

## The *Escherichia coli* *hemL* Gene Encodes Glutamate 1-Semialdehyde Aminotransferase

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Received 13 February 1991/Accepted 1 April 1991

$\delta$ -Aminolevulinic acid (ALA), the first committed precursor of porphyrin biosynthesis, is formed in *Escherichia coli* by the C<sub>5</sub> pathway in a three-step, tRNA-dependent transformation from glutamate. The first two enzymes of this pathway, glutamyl-tRNA synthetase and Glu-tRNA reductase, are known in *E. coli* (J. Lapointe and D. Söll, J. Biol. Chem. 247:4966–4974, 1972; D. Jahn, U. Michelsen, and D. Söll, J. Biol. Chem. 266:2542–2548, 1991). Here we present the mapping and cloning of the gene for the third enzyme, glutamate 1-semialdehyde (GSA) aminotransferase, and an initial characterization of the purified enzyme. Ethylmethane sulfonate-induced mutants of *E. coli* AB354 which required ALA for growth were isolated by selection for respiration-defective strains resistant to the aminoglycoside antibiotic kanamycin. Two mutations were mapped to min 4 at a locus named *hemL*. Map positions and resulting phenotypes suggest that *hemL* may be identical with the earlier described porphyrin biosynthesis mutation *popC*. Complementation of the auxotrophic phenotype by wild-type DNA from the corresponding clone pLC4-43 of the Clarke-Carbon bank (L. Clarke and J. Carbon, Cell 9:91–99, 1976) allowed the isolation of the gene. Physical mapping showed that *hemL* mapped clockwise next to *fhuB*. The *hemL* gene product was overexpressed and purified to apparent homogeneity. The pure protein efficiently converted GSA to ALA. The reaction was stimulated by the addition of pyridoxal 5'-phosphate or pyridoxamine 5'-phosphate and inhibited by gabaculine or aminooxyacetic acid. The molecular mass of the purified GSA aminotransferase under denaturing conditions was 40,000 Da, as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The enzyme has an apparent native molecular mass of approximately 80,000 Da, as determined by rate zonal sedimentation on glycerol gradients and molecular sieving through Superose 12, which indicates a homodimeric  $\alpha_2$  structure of the protein.

In the chloroplasts of plants and algae and a variety of photosynthetic and nonphotosynthetic bacteria, including *Escherichia coli*,  $\delta$ -aminolevulinic acid (ALA), the first committed precursor of porphyrin biosynthesis, is formed in a three-step reaction utilizing the five-carbon skeleton of glutamate (for recent reviews, see references 4 and 30). This C<sub>5</sub> pathway is summarized in Fig. 1. In the first step, which is also a crucial reaction in protein biosynthesis, glutamate is activated by glutamyl-tRNA synthetase to form Glu-tRNA (25). The aminoacyl-tRNA is the substrate of an NADPH-dependent reductase (Glu-tRNA reductase) which must recognize tRNA in a sequence-specific manner. The activated (by tRNA) carboxyl group of glutamate is then reduced to glutamate 1-semialdehyde (GSA) or its cyclic form 2-hydroxy-3-aminotetrahydropyran-1-one (21). Finally, GSA is the substrate for GSA aminotransferase, which transaminates GSA to ALA. The details of the reaction mechanisms of Glu-tRNA reductase and GSA aminotransferase are still subjects of intensive research.

Of the three enzymes, the most is known about glutamyl-tRNA synthetase, which has been purified from many organisms (for a recent review, see reference 30). Much less is known about Glu-tRNA reductase which has been purified and functionally characterized from *E. coli* and *Chlamydomonas reinhardtii* chloroplasts (6, 19). For several prokaryotic organisms, genes complementing the ALA-auxotrophic *E. coli* *hemA* mutant are believed to encode a structural component of that enzyme (1, 2). The third enzyme of the pathway, GSA aminotransferase, has been purified and

characterized from barley, cyanobacteria, and green algae (14, 18). The gene encoding the barley enzyme was characterized recently; the deduced amino acid sequence suggested a conserved peptide possibly involved in pyridoxal 5'-phosphate (PLP) binding (13). Complementation of the ALA-auxotrophic *hemL* mutation in *Salmonella typhimurium* yielded a gene with a DNA sequence homologous to the barley GSA aminotransferase clone, and physiological examination of bacteria transformed with the cloned gene provided evidence for functional identity (8). In *E. coli* the *popC* mutation is thought to be related to *hemL*, on the basis of their similar map positions and resulting phenotypes (9, 32). However, as the *popC* mutation is lost (28a), no direct evidence has been provided.

In order to obtain amounts of GSA aminotransferase sufficient to investigate its enzymatic mechanism and also to settle the question of the *popC* mutation in *E. coli*, we cloned the GSA aminotransferase gene by complementation of an *E. coli* strain auxotrophic for ALA. Overexpression of the gene allowed an easy purification of the enzyme, which has the properties of an aminotransferase dependent on PLP or pyridoxamine 5'-phosphate (PAP).

### MATERIALS AND METHODS

**Bacterial strains and plasmids.** The starting strain for mutagenesis studies was *E. coli* AB354 (HfrP012 *panD2 thr-1 leuB6 lacZ4 rpsL8 supE44*). *E. coli* DH5 $\alpha$  was used for the initial transformation and propagation of plasmids constructed and used in this study. *E. coli* BL21(DE3) carrying the T7 RNA polymerase gene behind an isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG)-inducible promoter (35) was

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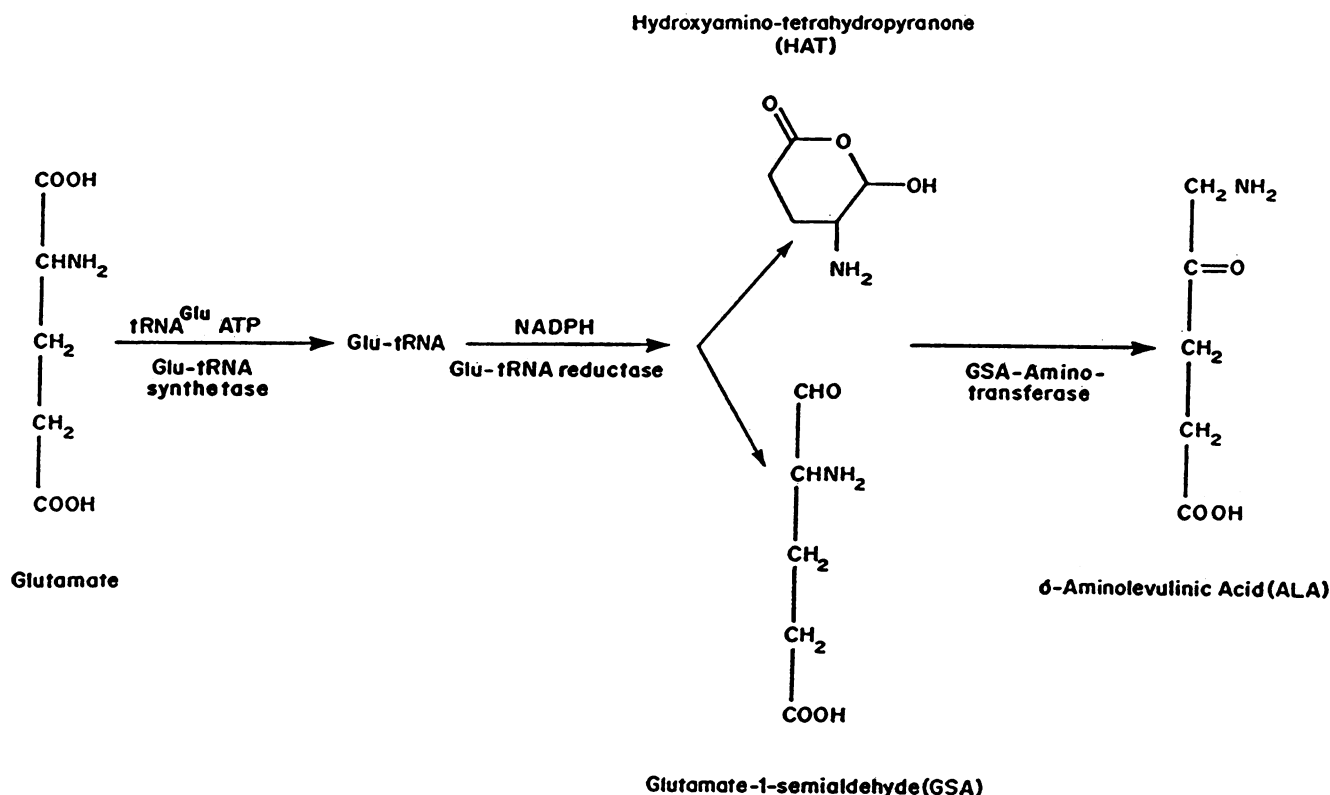


FIG. 1.  $C_5$  pathway of ALA formation. Although the identities of the enzymes and tRNA involved in ALA formation have been established, the precise mechanisms of the Glu-tRNA reductase and GSA aminotransferase reactions are unknown, and the chemical nature of the GSA intermediate is still a subject of discussion. Two of the proposed structures for the intermediate, the linear form (16) and a cyclic one (21), are shown.

used in protein overexpression of *hemL* as described below. *E. coli* JA200 contained plasmid pLC4-43 of the Clarke-Carbon gene library (7).

Plasmids pBluescript KS<sup>+</sup> and SK<sup>+</sup> (Stratagene Inc.) were used for the initial subcloning of the *hemL* gene, while plasmid pET3 (35) served as the vector for overexpression of the HemL protein. Plasmid pPC1 contains a *Bam*HI-*Pst*I fragment of the pLC4-43 insert cloned into the appropriate sites of pBluescript KS<sup>+</sup>. The overexpression vector pLI-popC contains the *Pst*I-*Dde*I fragment from the pPC1 insert cloned into the *Bam*HI site of pET3; the DNA fragments were blunt ended by treatment with Klenow polymerase before ligation.

**Recombinant DNA procedures.** Procedures for the construction and handling of recombinant DNAs were essentially as described by Maniatis et al. (26). DNA fragments were purified from agarose gel slices, using a GeneClean kit (Bio 101, Inc.). Transformation of *E. coli* cells was done by the method of Hanahan (15).

**GSA aminotransferase assay.** GSA aminotransferase activity was measured with GSA as the substrate as described previously (18). GSA synthesized by the method of Gough et al. (12) was kindly provided by C. G. Kannangara.

**High-pressure liquid chromatographic (HPLC) analysis of GSA→ALA conversion.** Enzyme assays were performed as described earlier (18). GSA (0.7  $\mu$ mol) were incubated for 30 min at 30°C in the presence of GSA aminotransferase (MonoQ fraction; 15  $\mu$ g of protein). The reaction products were isolated by Dowex chromatography, and the eluate (0.1 ml) was mixed with the same volume of buffer C (50 mM

NaH<sub>2</sub>PO<sub>4</sub>, 5 mM sodium dodecyl sulfate [SDS], 1% *tert*-amyl alcohol, adjusted to pH 3.0 with phosphoric acid and degassed). A Bondapak C18 reversed-phase column was preequilibrated overnight with buffer C. The sample (0.2 ml) was injected and separated at a flow rate of 2 ml/min at room temperature (28). The column effluent was monitored by determining  $A_{210}$ . Elution positions of the nonradioactive Glu, GSA, and ALA were determined in standard runs with pure material.

**Overexpression and purification of GSA aminotransferase.** *E. coli* BL21(DE3) transformed with plasmid pLIpopC was inoculated overnight with vigorous shaking at 37°C in 20 ml of M9ZB-Amp medium (casein hydrolysate N-Z-amine A [10 g/liter], NaCl [5 g/liter], NH<sub>4</sub>Cl [1 g/liter], KH<sub>2</sub>PO<sub>4</sub> [3 g/liter], (Na<sub>2</sub>HPO<sub>4</sub> [6 g/liter], glucose [4 g/liter], MgSO<sub>4</sub> [1 mM], ampicillin [0.2 g/liter]). A portion (10 ml) of the overnight culture was transferred to 1 liter of fresh M9ZB-Amp medium, and incubation was continued until a cell density of  $A_{600} \approx 0.8$  was reached. More ampicillin (0.2 g/liter) and IPTG (final concentration, 0.4 mM) were added, and the incubation was continued for another 3 h. The cells were harvested, washed with a solution consisting of 20 mM Tris (pH 7.5), 3 mM dithiothreitol (DTT), and 1 mM EDTA and resuspended in 10 ml of buffer A [100 mM 2-(*N*-morpholino)ethanesulfonic acid (MES)-phosphate (pH 6.8), 3 mM DTT, 0.2 mM phenylmethylsulfonyl fluoride (PMSF), 3% glycerol] including 1 mM EDTA. The suspension was cycled through three rounds of freezing and thawing before the cells were finally disrupted by sonication (5 bursts of 20 s at 35% of maximum strength with a Fisher sonicator model

300). The extract was centrifuged for 1.5 h at 4°C at 100,000 × *g*. The supernatant (S-100) was taken and could be stored for months at -80°C without any detectable loss of activity.

The S-100 fraction was dialyzed against buffer B (20 mM *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid [HEPES] [pH 7.9], 10 mM KCl, 3 mM DTT, 0.2 mM PMSF, 3% glycerol) and loaded with a flow rate of 1 ml/min and a concentration of 10 mg/ml of column volume onto a MonoQ 5/5 (1 ml) equilibrated with buffer B. After the column was washed extensively with buffer B, proteins were eluted with a linear gradient (20 ml) from 10 mM to 2 M KCl in buffer B. Most of the cellular proteins were eluted from the ion exchanger before GSA aminotransferase activity was recovered as a single peak around 650 mM KCl. All nucleic acids were retained on the column (nucleic acids require a higher ionic strength for elution) and therefore separated from the enzyme. The eluted enzyme fraction (MonoQ fraction) contained almost pure GSA aminotransferase when analyzed by SDS-polyacrylamide gel electrophoresis.

**SDS-polyacrylamide gel electrophoresis.** Protein samples were separated by electrophoresis on 10% polyacrylamide gels containing SDS as described by Laemmli (24). After electrophoresis, the proteins were visualized by Coomassie blue staining.

**Gel filtration and glycerol gradient centrifugation.** A complete description of the determination of the native molecular weight, the sedimentation coefficient, and the Stokes radius by molecular sieving and rate zonal sedimentation is given by Chen et al. (6). In these experiments, buffer A was used.

## RESULTS AND DISCUSSION

**Isolation and characterization of *hemL* mutants.** Mutants requiring ALA for growth (Ala<sup>-</sup>) were obtained in strain AB354. A total of 64 Ala<sup>-</sup> mutants were obtained. In addition, one mutant which requires both ALA and cysteine was found. With one exception, the Ala<sup>-</sup> mutants grew well on L agar supplemented with ALA and therefore do not belong to the class of ALA-requiring *hemB* mutants recently described (31). All Ala<sup>-</sup> mutants had a leaky phenotype. Preliminary mapping was done by conjugation experiments in which Ala<sup>-</sup> mutants were used as donors and an F<sup>-</sup> *hemA204* strain (GE1358) was the recipient. Of 13 mutants tested, 10 clearly did not have mutations in the *hemA* gene. Two of these mutant strains, GE1376 and GE1377, were selected for further mapping. P1 transduction experiments with both of these strains showed about 40% cotransduction between the mutated gene and *panD*. In analogy to a similarly characterized *hem* gene in *S. typhimurium* (9), we named the mutant gene *hemL* and the mutations in GE1376 and GE1377 *hemL206* and *hemL207*, respectively. On the basis of the similar map positions and resulting phenotypes, we surmised that the *popC* locus of *E. coli* (32) is the same as our *hemL* gene and is related to *S. typhimurium hemL* gene (9), which encodes GSA aminotransferase (8). In our previous search for Ala<sup>-</sup> mutants (31) using the same strategy, no *hemL* mutants were found whereas *hemA* mutants were abundant. The reason for this discrepancy is not understood, but different strains were used in the two studies.

**Cloning of the *hemL* gene by complementation of strain GE1377.** The *E. coli* map indicated that *popC* was located clockwise from the *fhu* locus. Braun and coworkers previously determined the arrangement of genes in the *fhu* locus contained on the pLC4-43 plasmid (10) and showed the order relative to neighboring genes to be *pan-fhuA-fhuB-metD*

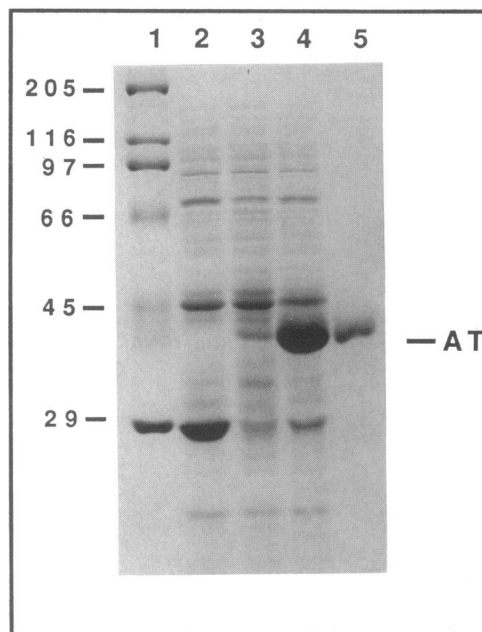


FIG. 2. SDS-polyacrylamide gel electrophoretic analysis of various S-100 preparations and of pure *E. coli* GSA aminotransferase. Proteins were run on a SDS-10% polyacrylamide gel followed by Coomassie blue staining. S-100 extracts (10 µg of protein) of the following origins were applied: BL21(DE3) transformed with pET3 (lane 2); BL21(DE3) transformed with pLIpopC without IPTG induction (lane 3) and after IPTG induction for overexpression (lane 4). Lane 5 contained purified GSA aminotransferase (AT) (MonoQ fraction, 5 µg). Lane 1 contains the following marker proteins: myosin (205 kDa), β-galactosidase (116 kDa), phosphorylase *b* (97 kDa), BSA (66 kDa), ovalbumin (45 kDa), and carbonic anhydrase (29 kDa).

(22). Since *popC* was believed to map clockwise of *fhuB* (3), we compared the restriction maps established by the groups of Braun (10) and Kohara (23) for this region of the *E. coli* chromosome. The detailed analysis of both maps was confirmed by restriction mapping of the DNA insert in the pLC4-43 plasmid. This revealed a unique *Bam*HI site and *Pst*I site just clockwise (using the direction of the *E. coli* map) of *fhuB*. This DNA fragment was isolated and ligated into the *Bam*HI-*Pst*I-cleaved pBluescript KS<sup>+</sup> vector DNA. The resulting plasmid pPC1 transformed strain GE1377 to ALA prototrophy and thus complemented the *hemL* mutation. Further restriction analysis of the complementing *Bam*HI-*Pst*I fragment indicated a unique *Dde*I site approximately 500 bp downstream of the *Bam*HI site. The resulting shorter *Dde*I-*Pst*I DNA fragment was also found to complement ALA auxotrophy of GE1377. Thus, the *Pst*I-*Dde*I fragment (approximately 2,100 bp) was used for the overexpression studies. While this paper was under review, the DNA sequence of the same *E. coli* locus was published (13a); a restriction map of this sequence agrees with the data given above.

**Overexpression and purification of the GSA aminotransferase.** Two clones were constructed with the *Pst*I-*Dde*I fragment in opposite orientations behind the T7 RNA polymerase promoter of pET3 (see Materials and Methods). Initial expression experiments in *E. coli* BL21(DE3) indicated that the desired protein is formed in significant quantities only when the *Dde*I end of the cloned fragment is near

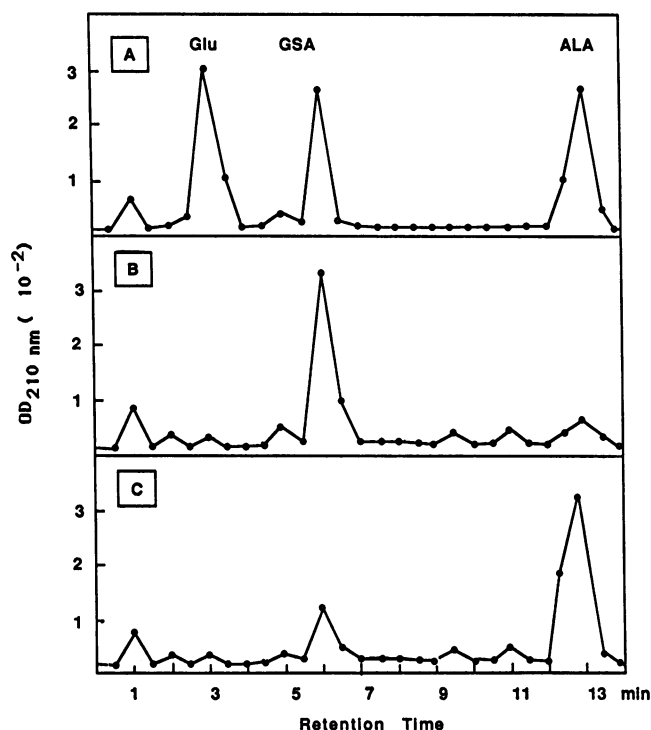


FIG. 3. In vitro GSA→ALA conversion by purified GSA aminotransferase. HPLC analysis of the in vitro-generated reaction products was carried out as described in Materials and Methods. (A) Unlabeled authentic markers of Glu, ALA, and GSA. (B) Product separation of a reaction of heat-inactivated GSA aminotransferase (MonoQ fraction, 15  $\mu$ g) with 50  $\mu$ M PLP and 0.7  $\mu$ mol of GSA. (C) Product separation of a reaction of GSA aminotransferase (MonoQ fraction, 15  $\mu$ g) with 50  $\mu$ M PLP and 0.7  $\mu$ mol of GSA.

the T7 RNA polymerase promoter, indicating that the cloned open reading frame is read in the direction *DdeI*→*PstI*.

Overexpression from the correct clone (pLlpopC) produced a substantial amount (up to 50% of total cellular protein) of a single protein (Fig. 2, lane 4). Bacteria carrying the vector without the inserted fragment did not overproduce the HemL protein and served as control for the specificity of the expression system (Fig. 2, lane 2). Bacteria containing the pLlpopC plasmid expressed low levels of the *hemL* gene product without IPTG induction (Fig. 2, lane 3). GSA aminotransferase assays of these S-100 extracts demonstrated a significant increase of enzyme activity related to the presence of the cloned *hemL* gene (Table 1).

For the purification of the overexpressed protein, we employed chromatography on the fast protein liquid chromatography (FPLC) resin MonoQ, a step which has been successful in our purification of GSA aminotransferase from *C. reinhardtii* (18). The overexpression product was recovered as a single peak (MonoQ fraction) which contained all GSA-converting enzymatic activity. SDS-polyacrylamide gel electrophoresis demonstrated the apparent homogeneity of the purified protein (Fig. 2, lane 5). Thus, large amounts of this protein can be easily obtained in a one-step purification method.

**Characterization of some enzymatic properties of the HemL protein.** For a final test of the enzymatic function of the overexpressed protein, we analyzed the products of the GSA aminotransferase reaction by HPLC (18). The employed

TABLE 1. Requirements of the GSA aminotransferase reaction<sup>a</sup>

Reaction mixture	ALA formed (nmol)
S-100 BL21(DE3)/pET3	0.5
S-100 BL21(DE3)/pLlpopC	70.0
MonoQ	10.8
MonoQ + PLP (5 $\mu$ M)	25.9
MonoQ + PAP (5 $\mu$ M)	25.1
MonoQ + gabaculine (25 $\mu$ M)	0.1
MonoQ + PLP (15 $\mu$ M) + gabaculine (25 $\mu$ M)	0.5
MonoQ + PAP (15 $\mu$ M) + gabaculine (25 $\mu$ M)	0.4
MonoQ + AOA (25 $\mu$ M)	4.4
MonoQ + PLP (15 $\mu$ M) + AOA (25 $\mu$ M)	9.4
MonoQ + PAP (15 $\mu$ M) + AOA (25 $\mu$ M)	8.5
MonoQ + AOA (250 $\mu$ M)	0.1
MonoQ + PLP (15 $\mu$ M) + AOA (250 $\mu$ M)	0.1
MonoQ + PAP (15 $\mu$ M) + AOA (250 $\mu$ M)	0.1

<sup>a</sup> Reaction mixtures (0.1 ml) containing S-100 fraction (20  $\mu$ g of protein) of IPTG-induced BL21(DE3) carrying the indicated plasmid or purified GSA aminotransferase (MonoQ fraction, 0.5  $\mu$ g) or the indicated compounds were preincubated under standard GSA aminotransferase assay conditions (see Materials and Methods) for 20 min at room temperature before GSA (75 nmol) was added and the incubation was continued for another 20 min. This was in the linear velocity range. A control reaction with heat-inactivated enzyme gave a background value of 5 nmol of ALA, which was subtracted from all determined values. AOA, aminooxyacetate.

reversed-phase column was calibrated with pure glutamate, GSA, and ALA (Fig. 3A). A control assay performed with heat-inactivated enzyme did not lead to any detectable GSA→ALA conversion (Fig. 3B), while the unheated MonoQ fraction enzyme gave significant conversion (Fig. 3C). This demonstrates directly that the protein encoded by the cloned *hemL* DNA is GSA aminotransferase.

Purified GSA aminotransferases from barley, *Synechococcus* species, and *C. reinhardtii* exhibited different properties regarding their cofactor utilization (5, 14, 18). While the barley enzyme transaminates in the absence of PLP or PAP (indicating a new mechanism of amino group donation and transfer) and the *Synechococcus* enzyme was stimulated by the presence of PAP, the green alga GSA aminotransferase was totally dependent on the presence of PLP. Moreover, other aminotransferases utilize different types of amino group donors with less substrate specificity (17, 20). To investigate the cofactor requirements of the purified *E. coli* GSA aminotransferase, we analyzed a number of compounds (Table 1). A basic level of transamination was achieved without further addition of PLP or PAP, perhaps as a result of the presence of a cofactor tightly bound to the enzyme. However, there was a twofold increase of GSA aminotransferase activity in the presence of 5  $\mu$ M PLP and PAP, making their role as cofactors probable. Higher amounts (up to 500  $\mu$ M) had no further stimulating or toxic effect. Other amine donors tested (e.g., L-alanine, L-glutamate, or L-asparagine) were not able to contribute to the reaction either in the presence or absence of PLP or PAP (data not shown). The transamination reaction is independent of the presence of ATP, NADPH, and  $Mg^{2+}$  (data not shown). These results indicate that the *E. coli* GSA aminotransferase belongs to the class of aminotransferases dependent on PLP or PAP.

A number of inhibitors of aminotransferases which exert their effect by interaction with the cofactor are known. Gabaculine, a potent inhibitor of different mammalian and bacterial aminotransferases (34) sequesters PLP. Our experiments show that purified *E. coli* GSA aminotransferase plus

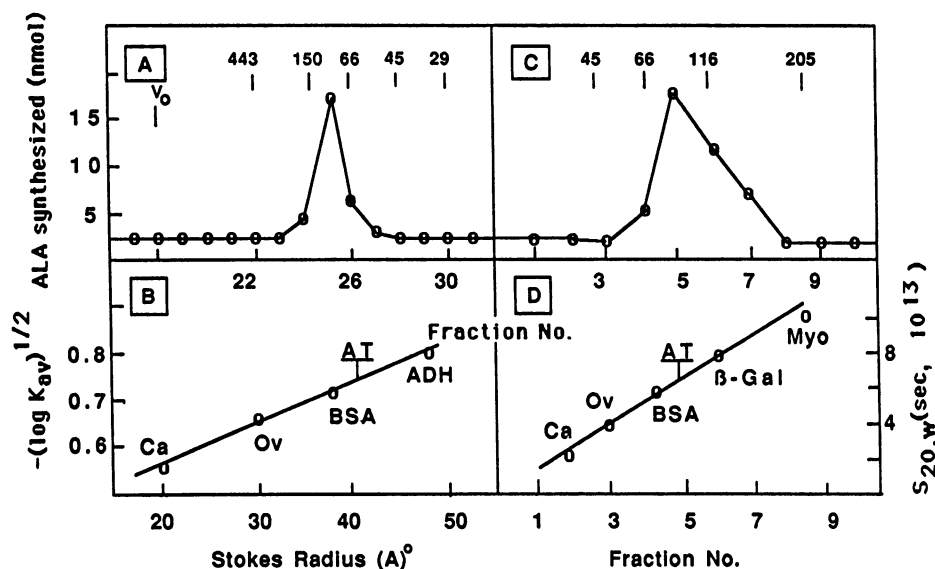


FIG. 4. Determination of the native molecular mass of purified GSA aminotransferase by estimation of the sedimentation coefficient and Stokes radius. (A) Estimation of the native molecular mass by gel filtration on Superose 12. GSA aminotransferase (MonoQ fraction, 10  $\mu$ g) was chromatographed through the FPLC column Superose 12 which had been precleaned with the following marker proteins: apoferritin (443 kDa); alcohol dehydrogenase (150 kDa); BSA (66 kDa); ovalbumin (45 kDa); carbonic anhydrase (29 kDa). Fractions (0.5 ml) were dialyzed and analyzed for GSA aminotransferase activity. (B) Estimation of the Stokes radius. For details, see reference 6. (C) Estimation of the native molecular mass by sedimentation through glycerol gradients. GSA aminotransferase (MonoQ fraction, 20  $\mu$ g) was sedimented through a 10 to 35% glycerol gradient. Fractions were dialyzed and analyzed for GSA aminotransferase activity. The marker proteins carbonic anhydrase (29 kDa), ovalbumin (45 kDa), BSA (66 kDa),  $\beta$ -galactosidase (116 kDa), and myosin (205 kDa) were combined and sedimented through a parallel gradient; their positions in the gradient were determined by SDS-polyacrylamide gel electrophoresis. (D) Estimation of the sedimentation coefficient (data from panel C). The mobility of the marker proteins versus their sedimentation coefficients is shown. Abbreviations: ADH, alcohol dehydrogenase; Ov, ovalbumin; Ca, carbonic anhydrase;  $\beta$ -Gal,  $\beta$ -galactosidase; Myo, myosin; AT, GSA aminotransferase.

of PLP or PAP is clearly inhibited by gabaculine (Table 1). Aminoxyacetate is an inhibitor of many plant transaminases (29) which may form an imine complex with the bound cofactor PLP (11). Compared with gabaculine, higher concentrations (250  $\mu$ M) were needed for total inhibition (Table 1).

**Molecular mass of *E. coli* GSA-aminotransferase.** SDS-polyacrylamide gel electrophoresis of the purified GSA aminotransferase (MonoQ fraction) showed a single band with an  $M_r$  of 40,000. It appears that the *E. coli* enzyme is similar in size to the *S. typhimurium* enzyme, for which a molecular mass of 45.3 kDa was deduced from the gene sequence (8). While this paper was under review, the DNA sequence of the GSA aminotransferase gene was published (13a); the derived amino acid sequence predicts a molecular mass of 45.4 kDa (13a). In order to define whether GSA aminotransferase acts as a monomer, we determined its native molecular mass under nondenaturing conditions. For this purpose, we employed two independent methods, rate zonal centrifugation in glycerol gradients (Fig. 4C and D) and gel filtration on the FPLC molecular sieving matrix Superose 12 (Fig. 4A and B). GSA aminotransferase activity in the glycerol gradients was found in a position between bovine serum albumin (BSA) (66 kDa) and  $\beta$ -galactosidase (116 kDa) (Fig. 4C). This agrees with the results of gel filtration experiments, which showed a sharp activity peak in the elution position between BSA and alcohol dehydrogenase (150 kDa) (Fig. 4A). A sedimentation coefficient ( $s_{20,w}$ ) of  $5.9 \pm 0.2$  and an apparent Stokes radius of  $4.2 \pm 0.2$  nm were deduced (33). Thus, an apparent native molecular mass of approximately  $80,000 \pm 5,000$  Da was calculated (6, 27). We

conclude that GSA aminotransferase is a functional dimer of 80,000 Da with an  $\alpha_2$  structure.

#### ACKNOWLEDGMENTS

We thank Elizabeth Verkamp and M. John Rogers for helpful discussions and critical reading of the manuscript and Dennis Diener for technical assistance. Moreover, we thank F. W. Studier for supplying the T7 RNA polymerase expression system and B. Bachmann for the gift of the *E. coli* JA200 containing pLC4-43.

L.L.I. holds an NSF predoctoral fellowship, and D.J. was a postdoctoral fellow of the Deutsche Forschungsgemeinschaft. This work was supported by grants from NIH, DOE, and the Icelandic Science Foundation.

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