Glutamyl-tRNA Reductase from *Escherichia coli* and Synechocystis 6803*

GENE STRUCTURE AND EXPRESSION

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In the cyanobacterium *Synechocystis* sp. PCC 6803 and in the enterobacterium *Escherichia coli* l-aminolevulinic acid (ALA) is formed from glutamyl-tRNA by the sequential action of two enzymes, glutamyl-tRNA reductase (GluTR) and glutamate-1-semialdehyde aminotransferase. *E. coli* hemA gene product has two GluTR proteins with sizes of 45 kDa (GluTR45) and 85 kDa (GluTR85) (Jahn, D., Michelsen, U., and Soll, D. (1991) *J. Biol. Chem.* 266, 2542–2548). The *hemA* gene, isolated from *E. coli* and several other eubacteria, has been proposed to encode a structural component of GluTR. Because of the inability to overexpress this gene in *E. coli*, we demonstrate directly GluTR function for the *E. coli* *hemA* gene product in *Bacillus subtilis*, *Salmonella typhimurium*, and *Chlorobium vibrioforme* but does not contain the amino acid sequence derived from the N terminus of the previously purified GluTR protein (Riehle, S., and Beale, S. I. (1981) *J. Biol. Chem.* 266, 9740–9745). These experiments are the first direct demonstration of GluTR activity of the *hemA* protein and provide further evidence for two pathways of ALA formation in prokaryotes.

δ-Aminolevulinic acid (ALA) is the first committed pre-

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1 The abbreviations used are: Ala, δ-aminolevulinic acid; Glu-tRNA, glutamyl-tRNA; GluTR, glutamyl-tRNA reductase; GluTR45, Glu-tRNA reductase, 45-kDa species; GluTR85, Glu-tRNA reductase, 85-kDa species; GSA, glutamate 1-semialdehyde; Hopes, N-3-hydroxy-ethylpiperazine N'-2-ethanesulfonic acid; HPLC, high performance liquid chromatography; kb, kilobase pair(s).

2 E. Verkamp and D. Jahn, unpublished results.

3 I. Schröder, personal communication.
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MATERIALS AND METHODS

General—Restriction endonucleases and DNA modification enzymes were obtained commercially and used according to the manufacturer’s specifications. E. coli tRNA<sup>74</sup> was obtained from Boehringer Mannheim. [1<sup>4</sup>C]Glutamate (specific activity 266 mCi/mmol) was a product of Du Pont-New England Nuclear. Chemically synthesized GSA (20) was a gift of Dr. C. G. Kannangara (Carlsberg Laboratory, Copenhagen, Denmark). pMR57 containing the E. coli hemA gene was described earlier (14).

Strains, Media, and Growth Conditions—E. coli strains were grown routinely on LB medium or M9 minimal medium (21). Strains SASSX41B (HfrF02A hemA41 metB1 recA1) and EV61 (thr-1 ara-14 leu2-3,5 glnB12 thi-18 thyA62 lac-proAB2 hisG4 rpsL31 kdgK51 rplS31 hdpK51 xyl-5-1 malG31 thi-1 recB2 recC22 sbeB15 sbeC201 ΔhemA Rac<sup>−</sup>) (14) were grown routinely on medium supplemented with ALA (50 μg/ml). Antibiotics (concentration) was added to growth media to maintain that ampicillin (0.1 μg/ml) and kanamycin (50 μg/ml). For GluTR assays, strain SASSX41B harboring the indicated plasmid was grown to late log phase in LB medium (500 ml) supplemented with either ampicillin (for pSH111) or ampicillin + ALA (for pBluescript). Strain EV61 was grown anaerobically in M9 minimal medium supplemented with glucose (0.2%), thiamine (1 μg/ml), kanamycin (50 μg/ml), and casamino acids (0.2%). Anaerobic condition was achieved by growing the culture without shaking in anaerobic jars with sealed one-liter bottles that were filled completely with freshly auto claved medium.

DNA Methods—Southern hybridizations and random primer probe preparations were performed according to published protocols (22). Synthetic DNA was purchased from Peninsula Laboratories (for pBluescript). DNA sequence was determined by the method of Sanger (23) with [α-<sup>32</sup>P]dATP. Nucleotide sequence data were analyzed by the University of Wisconsin Genetics Computer Group program (24).

Construction of the Yeast Overexpression Plasmid—Two primers covering the 5′ and 3′ region of the E. coli hemA gene were used to generate a DNA fragment by polymerase chain reaction containing the coding region of the gene of interest, appropriate restriction sites for cloning and a 5′ region ensuring correct and efficient initiation. Oligonucleotide 1 (5′-CTTACGGATCCTGTATAATACAAGCAGA-3′) corresponds to bases -64 to -38 of the hemA gene (14). A base mismatch in position -58 (C → G) generated a new BamHII site. Base changes in positions -48 (G → T) and -38 (A → G) generated a new yeast specific mRNA initiation sequence (ATAA-TAC) (25). Oligonucleotide 2 (5′-TAGGCGTAAATGGATCTTAACCC-3′) was complementary to bases 1272-1299 of the published sequence. Base changes in positions 1285 (C → T) and 1299 (C → G) created a second BamHII restriction site. The polymerase chain reaction reaction (0.1 ml) contained 0.1 μg of pMR57 DNA, 1 μg of each primer, 1.5 units of Taq DNA polymerase, 0.2 mM dNTPs in 20 mM Tris-Cl (pH 8.5), 40 mM KCl, and 2.5 mM MgCl<sub>2</sub>. During the first cycle of renaturation of the DNA fragment min at 94 °C was followed by 40 cycles of 1 min at 94 °C, 1 min at 55 °C, and 1.5 min at 72 °C. The generated DNA fragment was purified by phenol extraction, gel filtration through Sephadex-G50, and subsequent ethanol precipitation before the correct size was rescued phagemid preparation were used to transform competent SASSX41B cells to ALA prototrophy. The identity as GSA was confirmed by its ability to serve as a substrate for DEAE-cellulose chromatography (in the case of the yeast expression products) were dialyzed against Mono Q buffer containing 0.5 M KCl before gel filtration through Superose 12 as described previously (9). Fractions containing GluTR activity were dialyzed against assay buffer before enzyme activity was determined.

Isolation of the Synechocystis 6803 hemA Gene—A library of 6-9 kb Synechocystis 6803 genomic DNA fragments cloned into the EcoRI site of pApaII (Stratagene) was the gift of Dr. L. McIntosh (MSU-DOE Plant Research Laboratory, East Lansing, MI). Recombinant Bluescript phagemids were excised (33) and various dilutions of the rescued phagemid preparation were used to transform competent SASSX41B cells to ALA prototrophy. Samples were plated on LB/ampicillin plates and incubated at 37 °C to select for colonies that contained putative hemA-harboring plasmids.

RESULTS

Overexpression of the E. coli hemA Gene in S. cerevisiae—Significant overexpression of the cloned hemA gene in E. coli was unsuccessful despite the use of overexpression systems with different promoters and RNA polymerases. An example is seen in Fig. 1B where the expression of pMR57, a pUC-derived plasmid containing the hemA gene with its own promoter, resulted only in an approximately 4-fold increase in GluTR activity. Therefore we attempted to express this gene in the yeast S. cerevisiae, an organism which lacks the C<sub>3</sub> pathway and utilizes the Shemin pathway for ALA synthesis (34). The E. coli hemA gene was cloned into the yeast shuttle vector pVT103-U. The use of polymerase chain reaction the gene was engineered to ensure correct transcription and translation initiation in yeast (see "Materials and Methods"). S. cerevisiae cultures were transformed with plasmid containing the hemA gene in the correct (pMJ55) and reversed (pMJ3) orientation and with empty vector. Extracts prepared from the different cultures were partially purified by DEAE-cellulose chromatography. Enzyme assays of the different preparations clearly demonstrated the presence of GluTR activity only in the extract from S. cerevisiae transformed with the hemA gene in the correct orientation (Table I). This result was confirmed by the product analysis on HPLC (Fig. 2) which showed that the extracts contained GluTR activity capable of transforming Glu-tRNA into GSA. The authentic-
Fig. 1. Determination of the relative molecular mass of E. coli GluTR activities by gel filtration. Enzyme fractions (0.5 mg of protein), partially purified by two column steps (see "Materials and Methods"), from various sources were chromatographed through Superose 12 under conditions outlined under "Material and Methods." The column was calibrated before with the following protein markers: apoferritin (443 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), and carbonic anhydrase (29 kDa). Fractions (0.5 ml) were collected, dialyzed against assay buffer, and analyzed for GluTR activity. A, extract of E. coli K12. B, extract of E. coli K12 transformed with pMR57. C, extract of E. coli EV61 grown under anaerobic conditions. D, extract of yeast transformed with pMJ55.

TABLE I
Glutamyl-tRNA reductase activity in extracts of transformed yeast strains

| Plasmid used  | ALA formed | pmol |
|--------------|------------|------|
| No plasmid   | ND         |      |
| pVT103-U     | ND         |      |
| pMJ3         | ND         |      |
| pMJ55        | 25.0       |      |

Fig. 2. Conversion of E. coli [14C]Glu-tRNA → [14C]GSA by E. coli GluTR expressed in yeast. Further transformation to [14C]ALA was accomplished by adding E. coli GSA-AT. Calibration of the C18 reversed-phase column and HPLC analysis of the in vitro formed reaction products was performed as described (Jahn et al., 1991b). A, unlabeled authentic markers of Glu, GSA, and ALA were monitored by their absorbance at 210 nm. [14C]Glu-tRNA was identified after trichloroacetic acid precipitation by liquid scintillation counting (Chen et al., 1990). B, separation of the products from the reaction of the yeast expressed hemA product (DEAE-cellulose fraction, 50 pg of protein). C, same as B plus purified recombinant GSA aminotransferase (10 pg of protein).

shown in Fig. 1D, the relative molecular mass of the GluTR activity is approximately 45 kDa. Therefore, the E. coli hemA product is GluTR45. This is in line with the weak overexpression in the homologous system (see above) which also caused a slight increase of the GluTR45 activity (Fig. 1B).

Molecular Weight of the GluTR Activity Found in a hemA Strain of E. coli—Since the hemA gene product was identified as GluTR45 we wanted to ascertain that an E. coli strain in which the product of this gene was inactive lacked the GluTR45 activity. Therefore we analyzed an extract from E. coli strain EV61, which contains a gene disruption of hemA. Like other hemA strains (35), EV61 can grow under anaerobic conditions without ALA supplementation utilizing glucose as carbon source. Extracts from anaerobically grown E. coli EV61 contained GluTR activity. This activity was partially purified by chromatography on DEAE-cellulose and Mono Q before the preparation was subjected to gel filtration on Superose 12. As is clearly seen from Fig. 2C the GluTR activity of the hemA− strain has a molecular mass of approximately 85 kDa and presumably corresponds to the GluTR55 enzyme.

Isolation and Characterization of the Synechocystis 6803 hemA Gene—The hemA mutation in E. coli strain SASX41B confers a growth requirement for ALA. In order to isolate the hemA homolog from Synechocystis 6803, we transformed a λZapII clone bank of Synechocystis genomic DNA into E. coli SASX41B and selected for ALA prototrophy of SASX41B by the recombinant phagemids on LB/ampicillin plates. Nineteen complemented transformants were obtained by this pro-
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cedure. Plasmid DNA purified from these strains simultaneously conferred ampicillin resistance and ALA prototrophy to SASX41B cells upon re-transformation.

One plasmid, designated pSH11, was analyzed in detail, and a partial restriction endonuclease map of the 6.5-kb EcoRI insert of plasmid pSH 11 is depicted in Fig. 3. Total genomic DNA extracted from Synecochystis 6803 was double-digested to completion with BamHI and HindIII, size-fractionated on a 1% agarose gel, transferred to cellulose nitrate, and hybridized to the 32P-labeled DNA of the EcoRI insert of pSH11. As expected, this probe hybridized to two BamHI/HindIII fragments; a 2.2- kb fragment that corresponded to the fragment present on pSH11 and an approximately 4.3-kb fragment that would correspond to a fragment delimited by the HindIII site present on the insert and the next BamHI or HindIII site present on the Synecochystis genome (data not shown). Since no other hybridizing bands could be observed, these data are consistent with the presence of one copy of this gene in the Synecochystis genome.

In order to localize the hemA gene within the 6.5-kb EcoRI fragment, subclones were constructed that contained either the 4.0-kb EcoRI/Hind III fragment (pSH12) or the 2.5-kb EcoRI/HindIII (pSH13) in pBluescript (Fig. 3). Neither plasmid complemented the hemA mutation, indicating that HindIII cleaves the DNA within the region required for expression of the putative Synecochystis hemA gene. A 1.4-kb TfiI fragment (Fig. 3), subcloned into the EcoRV site of pBluescript SK+ to construct plasmid pSH17, complemented the hemA mutation and therefore contained the hemA gene.

DNA sequence analysis identified an open reading frame capable of encoding a polypeptide of 427 amino acids with a molecular weight of 47,525. The DNA sequence encoded with GenBank (accession number M84218). The deduced amino acid sequence exhibited substantial similarity to the HemA polypeptides reported from E. coli, deposited with GenBank (accession number M84218). As both glutamyl-tRNA synthetase (36) and GluTR are able to recognize tRNA in a sequence-specific manner (3). As both glutamyl-tRNA synthetase (36) and GluTR are able to recognize tRNA (3), it will be interesting to determine their respective tRNA identities (i.e. which nucleotides in tRNA are important for specific recognition by these proteins). The sequence comparison of the presently known GluTR enzymes (Fig. 4) reveals some highly conserved regions. For instance, there is a sequence of 23 amino acids (positions 99-121) in which 20 amino acids are conserved. Within this region, a mutation of Cys to Tyr in B. subtilis leads to loss of enzyme activity resulting in a hemA phenotype (18).

A second question concerns the regulation of this enzyme. Clearly, as demonstrated by the hemA phenotype, GluTR is the crucial enzyme for ALA formation in aerobically grown E. coli cells. There is currently no information on the extent of transcriptional and translational regulation of the hemA gene. A number of experiments have indicated that regulation by intermediates of later steps in the heme pathway is certainly a possibility (37). In addition, the fact that good overexpression in E. coli is not observed may indicate that proteolysis may regulate the level of GluTR as is the case for ALA-synthase, the corresponding enzyme in the Shemin pathway in mammals (38).

The different sizes of the known GluTR enzymes also merit further study. Although the five bacterial proteins for which the gene sequences are known are very similar (see Fig. 4) the enzyme recently purified from Synecochystis 6803 (10) shows a size inconsistent with that of the protein deduced from our DNA sequence. In addition, the 42-amino acid N-terminal sequence determined for the Synecochystis enzyme is not represented in the gene sequence reported here. It may also be pertinent to note that the Synecochystis 6803 GluTR is relatively abundant, as evidenced by a 370-fold purification factor (10), whereas the enzymes from E. coli and C. reinhardtii require a 5-10-fold higher factor for obtaining homogeneous preparations (9, 11). The difference in these results may be resolved by further characterization of Synecochystis GluTR enzyme preparations or may point to the existence of two reductase activities in this organism.

Why are there two GluTR activities in E. coli? The observation of two GluTR activities has so far only been reported for E. coli. The demonstration that hemA encodes GluTR45 and that a hemA+ strain contains only GluTR85 suggests that there are two pathways for ALA formation in E. coli. In line with this notion is the fact that hemL strains of E. coli lacking GSA aminotransferase display a leaky phenotype regarding ALA auxotrophy, implying the presence of another aminotransferase which can utilize GSA or the existence of a compensatory pathway for ALA formation (30). It may also be pertinent that there are two pools of ALA found in cucumber chloroplasts (39), which may be due to different pathways for their formation.

FIG. 3. Partial restriction enzyme map of the genomic DNA insert present in plasmids pSH11, pSH12, and pSH13 and the Synecochystis hemA gene subcloned in pSH17. The DNA sequence at the 5' fusion junction between the Synecochystis hemA gene (bold) and the polylinker of pSK+ are shown. The open boxes represent vector sequences of pBluescript SK+.

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Fig. 4. Comparison of the GluTR amino acid sequences deduced from the hema DNA sequences of Synechocystis 6803 (this work), E. coli (12), S. typhimurium (17), C. vibrioforme (19), and B. subtilis (18).
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