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Identification of the Enzymatic Basis for δ-Aminolevulinic Acid Auxotrophy in a *hemA* Mutant of *Escherichia coli*

YAEL J. AVISSAR AND SAMUEL I. BEALE*

Division of Biology and Medicine, Brown University, Providence, Rhode Island 02912

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The *hemA* mutation of *Escherichia coli* K-12 confers a requirement for δ-aminolevulinic acid (ALA). Cell extract prepared from the *hemA* strain SASX41B was incapable of producing ALA from either glutamate or glutamyl-tRNA, whereas extract of the *hem*$^+$ strain HB101 formed colorimetrically detectable amounts of ALA and transferred label from 1-[^{14}C]glutamate and 3,4-[^{3}H]glutamyl-tRNA to ALA. Extracts of both strains converted glutamate-1-semialdehyde to ALA and were capable of aminoacylating tRNA^{Glu}. Glutamyl-tRNA formed by extracts of both strains could be converted to ALA by the extract of *hem*$^+$ cells. The extract of *hemA* cells did not convert glutamyl-tRNA formed by either strain to ALA. However, the *hemA* cell extract, when supplemented in vitro with glutamyl-tRNA dehydrogenase isolated from *Chlorella vulgaris* cells, formed about as much ALA as did the unsupplemented *hem*$^+$ cell extract. We conclude from these observations that the enzyme activity that is lacking in the ALA auxotrophic strain carrying the *hemA* mutation is that of glutamyl-tRNA dehydrogenase.

Several *hemA* mutants of *Escherichia coli* K-12 have been described. These mutants are auxotrophs requiring the heme precursor δ-aminolevulinic acid (ALA) for growth on minimal media. The *hemA* locus has been mapped at 27 min on the *E. coli* genomic linkage map (2, 8, 20, 21). Another mutation causing an ALA requirement has been variously named *hem*$^-$ and *popC* (3, 18, 30) and was mapped at 4 min on the *E. coli* K-12 chromosome.

It has been assumed that ALA auxotrophy in the *hemA* mutation is conferred by the absence of ALA synthase (succinyl coenzyme A:glycine C-succinyl transferase [decarboxylating] EC 2.3.1.37) activity, since this enzyme was believed to be the only one catalyzing ALA formation in *E. coli* and other bacteria. This assumption was supported by the observation that *hemA* mutants transformed by plasmids carrying the ALA synthase gene from other organisms regained the ability to grow on media without added ALA (11, 24).

A second route for ALA formation has been characterized in plants and algae, wherein the intact five-carbon skeleton of glutamate is converted to ALA (4, 5, 7, 15, 28). In this pathway (Fig. 1), the α-carboxyl group of glutamate is first activated by ligation to tRNA^{Glu}. The activated glutamate is next reduced to glutamate-1-semialdehyde (GSA) or a closely related compound. The GSA is then converted to ALA through an aminotransferase reaction. Although originally thought to be restricted to plants and algae, the five-carbon ALA biosynthetic pathway has recently been reported to occur in several species of bacteria (6, 16, 17, 19; S. Rieble, J. G. Ormerod, and S. I. Beale, Plant Physiol. **86**:S-60, 1988; Y. J. Avissar, J. G. Ormerod, and S. I. Beale, Arch. Microbiol., in press).

Recently, the *hemA* gene of *E. coli* was isolated, and its sequence was determined (J.-M. Li, S. D. Cosloy, and C. S. Russell, J. Cell Biol. **107**:617a, 1988). The sequence was reported to bear no resemblance to previously characterized ALA synthase genes from other organisms (14, 27), even though the latter genes show considerable similarity among themselves. It was also demonstrated that a transformed *hemA E. coli* strain synthesized ALA in vivo and in vitro from glutamate via the five-carbon pathway, rather than from glycine via the ALA synthase-catalyzed reaction (11a; Li et al., J. Cell Biol. **107**:617a). The cells had been transformed by a multicopy plasmid carrying DNA derived from a *hem*$^+$ *E. coli* strain.

We have examined ALA formation in extracts of *hem*$^+$ and *hemA* strains of *E. coli* and have determined that *hem*$^+$ cells form ALA solely via the five-carbon pathway. By measuring individual steps in the conversion of glutamate to ALA and by supplementing in vitro incubation mixtures with isolated enzymes from *Chlorella vulgaris*, we determined that the ALA auxotrophy in the strain carrying the *hemA* mutation is caused by the absence of glutamyl-tRNA dehydrogenase activity.

MATERIALS AND METHODS

Organisms. *E. coli* ALA-auxotrophic strain SASX41B carrying the *hemA* mutation was obtained from the *E. coli* Genetic Stock Center, Department of Biology, Yale University, New Haven, Conn. *E. coli* HB101 (*hem*$^+$) was obtained from W. E. Tapprich, Brown University.

Strain HB101 cells were grown in LB medium or M9CA medium (12). Strain SASX41B cells were grown in these media supplemented with 20 μM ALA. Cultures were grown in Erlenmeyer flasks on a rotary shaker (200 rpm) at 37°C.

Preparation of cell extracts. *E. coli* cultures were harvested in late-exponential growth phase by centrifugation at 10,000 × *g* for 5 min at 2°C and washed twice with extraction buffer [100 mM *N*-tris(hydroxymethyl)methylglycine (Tricine) (pH 7.9), 0.3 M glycerol, 15 mM $MgCl_2$, 3 mM dithiothreitol]. The cell pellet was weighed and suspended in 4 ml of extraction buffer per g of cells. Approximately one-third volume of glass powder (5-μm diameter) was added, and the suspension was sonicated with an MSE sonic disruptor at 0 to 4°C for eight 15-s periods with 30-s cooling intervals. The suspension was centrifuged at 10,000 × *g* for 10 min at 2°C. Low-molecular-weight materials were removed from the supernatant by passage through a column of Sephadex G-25 previously equilibrated with assay buffer (50 mM Tricine [pH 7.9], 1 M glycerol, 15 mM $MgCl_2$, 1 mM dithiothreitol).

* Corresponding author.

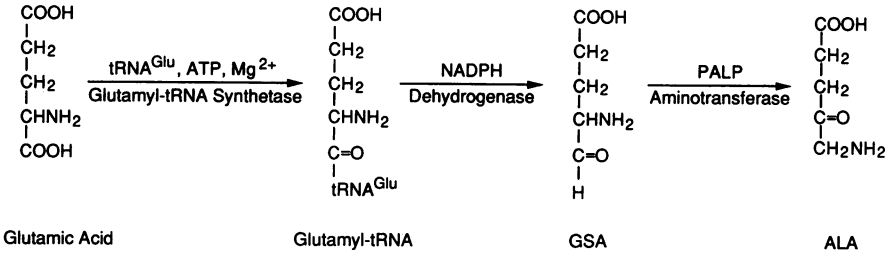

FIG. 1. Proposed biosynthetic sequence of ALA formation from glutamate via the RNA-dependent five-carbon pathway. The cofactor requirements, enzyme activities, and intermediate products are illustrated. GSA, the reduced intermediate illustrated, is one of several structures proposed for the immediate precursor of ALA.

High-speed supernatant was obtained by centrifugation of the extract at 264,000 × *g* for 90 min at 2°C. The extract was used directly or stored at −75°C until used for enzyme assays.

Chlorella enzyme fractions catalyzing each of the three steps of ALA formation from glutamate were prepared by using serial affinity chromatography and identified as described previously (1, 1a).

RNA extraction and purification. *Chlorella* tRNA, prepared and purified by phenol-chloroform extraction and DEAE-cellulose chromatography as previously described (22), and commercial *E. coli* tRNAs were dissolved in small volumes of buffer (10 mM Tris hydrochloride [pH 7.5], 10 mM magnesium acetate, 100 mM NaCl, 1 mM dithiothreitol) to a final concentration of 250 A_{260} units per ml (*Chlorella* tRNA), 31 A_{260} units per ml (*E. coli* $tRNA^{Glu}$), or 63 A_{260} units per ml (*E. coli* tRNA mixture) and stored at −20°C in small working samples.

Enzyme assays. (i) Assays of ALA synthesis from labeled glutamate. Assays were performed in 1.5-ml microcentrifuge tubes. A 15-min preincubation of the extract (100 to 170 μl in assay buffer) was carried out in the presence or absence of 0.25 μg of RNase A or 5 μM gabaculine. The incubation mixture was adjusted to 250 μl (final volume) with assay buffer and contained the cell extract plus 50 μM glutamate containing 10 μCi of 3,4-[^{3}H]glutamate or 1 μCi of 1-[^{14}C]glutamate, 1 mM NADPH, 5 mM ATP, 5 mM levulinate, and 20 μM pyridoxal phosphate (PALP). Incubation was at 37°C for 1 h. In assays of ALA synthesis from unlabeled glutamate, similar conditions were employed with the sole difference that radioactive glutamate was omitted and the glutamate concentration was 1 mM.

(ii) ALA synthase. ALA synthase activity was assayed in a similar volume of extract in the same buffer containing (after the preincubation) 50 μM glycine (containing 1 μCi of 2-[^{14}C]glycine), 50 μM succinate, 0.24 mM PALP, 0.34 mM coenzyme A, 8 mM ATP, and 5 mM levulinate. Nonincubated controls underwent only the preincubation before the addition of the substrates and cofactors. Incubations were terminated by the addition of 100 μl of 1 M citric acid, 250 μl of 10% (wt/vol) sodium dodecyl sulfate, and (for the radioactive assays only) 20 nmol of unlabeled carrier ALA. The resulting mixture was heated at 95°C for 2 min, cooled rapidly to room temperature, and centrifuged in an Eppendorf microcentrifuge at 13,500 × *g* for 2 min to remove precipitated material. ALA in the supernatant was purified by ion-exchange chromatography and solvent extraction as previously described (1). For the colorimetric estimation of the ALA produced, the ALA was quantitated spectrophotometrically at 553 nm after reaction with ethylacetoacetate and Ehrlich-Hg reagent (13, 26).

(iii) Aminoacylation. Ligation of glutamate to tRNA was assayed by measuring the amount of radioactive material, precipitable by cold trichloroacetic acid, that was produced during incubation of cell extracts with tRNA and 3,4-[^{3}H]glutamate in the presence of ATP, as described previously (1, 22). The incubation time and the protein concentration were reduced to make the extent of the reaction dependent on time and enzyme concentration.

(iv) ALA formation from glutamyl-tRNA. ALA formation from glutamyl-tRNA was assayed as previously described (1). 3,4-[^{3}H]glutamyl-tRNA substrate was prepared by the method of Schneegurt et al. (23) with 0.62 A_{260} unit of *E. coli* $tRNA^{Glu}$ and 50 μl of *E. coli* cell extract (0.80 to 0.84 mg of protein) in 200 μl of incubation mixture containing 5 mM ATP and 5 μM glutamate (containing 10 μCi of 3,4-[^{3}H]glutamate). The mixture was incubated for 20 min at 37°C. The reaction was stopped by the addition of 400 μl of termination buffer [0.1 M 2-(*N*-morpholino)ethanesulfonic acid (pH 5.8), 10 mM $MgCl_2$, 0.1 M glutamate]. The solution was first extracted with an equal volume of phenol that was preequilibrated with termination buffer and then with an equal volume of chloroform-isoamyl alcohol (24:1 [vol/vol]), and the nucleic acids were precipitated overnight at −20°C after the addition of 0.1 volume of 20% (wt/vol) sodium acetate and 2.5 volumes of absolute ethanol. The precipitate was collected by centrifugation, washed with absolute ethanol, and dried in vacuo. For use as a substrate in ALA formation assays, the precipitated 3,4-[^{14}C]glutamyl-tRNA was dissolved in assay buffer immediately before use and added to the incubation. The incubation mixture contained the indicated cell extract in assay buffer, 3,4-[^{14}C]glutamyl-tRNA, 1 mM glutamate, 5 mM levulinate, 20 μM PALP, and 1 mM NADPH. Incubation was for 1 h at 37°C for *E. coli* extracts and at 30°C for *Chlorella* extracts or mixtures of *E. coli* extracts and *Chlorella* enzyme fractions.

(v) Conversion of GSA to ALA. Conversion of GSA to ALA was assayed with chemically prepared GSA (9, 10) by the method of Hoober et al. (9) with minor modifications (1a). The ALA produced was purified by ion-exchange chromatography and solvent extraction as described previously (1) and then quantitated colorimetrically. Control reactions, in which cell extract was added at the end of the incubation period, were carried out to control for nonenzymatic conversion of GSA to ALA (9).

Materials. Chemically synthesized GSA was a generous gift from C. G. Kannangara (Carlsberg Laboratory, Copenhagen, Denmark), *E. coli* $tRNA^{Glu}$ type II was purchased from Boehringer Mannheim Biochemicals, *E. coli* tRNA mixture and pancreatic RNase A were from Sigma Chemical Co., 3,4-[^{3}H]glutamate and 1-[^{14}C]glutamate were from Du Pont, NEN Research Products, gabaculine was from Fluka, and all other reagents were from Fisher Scientific Co., Sigma, or Research Organics.

TABLE 1. Incorporation of label into ALA from 1-[^{14}C]glutamate and 2-[^{14}C]glycine by extract of *hem*$^+$ *E. coli* cells[a]

Labeled substrate	Preincubation mixture	Incubation time (min)	ALA formation (cpm)
1-[^{14}C]Glu	Buffer	0	50
1-[^{14}C]Glu	Buffer	60	30,770
1-[^{14}C]Glu	RNase	60	50
2-[^{14}C]Gly	Buffer	0	60
2-[^{14}C]Gly	Buffer	60	20
2-[^{14}C]Gly	RNase	60	150

[a] Preincubation was at 37°C for 20 min in 200 μl of assay buffer containing 170 μl of extract from strain HB101 cells and, where indicated, 0.25 μg of RNase A. Incubation was started by the addition of 50 μl of assay buffer containing 1 μCi of 1-[^{14}C]glutamate and a substrate-cofactor mixture giving a final concentration of 1 mM NADPH, 5 mM ATP, 5 mM levulinate, 20 μM PALP, and 50 μM glutamate or containing 1 μCi of 2-[^{14}C]glycine and a substrate-cofactor mixture giving a final concentration of 50 μM glycine, 50 μM succinate, 0.24 mM PALP, 0.34 mM coenzyme A, 8 mM ATP, and 5 mM levulinate. Incubation was at 37°C for the indicated time periods.

RESULTS

Conversion of glutamate but not glycine to ALA by extract of *hem*$^+$ cells. The cell extract of *E. coli* HB101 was capable of forming labeled ALA from 1-[^{14}C]glutamate but not from 2-[^{14}C]glycine (Table 1). Colorimetrically detectable amounts of ALA were formed during the incubation (Table 2). The reaction product was identified by the visible absorption spectrum of the product formed after reaction with ethylacetoacetate and Ehrlich reagent, which was identical to that of the product formed by authentic ALA (data not shown). Both label transfer from glutamate to ALA and the appearance of ALA in the colorimetric assay were completely blocked by preincubation of the cell extract with RNase A. The rate of ALA formation was approximately linear during the 60-min incubation period (Table 2). The reaction was stimulated by addition of *E. coli* tRNA^{Glu} and inhibited by gabaculine.

After low-molecular-weight components were removed by Sephadex G-25 gel filtration, the reaction was dependent on added reduced pyridine nucleotide (Table 2). NADPH was more effective than NADH at 1 mM. Omission of ATP or glutamate from the incubation mixture significantly lowered ALA formation, but omission of PALP had no measurable effect.

TABLE 2. Requirements for ALA synthesis from glutamate in extract of *hem*$^+$ *E. coli* cells[a]

Preincubation addition	Incubation mixture	Incubation time (min)	ALA formation (nmol)
None	Complete	0	0.00
None	Complete	30	0.87
None	Complete	60	1.78
None	Complete	90	1.77
None	Without ATP	60	0.27
None	Without glu	60	0.68
None	Without PALP	60	1.78
None	Without NADPH	60	0.00
None	Without NADPH, with NADH	60	1.03
0.16 A_{260} unit of *E. coli* tRNA^{Glu}	Complete	60	2.10
0.25 μg of RNase A	Complete	60	0.04
5 μM gabaculine	Complete	60	0.18

[a] Preincubation was at 37°C for 20 min in 225 μl of assay buffer containing 200 μl (3.2 mg of protein) of Sephadex G-25 gel-filtered extract of strain HB101 cells plus the indicated additions. Incubation was started by the addition of 25 μl of substrate-cofactor mixture giving a final concentration of 1 mM each NADPH (or NADH) and glutamate, 5 mM each ATP and levulinate, and 20 μM PALP, with the indicated variations. Incubation was at 37°C for the indicated time periods.

TABLE 3. ALA formation from glutamate by extracts of *hem*$^+$ and *hemA E. coli* cells[a]

Cell extract source (genotype)	Preincubation mixture	Incubation mixture	ALA formation (cpm)
hem$^+$	Buffer	Unincubated	100
hem$^+$	Buffer	Complete	1,550
hem$^+$	RNase	Complete	50
hem$^+$	Buffer	With tRNA	2,040
hemA	Buffer	Unincubated	60
hemA	Buffer	Complete	60
hemA	RNase	Complete	60
hemA	Buffer	With tRNA	60

[a] All samples contained 200 μl of high-speed supernatant (720 μg of protein) from extract of strain HB101 (*hem*$^+$) or strain SASX41B (*hemA*) cells and were preincubated at 37°C for 20 min with or without 0.25 μg of RNase A as indicated. Incubation was started by the addition of 1 μCi of 1-[^{14}C]glutamate in 50 μl of a substrate-cofactor mixture, resulting in a final concentration of 50 μM glutamate, 1 mM of NADPH, 5 mM each ATP and levulinate, 20 μM PALP, and, when indicated, 0.16 A_{260} units of *E. coli* tRNA^{Glu}. Incubation was at 37°C for 1 h.

Comparison of ALA formation from glutamate by extracts of *hem*$^+$ and *hemA* cells. In contrast to the results with the *hem*$^+$ cell extract, the extract of *hemA* cells was unable to form labeled ALA from 1-[^{14}C]glutamate, either in unsupplemented incubation mixture or when supplemented with *E. coli* tRNA^{Glu} (Table 3).

Formation of glutamyl-tRNA by extracts of *hem*$^+$ and *hemA* cells. Extracts of both cell types were capable of forming glutamyl-tRNA (Table 4). Per milligram of protein in the incubation mixture, the *hem*$^+$ cell extract was more active than the extract of the *hemA* cells. The aminoacylation reaction was inhibited by preincubation with RNase A and stimulated by addition of *E. coli* tRNA mixture or tRNA^{Glu}.

ALA formation from glutamyl-tRNA by extracts of *hem*$^+$ and *hemA* cells. ALA formation from glutamyl-tRNA was assayed by using ^{3}H-labeled substrate glutamyl-tRNA pro-

TABLE 4. Aminoacylation of tRNA^{Glu} by extracts of *hem*$^+$ and *hemA E. coli* cells[a]

Cell extract source (genotype)	Preincubation mixture	Incubation mixture	Glu-tRNA formation (cpm)	Net sp act (nmol mg of protein^{-1} h^{-1})
hem$^+$	Buffer	Unincubated	1,180	
hem$^+$	Buffer	Complete	3,490	
hem$^+$	RNase	Complete	1,160	
hem$^+$	Buffer	With tRNA mixture	22,990	1,420
hem$^+$	Buffer	With tRNA^{Glu}	84,340	5,200
hemA	Buffer	Unincubated	910	
hemA	Buffer	Complete	2,250	
hemA	RNase	Complete	970	
hemA	Buffer	With tRNA mixture	11,520	770
hemA	Buffer	With tRNA^{Glu}	48,110	3,220

[a] All samples were preincubated for 30 min at 37°C in assay buffer with or without 0.1 μg of RNase A as indicated. Incubations were started by the addition of the substrates and ATP. Complete incubation mixture contained, in 100 μl assay buffer, 1 μl of enzyme extract (high-speed supernatant, 3.5 to 3.8 μg of protein) from strain HB101 (*hem*$^+$) or strain SASX41B (*hemA*) cells, 5 μCi (100 μM) of 3,4-[^{3}H]glutamate, 5 mM ATP, and 1.25 A_{260} units of *E. coli* tRNA mixture or 0.16 A_{260} units of *E. coli* tRNA^{Glu}, as indicated. Incubation was at 37°C for 1.25 min.

TABLE 5. ALA formation from glutamyl-tRNA by extracts of *hem*$^+$ and *hemA* *E. coli* cells[a]

Cell extract source (genotype) for:		ALA formation (cpm)
Glu-tRNA formation	ALA formation assay	
hem$^+$	None	50
hem$^+$	*hem*$^+$	1,210
hem$^+$	*hemA*	30
hemA	None	50
hemA	*hem*$^+$	1,300
hemA	*hemA*	30

[a] 3,4-[^{3}H]Glutamyl-tRNA substrate was prepared by using extract from either strain HB101 (*hem*$^+$) or strain SASX41B (*hemA*) cells as described in the text. Incubations were started by the addition of 20 μl of assay buffer containing 12,746 cpm (*hem*$^+$ extract generated) or 10,745 cpm (*hemA* extract generated) of 3,4-[^{3}H]glutamyl-tRNA. The complete incubation contained, in 250 μl of assay buffer, 200 μl of enzyme extract (high-speed supernatant, 720 μg of protein), 1 mM NADPH, 20 μM PALP, and 5 mM levulinate. Incubation was at 37°C for 1 h.

duced by extracts of both *hem*$^+$ and *hemA* cells. Extract of *hem*$^+$ cells converted glutamyl-tRNA produced by both cell extracts to ALA to about the same extent, whereas extract of *hemA* cells was unable to convert either substrate to ALA (Table 5).

Conversion of GSA to ALA by extracts of *hem*$^+$ and *hemA* cells. Extracts of *hem*$^+$ and *hemA* cells were both able to convert GSA to ALA, although the *hemA* cell extract was somewhat less active than the *hem*$^+$ cell extract (Table 6).

Complementation of ALA-forming activity in extract of *hemA* cells by *Chlorella* enzyme fractions. The *Chlorella* enzyme fractions were prepared as described previously (1, 1a). Supplementation of the extract of *hemA* *E. coli* cells with the *Chlorella* enzyme fractions indicated that the only enzyme fraction capable of restoring ALA-forming activity was the one containing the enzyme glutamyl-tRNA dehydrogenase (Table 7). None of the *Chlorella* enzyme fractions catalyzed ALA formation alone, although all three enzymes catalyzed ALA formation when incubated together. The ALA-forming capacity of the *hemA* cell extract when supplemented with the *Chlorella* dehydrogenase-containing enzyme fraction was approximately equal to that of unsupplemented extract of *hem*$^+$ cells.

DISCUSSION

Cell extracts of the *hem*$^+$ *E. coli* strain HB101 incorporated label into ALA from 1-[^{14}C]glutamate but did not incorporate label to any appreciable extent from 2-[^{14}C] glycine. In vitro ALA formation was completely dependent

TABLE 6. ALA formation from GSA by extracts of *hem*$^+$ and *hemA* *E. coli* cells[a]

Cell extract source (genotype)	ALA formation (nmol)	Net sp act (nmol mg protein^{-1} h^{-1})
hem$^+$ (control)	1.16	
hem$^+$	6.94	40
hem$^+$ (control)	1.29	
hemA	4.32	24

[a] Incubation mixtures contained 100 μl of extract (high-speed supernatant) containing 0.18 (strain HB101, *hem*$^+$) or 0.35 (strain SASX41B, *hemA*) mg of protein, 20 μM PALP, 64 μM GSA, and 5 mM levulinate in 225 μl of assay buffer. Incubation was at 30°C for 20 min. Control reactions were carried out to measure nonenzymatic conversion of GSA to ALA. In the control reactions the cell extract was added at the end of the incubation.

TABLE 7. ALA formation from glutamate by extracts of *hemA* *E. coli* cells complemented by *Chlorella* enzyme fractions[a]

Cell extract source (genotype)	*Chlorella* enzyme(s) added	ALA formation (cpm)
None	Glu-tRNA synthetase	120
None	Dehydrogenase	130
None	Aminotransferase	60
None	All three enzymes	10,380
hemA	None, unincubated	60
hemA	None	70
hemA	Glu-tRNA synthetase	110
hemA	Dehydrogenase	4,360
hemA	Aminotransferase	110
hem$^+$	None, unincubated	70
hem$^+$	None	4,090

[a] Incubations contained in 250 μl of assay buffer 150 μl of *E. coli* HB101 (*hem*$^+$) or SASX41B (*hemA*) cell extract (high-speed supernatant, 0.52 μg of protein), 50 μl of *Chlorella* extract fractions containing the indicated enzyme activities (glutamyl-tRNA synthetase, 115 μg of protein; dehydrogenase, 65 μg of protein; aminotransferase, 565 μg of protein), 1 mM NADPH, 5 mM each ATP and levulinate, 20 μM PALP, 0.16 A_{260} units of *E. coli* tRNA^{Glu}, and 10 μM glutamate containing 1 μCi of 1-[^{14}C]glutamate. Incubation was at 30°C for 1 h.

on the addition of reduced pyridine nucleotide to the incubation mixture and partially dependent on added glutamate and ATP. As with the *Chlorella* enzyme system, dependence on added PALP could not be demonstrated at this stage of purification (1a). The fact that the label from C-1 of glutamate is incorporated and the sensitivity of that incorporation to RNase indicate that the precursor of ALA is the complete carbon skeleton of glutamate and that RNA is involved in the reaction sequence. The substrate and cofactor requirements for ALA formation and the sensitivity of the reaction to RNase and the aminotransferase inhibitor gabaculine are consistent with the conclusion that the biosynthetic steps between glutamate and ALA in *E. coli* are similar to those of the five-carbon pathway that was originally characterized in plant and algal species (4, 5, 7, 15, 28) and also recently found in other bacteria, including protosynthetic and methanogenic species (6, 16, 17, 19; Rieble et al., Plant Physiol. **86**:S-60; Avissar et al., in press). Because glycine is not converted to ALA, ALA synthase activity is apparently lacking in *E. coli*. It can therefore be concluded that *E. coli* HB101, a commonly used laboratory strain, utilizes the five-carbon pathway for ALA biosynthesis. This conclusion is consistent with the results of Li et al. (11a; J. Cell Biol. **107**:617a), who reported that intact cells and cell extracts of a *hemA* strain of *E. coli*, which had been transformed to ALA prototrophy by a multicopy plasmid bearing DNA from a *hem*$^+$ strain, formed ALA from glutamate but not from glycine.

The exclusive operation of the five-carbon pathway for ALA biosynthesis in *E. coli* is also consistent with the placement of this species within the γ subgroup of purple bacteria (29). Another member of the γ subgroup, *Chromatium vinosum*, was found to form ALA exclusively via the five-carbon ALA route (16; Avissar et al., in press). It is of interest that, although *hem*$^+$ cells form ALA via the five-carbon route, *hemA* cells, when transformed by a plasmid carrying a gene from another species coding for ALA synthase, are capable of using the gene product to form ALA and thereby escape ALA auxotrophy (11, 24). Thus, the existence of the five-carbon pathway in *hem*$^+$ *E. coli* cells cannot be attributed to a metabolic or physiological incom-

patibility (e.g., lack of appropriate precursor) with the ALA synthase reaction.

The identification of the five-carbon ALA biosynthetic route in *E. coli* raised the question of the biochemical character of the defect conferred by the *hemA* mutation, which results in ALA auxotrophy. Inability to form ALA could, in principle, result if any one of the three enzymes involved in the conversion of glutamate were missing or inactive or if the required tRNA molecule were missing. Extracts of cells bearing the *hemA* mutation can be tested for the activity of each of these enzymes in partial reactions, and the possible lack of tRNA can be assessed by adding glutamyl-tRNA in vitro. Also, extracts of the mutant can be supplemented with isolated enzyme components from other species to test for in vitro complementation of the mutation.

The assay of the complete reaction sequence indicated that the addition of $tRNA^{Glu}$ did not restore activity to the extract obtained from the mutant. Assay of the partial reactions involved in the ALA biosynthetic sequence indicated that the extract obtained from *hemA* cells is capable of aminoacylating $tRNA^{Glu}$ and producing a glutamyl-tRNA molecule that can serve as substrate for ALA production in extracts of hem^+ cells. However, the extract of *hemA* cells is incapable of producing ALA from either glutamate or glutamyl-tRNA. Inability to produce ALA from glutamyl-tRNA may result from either an inactive dehydrogenase or an inactive aminotransferase, or both. The ability of extract from *hemA* cells to catalyze the conversion of GSA to ALA allows the conclusion that the enzyme activity that is lacking in the mutant is the dehydrogenase.

Further evidence supporting this conclusion is provided by the results of heterologous reconstitution experiments with isolated enzyme fractions of the *Chlorella* ALA-forming system that were characterized previously (1). The ability to produce ALA from glutamate was regained in *hemA* cell extracts only upon supplementation with the *Chlorella* fraction containing glutamyl-tRNA dehydrogenase activity. Supplementation with other *Chlorella* fractions, containing glutamyl-tRNA synthetase and GSA aminotransferase activities, did not restore ALA-forming ability to *hemA* cell extracts.

Our results are consistent with the *hemA* mutation being an actual deletion of the structural gene for the dehydrogenase or a mutation causing a loss of its activity, but they are also consistent with a regulatory mutation affecting the expression of the dehydrogenase gene.

Although the enzyme activities of the other two enzymes involved in ALA biosynthesis were present in extract of *hemA* cells, their activities were somewhat lower than those in hem^+ cell extracts. These differences may be attributable to the slightly lower growth rate of the mutant, even in the presence of 20 μM ALA. Alternatively, they may be due to coordinate regulation of the enzymes involved in ALA biosynthesis, resulting in a lower cellular content of the glutamyl-tRNA synthetase and the aminotransferase when the dehydrogenase is not produced, or a lower rate of synthesis of these enzymes in the presence of exogenous ALA. Another possibility is that *hemA* is a regulatory mutation that affects the cellular levels of all three enzymes, with glutamyl-tRNA dehydrogenase being most affected.

The lowered activity of the glutamyl-tRNA synthetase in *hemA* cells is probably not a direct consequence of the absence of ALA-forming activity, because it is likely that ALA biosynthesis diverts only a small portion of the total glutamyl-tRNA produced and therefore would not be expected to exert major regulatory influence on the activity of this enzyme. The higher in vitro specific activity of glutamyl-tRNA synthetase with $tRNA^{Glu}$ as a substrate, compared with the activity measured with a tRNA mixture as a substrate, can be ascribed to the inhibitory effect of various tRNAs, present in the mixture, on the aminoacylation of $tRNA^{Glu}$ (22) or to the activities of other aminoacyl-tRNA synthetases in the cell extract in the absence of their specific tRNAs (25).

The presence of the five-carbon pathway in *E. coli* suggests that, in addition to *hemA*, there may be other sites on the genome affecting enzyme activities involved in ALA biosynthesis, which may be mutated to give rise to ALA auxotrophs. The *popC* mutation (18) may be one of these. However, it appears likely that the pathways of ALA biosynthesis and protein biosynthesis share the same glutamyl-tRNA and its specific synthetase, thus making mutations in the genes coding for either of these two components unlikely to specifically affect ALA biosynthesis. In the cyanobacterium *Synechocystis* sp. strain PCC 6803, for example, the tRNA involved in ALA synthesis is also used for protein synthesis (23). The aminotransferase involved in the last step of ALA production may be specific to this reaction sequence. Further work is required for the characterization of existing mutants (e.g., *popC*) and for the isolation and characterization of additional ALA auxotrophs before these questions can be answered.

In summary, cell extracts obtained from *E. coli* SASX41B carrying the *hemA* mutation are incapable of catalyzing the conversion of glutamate or glutamyl-tRNA to ALA, whereas they do form glutamyl-tRNA and convert GSA to ALA. These results indicate that the *hemA* mutation confers a deficiency in glutamyl-tRNA dehydrogenase—the enzyme catalyzing the conversion of glutamyl-tRNA to GSA or a physiologically equivalent intermediate that is the immediate precursor to ALA. This mutant, although isolated 20 years ago (20), has now been biochemically characterized for the first time.

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