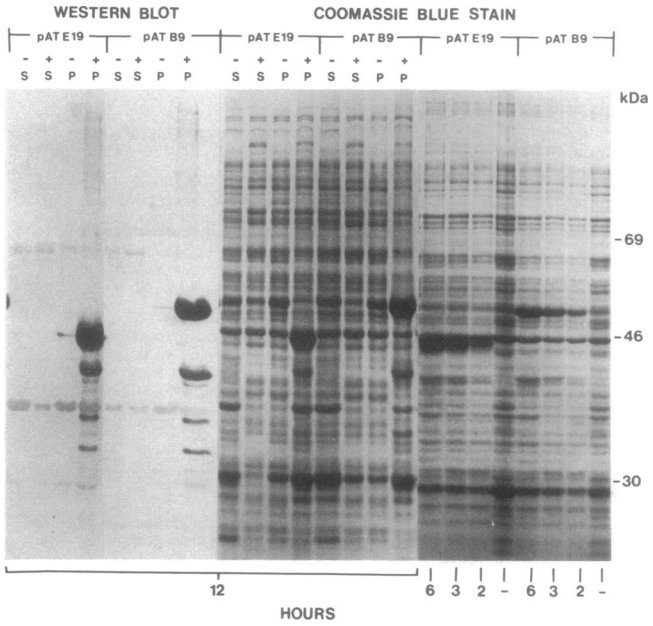


FIG. 4. Polypeptide patterns of extracts from *E. coli* cells transformed with plasmids pATB9 and pATE19 containing the cDNA genes encoding pre-GSA aminotransferase and GSA aminotransferase, respectively. (*Right*) Transformants were grown with the inducer IPTG for 0, 2, 3, and 6 hr. Increasing amounts of a band with a molecular mass of 50 kDa is seen in the cells containing the pATB9 plasmid and an increasingly prominent band at 45 kDa is seen in cells with the pATE19 plasmid. (*Center*) Pellets (P) and supernatants (S) of cells induced (+) for 12 hr are compared with uninduced controls (−). Recombinant gene products are present in extracts from pellets of induced cells. An immunoblot of the central panel with an antibody to the barley enzyme identifies the precursor and mature form of barley GSA aminotransferase as predominant bands.

Very little GSA aminotransferase activity is found in the pellets of the IPTG-induced SG 13009 cells in spite of the presence of large amounts of immunologically detectable GSA aminotransferase protein. The inactive protein is located in inclusion bodies, which could be solubilized by urea, SDS, or NaOH (pH 11.0) but not activated by dialysis.

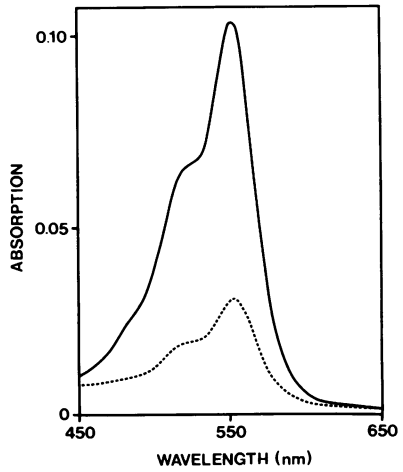


FIG. 5. Absorption spectra of ALA-derived pyrrole reacted with Ehrlich's reagent. ALA was synthesized from GSA with extracts from transformed *E. coli* cells induced with IPTG (solid line) and uninduced transformants (dotted line). The cells had been transformed with pATE19.

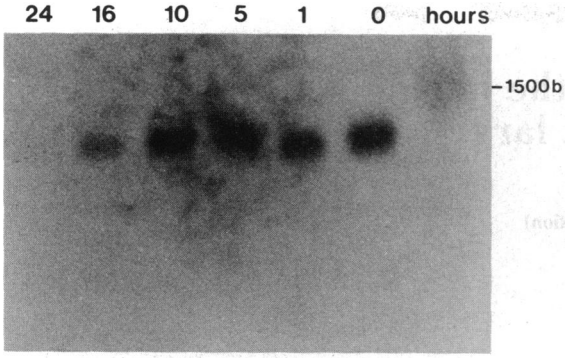


FIG. 6. Poly(A) RNA was isolated from 5-day-old dark grown barley seedlings illuminated from 0 to 24 hr and probed in a Northern blot with the PCR-generated DNA fragment of the GSA aminotransferase cDNA. b, Bases.

Amounts of Steady-State GSA Aminotransferase mRNA During Greening. A poly(A) RNA species of 1800 bp hybridized to the fragments cloned from the PCR product (Fig. 6). The amount of mRNA detected in leaves grown in darkness and in seedlings greened for 10 hr in the light is about the same. Thereafter, the mRNA decreases significantly and at 24 hr only traces are detectable.

DISCUSSION

GSA aminotransferase is functionally an aminomutase, which transfers the amino group on carbon 2 of glutamate semialdehyde to the neighboring carbon atom (i.e., carbon 5 of ALA). This apparent intramolecular amino group transfer in the same carbon skeleton differs from that of other aminotransferases in which an amino acid donates an amino group to a keto acid. It is therefore not surprising that no obvious primary structure similarities were found between GSA aminotransferase from barley and other aminotransferases. On the other hand, many aminotransferases have a molecular size resembling that of the barley enzyme. Examples are the cytosolic pig aspartate aminotransferase with 46.3 kDa (30), *E. coli* aspartate aminotransferase with 43 kDa, *E. coli* aromatic aminotransferase with 43 kDa (31), rat serine-pyruvate aminotransferase with 43.2 kDa (32), *E. coli* 7,8-diaminopelargonic acid aminotransferase with 47.4 kDa (33), human ornithine aminotransferase with 45 kDa (34), and rat tyrosine aminotransferase with 47 kDa (35). A molecular mass of 80 kDa was determined for GSA aminotransferase by gel filtration of barley stroma proteins (36), which indicates a dimer of two 46-kDa subunits for the active enzyme. Thus, the barley enzyme is like many aminotransferases in that it seems to function as a dimer of identical subunits (37, 38). Its mechanism of function can be investigated when overexpression of active enzyme has been achieved.

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1. Gibson, K. D., Laver, W. G. & Neuberger, A. (1958) *Biochem. J.* **70,** 71–81.
2. Kannangara, C. G., Gough, S. P., Bruyant, P., Hoober, J. K., Kahn, A. & von Wettstein, D. (1988) *Trends Biochem. Sci.* **13,** 139–143.
3. Kannangara, C. G., Gough, S. P., Oliver, R. P. & Rasmussen, S. K. (1984) *Carlsberg Res. Commun.* **43,** 417–437.
4. Schön, A., Krupp, G., Gough, S., Berry-Lowe, S., Kannangara, C. G. & Söll, D. (1986) *Nature (London)* **322,** 281–284.
5. Bruyant, P. & Kannangara, C. G. (1987) *Carlsberg Res. Commun.* **50,** 99–109.
6. Houen, G., Gough, S. P. & Kannangara, C. G. (1983) *Carlsberg Res. Commun.* **48,** 567–572.
7. Kannangara, C. G. & Schouboe, A. (1985) *Carlsberg Res. Commun.* **50,** 179–191.
8. Hoober, K., Kahn, A., Ash, D. E., Gough, S. & Kannangara, C. G. (1988) *Carlsberg Res. Commun.* **53,** 11–25.
9. Gough, S. P., Kannangara, C. G. & Bock, K. (1989) *Carlsberg Res. Commun.* **54,** 99–108.
10. Kannangara, C. G. & Gough, S. P. (1978) *Carlsberg Res. Commun.* **43,** 185–194.
11. Wang, W.-Y., Gough, S. P. & Kannangara, C. G. (1981) *Carlsberg Res. Commun.* **46,** 243–257.
12. Grimm, B., Bull, A., Welinder, K. G., Gough, S. P. & Kannangara, C. G. (1989) *Carlsberg Res. Commun.* **54,** 67–79.
13. Mau, Y.-H. L. & Wang, W. Y. (1988) *Plant Physiol.* **86,** 793–797.
14. Young, R. A. & Davis, R. W. (1983) *Science* **222,** 778–782.
15. Huynh, T. V., Young, R. A. & Davis, R. W. (1985) in *DNA Cloning*, ed. Glover, D. M. (IRL, Oxford), Vol. 1, pp. 49–76.
16. Maniatis, T., Fritsch, E. F. & Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab., Cold Spring Harbor, NY).
17. Villarejo, M. R. & Zabin, I. (1974) *J. Bacteriol.* **120,** 466–474.
18. Gottesman, S., Halpern, E. & Trisler, P. (1981) *J. Bacteriol.* **148,** 265–273.
19. Saiki, R. K., Gelfand, D. H., Stoffel, S., Scharf, S. J., Higuchi, R., Horn, G. T., Mullis, K. B. & Erlich, H. A. (1988) *Science* **239,** 487–491.
20. Benton, W. D. & Davis, R. W. (1977) *Science* **196,** 180–182.
21. Apel, K. & Kloppstech, K. (1978) *Eur. J. Biochem.* **85,** 581–588.
22. Sanger, F., Nicklen, S. & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. USA* **74,** 5463–5467.
23. Bujard, H., Genz, R., Lanzer, M., Stueber, D., Mueller, M., Ibrahimi, I., Haeuptle, M. T. & Dobberstein, B. (1987) in *Methods of Enzymology*, ed. Ray, W. (Academic, New York), Vol. 155, pp. 416–432.
24. Marston, F. A. O. (1987) in *DNA Cloning*, ed. Glover, D. M. (IRL, Oxford), Vol. III, pp. 59–88.
25. Mauzerall, D. & Granick, S. (1956) *J. Biol. Chem.* **219,** 435–446.
26. Köster, W. & Braun, V. (1986) *Mol. Gen. Genet.* **204,** 435–442.
27. Powell, K. A., Cox, R., McConville, M. & Charles, H. P. (1973) *Enzyme* **16,** 65–73.
28. Li, J.-M., Brathwaite, O., Cosloy, S. D. & Russel, C. S. (1989) *J. Bacteriol.* **171,** 2547–2552.
29. Kosak, M. (1984) *Nucleic Acids Res.* **12,** 857–872.
30. Ovchinnikov, Y. A., Egorov, C. A., Aldanova, N. A., Feigina, M. Y., Lipkin, V. M., Abdulaev, N. G., Grishin, E. V., Kiselev, A. P., Modyanov, N. N., Braunstein, A. E., Polyanovsky, O. L. & Nosikov, V. V. (1973) *FEBS Lett.* **29,** 31–34.
31. Fotheringham, I. G., Dacey, S. A., Taylor, P. P., Smith, T. J., Hunter, M. G., Finlay, M. E., Primrose, S. B., Parker, D. M. & Edwards, R. M. (1986) *Biochem. J.* **234,** 593–604.
32. Oda, T., Miyajima, H., Suzuki, Y. & Ichiyama, A. (1987) *Eur. J. Biochem.* **168,** 537–542.
33. Otsuka, A. J., Buoncristiani, M. R., Howard, P. K., Flamm, J., Johnson, C., Yamamoto, R., Uchida, K., Cook, C., Ruppert, J. & Matsuzaki, J. (1988) *J. Biol. Chem.* **263,** 19577–19585.
34. Muekler, M. M. & Pitot, H. C. (1985) *J. Biol. Chem.* **260,** 12993–12997.
35. Grange, T., Guenet, C., Dietrich, J. B., Chasserot, S., Fromont, M., Befort, N., Jami, J., Beck, G. & Piotet, T. (1983) *J. Mol. Biol.* **184,** 347–350.
36. Kannangara, C. G., Gough, S. P. & Girnth, C. (1981) in *Photosynthesis V*, ed. Akoyunoglou, G. (Balaban Int. Sci. Ser., Philadelphia), pp. 117–127.
37. Sonderegger, P., Jaussi, R., Christen, P. & Gehring, H. (1982) *J. Biol. Chem.* **257,** 3339–3345.
38. Kim, Y. T. & Churchich, J. E. (1989) *Eur. J. Biochem.* **181,** 397–401.