Regulation of Heme Biosynthesis in *Salmonella typhimurium*: Activity of Glutamyl-tRNA Reductase (HemA) Is Greatly Elevated during Heme Limitation by a Mechanism Which Increases Abundance of the Protein

LI YING WANG, LARISSA BROWN, MEENAL ELLIOTT, AND THOMAS ELLIOTT*

Department of Microbiology and Immunology, West Virginia University Health Sciences Center, Morgantown, West Virginia 26506

Received 23 December 1996/Accepted 11 February 1997

In *Salmonella typhimurium* and *Escherichia coli*, the *hemA* gene encodes the enzyme glutamyl-tRNA reductase, which catalyzes the first committed step in heme biosynthesis. We report that when heme limitation is imposed on cultures of *S. typhimurium*, glutamyl-tRNA reductase (HemA) enzyme activity is increased 10- to 25-fold. Heme limitation was achieved by a complete starvation for heme in *hemB, hemE*, and *hemH* mutants or during exponential growth of a *hemL* mutant in the absence of heme supplementation. Equivalent results were obtained by both methods. To determine the basis for this induction, we developed a panel of monoclonal antibodies reactive with HemA, which can detect the small amount of protein present in a wild-type strain. Western blot (immunoblot) analysis with these antibodies reveals that the increase in HemA enzyme activity during heme limitation is mediated by an increase in the abundance of the HemA protein. Increased HemA protein levels were also observed in heme-limited cells of a *hemL* mutant in two different *E. coli* backgrounds, suggesting that the observed regulation is conserved between *E. coli* and *S. typhimurium*. In *S. typhimurium*, the increase in HemA enzyme and protein levels was accompanied by a minimal (less than twofold) increase in the expression of *hemA-lac* operon fusions; thus HemA regulation is mediated either at a posttranscriptional step or through modulation of protein stability.

In *Salmonella typhimurium* and *Escherichia coli*, heme is essential both for respiration and in defense against the toxic oxygen metabolite H$_2$O$_2$. Heme $b$ (Fe protoporphyrin IX or protopoheme) and various modified hemes are cofactors for a number of cytochromes as well as two catalases (2, 13, 34). The heme biosynthetic pathway also branches to produce two other tetrapyrroles: siroheme, the cofactor for sulfite and nitrite reductases (31, 47), and cobalamin (vitamin B$_{12}$). *S. typhimurium* synthesizes cobalamin de novo under anaerobic or low-oxygen growth conditions (1, 37). Thus, the products of the branched heme biosynthetic pathway have a variety of functions related to oxygen, respiration and electron transfer.

The biochemistry of heme synthesis is well established, and, with the exception of the initial reactions leading to 5-aminolevulinic acid (ALA), the pathway is conserved among all organisms that make heme (7, 16, 29). However, two different mechanisms have been found for the synthesis of ALA in nature: either by a $C_5$ route from glutamate or by a $C_4$ route from succinyl coenzyme A and glycine (6, 27). *S. typhimurium* and *E. coli* use the $C_4$ route (4, 20, 32, 36). The key $C_4$ enzyme glutamyl-tRNA reductase converts charged glutamyl-tRNA$^{\text{Glu}}$ to glutamate-1-semialdehyde (GSA) or its cyclic form (Fig. 1). GSA is then converted to ALA by the *hemL*-encoded enzyme, glutamate-1-semialdehyde aminotransferase (reviewed in references 7 and 29); a nonenzymatic pH-dependent conversion of GSA to ALA is also observed in vitro (25). Since only a small fraction of the charged tRNA$^{\text{Glu}}$ of the cell is used to make heme, the reductase reaction is considered to be the first committed step in heme and tetrapyrrole biosynthesis.

Heterologous expression in *Saccharomyces cerevisiae* (45) and tRNA$^{\text{Glu}}$ substrate specificity studies (5) showed that the *hemA* gene encodes glutamyl-tRNA reductase. Null mutations in *hemA* cause a severe ALA auxotrophy in *S. typhimurium*, confirming the central role of HemA in the pathway (18, 21). Both the 46-kDa HemA protein and another glutamyl-tRNA reductase of 85 kDa have been purified from *E. coli* cells; the origin and metabolic role of the latter enzyme are unknown (26).

Indirect evidence has strongly suggested that the synthesis of heme is regulated in enteric bacteria (7). First, the levels of heme found in the membrane vary depending on the mode of growth (see, e.g., references 21, 24, and 38). Second, it was found that the amount of heme and, in particular, the glutamyl-tRNA reductase activity in *E. coli* can be increased dramatically by treatment with certain thiols and that this increase is blocked by chloramphenicol (28). Third, it is commonly observed that *E. coli* strains carrying multicopy plasmids encoding heme proteins (whether a catalase, cytochrome, or hemoglobin) are visibly red and may overproduce heme as much as 10- to 20-fold (see, e.g., references 23, 29a, and 48).

It is likely that ALA synthesis determines the rate of heme synthesis in *E. coli*. Strains carrying cloned *hemA* genes of various species excrete ALA and have a fluorescent red phenotype due to tetrapyrrole overproduction (12, 14, 32), while cells overproducing the *hemL* and HemB enzymes do not have a fluorescent phenotype (14). This suggests that additional HemA enzyme increases the flux through the pathway but additional HemL or HemB does not. ALA production in organisms other than the enteric bacteria is known to be regulated at the levels of both gene expression and enzyme activity.
(see, e.g., references 31a and 51). In previous work, we examined the expression of hemA-lac operon fusions during heme limitation and found only modest effects on expression (15). Furthermore, this effect was very small unless pyruvate was present in the Luria-Bertani (LB) medium. Neither the extent of regulation nor the involvement of arcA observed in a previous study (17) could be confirmed.

In this work, heme regulation has been investigated by direct analysis of the glutamyl-tRNA reductase (HemA) enzyme activity present in crude extracts of S. typhimurium. We demonstrate that HemA activity is elevated substantially (10- to 25-fold) when cells are limited for heme. Heme limitation was achieved either by complete starvation of mutants blocked at various steps of the pathway after ALA (Fig. 1B) or by leaky growth of a hemL mutant. A glutathione-S-transferase (GST)–HemA fusion protein containing all but the N-terminal 23 amino acids of HemA was overproduced and was used to elicit a panel of monoclonal antibodies that react specifically with HemA. Western blot (immunoblot) analysis confirmed that HemA protein abundance is increased in parallel with its enzymatic activity during heme limitation.

### MATERIALS AND METHODS

#### Bacterial strains and growth of cultures

The bacterial strains used in this study are listed in Table 1. All S. typhimurium strains are isogenic with the wild-type strain LT-2 except for the indicated mutations. Details of the pathway (40) and properties are given in the references listed in Table 1. The hemL mutant strain TE472 is a deletion mutant lacking nearly all of the hemL gene. The hemA60 mutant strain TE719 carries a point mutation that maps to the C terminus of hemA; the hemA::Kan insertion in strain TE739 is at the NheI site at codon 161 of hemA (10). The hemA insertion strain carries plasmid pTE367 to provide prfA, an essential function (18, 22). The Mud-J insertions in the hemB, hemE, and hemH genes were characterized previously but have not been localized precisely within the respective genes (49).

All cultures were grown at 37°C in either LB medium (43) or modified (9) minimal MOPS (morpholinepropanesulfonic acid) medium (35) containing 0.2% glycerol as the carbon source. Plates were prepared with nutrient agar (Difco) plus 5 g of NaCl per liter or with NCE medium (8) plus 0.2% glycerol as the carbon source. Heme was prepared as described and referenced (49) and used at 10 μg/ml. ALA was used at 2 μM in minimal medium (21).

Starvation of hmb, hemL, and hemH strains was carried out as follows. Overnight cultures grown in LB medium with heme were diluted 1:100 and grown in 25 ml of LB medium plus heme to an optical density at 600 nm (OD_{600}) of 0.5. The cells were collected by centrifugation, washed with LB medium, and resuspended in 250 ml of LB medium prewarmed to 37°C. Growth was continued for 3 h before harvest. The terminal OD_{600} was ~0.3. For adaptation of the hemL mutant strain TE472, cells were first grown overnight in minimal MOPS-glycerol with 2 μM ALA, diluted 1:10 into the same medium, and grown to an OD_{600} of 0.4. Flasks were rapidly chilled in ice-water and stored at 4°C overnight. Cells (37.5 ml) were centrifuged and resuspended in a final volume of 400 ml of minimal MOPS-glycerol medium and split into two parts, and to one portion ALA was added to 2 μM. For the experiment in which the hemL mutant strain TE472 was grown without ALA by serial dilution (see Results), growth was stopped at 12 h by chilling the flask in ice-water, cells were stored overnight at 4°C, and growth was resumed the next day by returning the flask to 37°C. Control experiments showed this procedure had negligible effects on the growth curve.

#### Preparation of cell extracts

Cultures were grown as described above or (for the wild type) from a 1:100 dilution of an overnight culture which was grown to

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. typhimurium</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LT-2</td>
<td>Wild type</td>
<td>Lab collection</td>
</tr>
<tr>
<td>TE719</td>
<td>hemA60</td>
<td>TTI1991 (21)</td>
</tr>
<tr>
<td>TE1303</td>
<td>hemE1 env-53</td>
<td>From SAST40 (17a, 21)</td>
</tr>
<tr>
<td>TE2504</td>
<td>hemE509::Mud-J env-53 zde-1858::Tn10d-Tet hemA+</td>
<td>49</td>
</tr>
<tr>
<td>TE2695</td>
<td>hemB479::Mud-J env-53 zde-1858::Tn10d-Tet hemA+</td>
<td>49</td>
</tr>
<tr>
<td>TE2698</td>
<td>hemH465::Mud-J env-53 zde-1858::Tn10d-Tet hemA+</td>
<td>49</td>
</tr>
<tr>
<td>TE2701</td>
<td>hemB479::Mud-J env-53 zde-1858::Tn10d-Tet hemA60</td>
<td>49</td>
</tr>
<tr>
<td>TE3726</td>
<td>LT-2/pTE367</td>
<td>18</td>
</tr>
<tr>
<td>TE3739</td>
<td>hemA702::Kan/ pTE367</td>
<td>18</td>
</tr>
<tr>
<td>E. coli</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DH5α</td>
<td>K-12 F− λ− endA1 hsdR17 (rK− mK+) supE44 thi-1 recA1 glyA96 (Nal') relA1 ΔlacZYA-argF169 (Δ889lacZΔM15)</td>
<td>P. Higgins</td>
</tr>
<tr>
<td>MC4100</td>
<td>K-12 F− λ− ΔlacI1939 Δ(lacI-1939, argF169)U169 fbl-5301 relA1 rpsL150 deoC1 ptsF25 rbsR</td>
<td>J. S. Parkinson</td>
</tr>
<tr>
<td>TE5814</td>
<td>MC4100 hemA41</td>
<td>15</td>
</tr>
<tr>
<td>TE6160</td>
<td>MC4100 hemL::Kan (EcoRI)</td>
<td>This study</td>
</tr>
<tr>
<td>MG1655</td>
<td>K-12 F− λ− prototroph</td>
<td>D. Bick</td>
</tr>
<tr>
<td>TE4288</td>
<td>MG1655 hemL::Kan (EcoRI)</td>
<td>This study</td>
</tr>
<tr>
<td>BL21(DE3)</td>
<td>B F− hsdS gal (ΔlacI− lacP− rpsL::T7 gene 1)</td>
<td>F. W. Studier</td>
</tr>
</tbody>
</table>
a final OD_{600} of 0.4. The cultures were chilled, and the cells were recovered by centrifugation, washed several times, and finally resuspended in 1/100 volume of assay buffer (150 mM Tricine [pH 7.9], 0.3 M glycerol, 20 mM MgCl₂, 1 mM dithiothreitol, 20 mM pyridoxal phosphate) also containing 200 μM phenylmethylsulfonyl fluoride. The cells were disrupted by passage through a French press; extracts were clarified by centrifugation at 11,000 x g for 10 min at 4°C, supplemented with 200 μM phenylmethylsulfonyl fluoride, and stored in aliquots at −70°C. Protein concentrations were 1 to 3 mg/ml as determined by an assay with bovine serum albumin as the standard.

Preparation of the substrate. Purified E. coli tRNA^{Glu} was obtained from Sigma (R-6591) and charged with [3H]glutamate by the method of Schneegurt et al. (41). Ten absorbance units (260 nm) of tRNA (manufacturer) were dissolved in 100 μl of trichloroacetic acid-precipitable material was extracted with phenol and then with chloroform–isoamyl alcohol (24:1). Aliquots of extracts were clarified by centrifugation, washed several times, and finally resuspended in 1/100 volume of 1 M citric acid, 250 mM NaCl–1 mM dithiothreitol and stored frozen at −20°C. (Buffers for charging reactions were prepared in diethylpyrocarbonate-treated water.) Charging was carried out for 15 min at 37°C in 100 μl of buffer containing 25 mM Tris-HCl [pH 7.5], 5 mM MgCl₂ and, in addition, 15 μl (1.5 absorbance units) of tRNA^{Glu}, 25 μCi of [2,3,4-3H]glutamate (Amersham; 49 Ci/mmol, 1 μCi/μl), 5 μM unlabeled glutamate, 5 mM ATP, and 9 μl (18 μg) of a crude extract containing glutamyl-tRNA synthetase. The final specific activity of labeled glutamyl-tRNA synthesis was about 20,000 cpm/mmol, assuming a counting efficiency of 40%. The synthetase was prepared from E. coli HB101 overexpressing E. coli glutamyl-tRNA synthetase (pLQ7611-LnynJ) (10) and centrifuged at 150,000 x g for 90 min. In some experiments, 8 μg of purified synthetase was used (a kind gift of J. LaPointe). In either case, the total incorporation of [3H]glutamate into cold trichloroacetic acid-precipitable material was ~180 pmol (4 x 10⁵ cpm). Reactions were terminated by addition of 2 volumes of 0.1 M morpholinethanesulfonic acid (MES; pH 5.8)–10 mM MgCl₂–10 mM glutamate. The products were extracted with phenol and then with chloroform–isooctyl alcohol (24:1). Aliquots were ethanol precipitated after addition of 1/10 volume of 3 M sodium acetate (pH 5.2). Charged tRNA was stored as an ethanol precipitate at −20°C and was stable for several weeks.

Glutamyl-tRNA reductase (HemA) enzyme assay. After the charging reaction, glutamyl-tRNA^{Glu} was used for a direct assay of HemA. The charged tRNA substrate was recovered by centrifugation, dried briefly, and resuspended in assay buffer. Each reaction mixture contained 100,000 cpm of substrate and, in addition, 2 mM NADPH, 5 mM levulinic acid (to inhibit HemB), 2 μl of RNasin (Promega) and 50 to 150 μl of extract containing 50 to 450 μg of protein in a final volume of 250 μl. Incubation was carried out for 60 min at 37°C. The reactions were terminated by the addition of 50 μl of 1 M citric acid, 250 μl of 10% sodium dodecyl sulfate, and 20 μl of 1 mM unlabeled ALA. The mixtures were heated at 95°C for 2 min, cooled, and microcentrifuged. ALA and GSA product in the supernatant was purified by ion-exchange chromatography on Dowex 50W-X8 (Na+), and the eluate was derivatized with ethyl acetocetate and extracted into ether exactly as described previously (20), except that the pH 4.25 wash was omitted. Radioactivity was determined by liquid scintillation counting in ScintiVerse II (Fisher).

We characterized the assay with respect to dependence on the amount of extract added (the genetic requirements for activity are described in Results). Figure 2 shows that formation of the product was linearly dependent on the amount of extract added over the range assayed (up to 1 mg of total protein per ml). The extract analyzed in this experiment was derived from a starved hemB mutant (see Results) and contained a high level of activity. A similar linear dependence on the amount of extract was obtained with extracts of low activity from wild-type cells (data not shown).

Overexpression of GST-HemA hybrid protein. A derivative of the S. typhimurium hemE gene which carries a BamHI linker upstream of codon 24 was inserted into the GST fusion vector pGSTag (39). The insert is bounded on the downstream side by an EcoRI site placed just beyond the hemE TAG codon by using PCR. This construct produces large amounts of GST-HemA fusion protein after induction of the tac promoter with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Crude extracts were prepared by disruption in a French press and clarified by centrifugation; the fusion protein was found almost entirely in the pellet, from which it could be released by washing with 20 mM Tris-HCl (pH 8.0)–1 mM EDTA–100 mM NaCl–0.5% Nonidet P-40–1% Sarkosyl.

Generation of monoclonal antibodies. A solubilized cell extract containing the GST-HemA fusion protein was dialyzed extensively against phosphate-buffered saline and used as the immunizing antigen. BALB/c mice were initially immunized with extract emulsified with complete Freund’s adjuvant by the intramuscular and subcutaneous routes and then given four boosts of antigen without the adjuvant, at 4-day intervals. Cells harvested from inguinal and popliteal lymph nodes and spleens the day after the final boost were hybridized with the nonsecreting myeloma partner P3X63.Ag8.653 as previously described (30) and placed in hyposynthetic-aminopterin-thymidine-containing medium. Supernatants from wells containing growth were screened 3 weeks later for binding to the GST-HemA extract in an enzyme-linked immunosorbent assay. Wells containing antibodies reactive with GST were eliminated by screening against an extract prepared from cells containing the parent vector pGSTag and induced for GST expression with IPTG. The specificity of antibodies was further confirmed by Western blot as well as immunoprecipitation analysis. Screening was done initially with the extract used for immunizations and subsequently with an extract containing overexpressed truncated HemA Q369Am protein. Appropriate hybridomas were subclassed by limiting dilution to establish monoclonals. The isotypes of anti-HemA antibodies were determined in an ELISA with a panel of biotin-conjugated isotype-specific antibodies (Southern Biotechnology).

Immunological detection of proteins. Techniques for Western blotting have been described in detail previously (11). The primary antibody was a mouse monoclonal anti-HemA antibody of the γ1 isotype, which was detected by sequential application of biotin-conjugated goat anti-mouse immunoglobulin G1 followed by streptavidin-conjugated horseradish peroxidase (Southern Biotechnology) and was finally visualized by enhanced chemiluminescence (Amersham).

β-Galactosidase assays. Cells were centrifuged and resuspended in Z-buffer (100 mM NaPO₄ [pH 7.0], 10 mM KCl, 1 mM MgSO₄) and then permeabilized by treatment with sodium dodecyl sulfate and chloroform (33). Assays were performed in Z-buffer containing 50 mM β-mercaptoethanol by a kinetic method using a plate reader as described previously (11). Activities (ΔOD₄₂₀ per minute) are normalized to the cell density in the assay.

RESULTS

Graphic illustration of heme regulation. A hem mutant defective in uroporphyrinogen III synthase (hemD) or in any subsequent step of the heme pathway will accumulate tetapyrrole(s) before the block. The accumulated intermediates can be visualized because of the red fluorescence of porphyrins under UV light. We have observed that in colonies of such hem mutants, the intensity of the red fluorescence and thus the level of tetrapyrrole accumulation become settled. Therefore, the heme concentration is decreased. This control of heme precursor synthesis by the product of the pathway can be simply visualized as shown in Fig. 3. A plate test of the growth response of a hemE mutant (defective in uroporphyrinogen decarboxylase) was observed on selective medium results in the establishment of a radial concentration gradient. As the heme concentration drops below the threshold for effective supplementation, starvation ensues and growth fails. Examination of the plate under UV light shows that the fluorescence of the accumulated uroporphyrin is much greater in the region where cells are starving for heme. In visible light, the orange uroporphyrin can also be seen as a ring at the periphery of the spot of cell growth.

Assay of glutamyl-tRNA reductase (HemA). To investigate the basis for the apparent regulatory effect of heme limitation, we assayed the activity of HemA, the first committed enzyme in the pathway (see Materials and Methods for details). The assay uses E. coli tRNA^{Glu} charged with [3H]glutamate as the substrate. After the reaction, the product is purified by ion-exchange chromatography, and a pyrrole derivative is formed.
and then extracted into ether. Given the high specific activity of the labeled glutamate used to prepare glutamyl-tRNA Glu and the low background (≈50 cpm), we can easily detect 5 fmol of product in this assay. Figure 4 shows that the activity of an extract of the wild-type strain LT-2 was about 100 fmol/mg of protein in this assay whereas the activity of an extract of a hemA mutant (hemA60) was very low and a hemA::Kan insertion mutant had no detectable activity (data not shown). Cultures for this experiment were grown with 2 mM ALA present; identical results were obtained when the wild type was grown in medium lacking ALA.

The assay does not distinguish whether the product was GSA or ALA (Fig. 1A) (see the discussion in reference 42). One reason is that at the pH of the assay, GSA is converted to ALA (and other products) at a high rate by a spontaneous, nonenzymatic mechanism (25). In addition, because we measured incorporated radioactivity rather than determining ALA colorimetrically (20), the purification allows both GSA and ALA to be counted as product. These factors account for the observation that the activity is mostly independent of hemL function (Fig. 4). We explain the slight decrease in the amount of product seen in hemL mutants by postulating that the accumulation of GSA may inhibit the HemA reaction. Recovery of GSA may also be inefficient compared to that of ALA. The activity of the HemL enzyme is about 10^4-fold higher than that observed in this assay and should not be rate limiting (20). To show that the HemA activity is actually rate limiting for product formation in all the extracts we examined, we included gabaculine, an inhibitor of HemL activity (42), in replicate assays. Gabaculine inhibits extracts of wild-type cells by about 50% but does not inhibit extracts of a hemL mutant at all. The activity observed in reaction mixtures containing gabaculine is not dependent on enzymatic conversion of GSA to ALA and thus is a specific measure of HemA activity.

Glutamyl-tRNA reductase (HemA) enzyme activity increases substantially in heme-starved cells. To examine the influence of starvation for heme on HemA enzyme activity, three strains were constructed and assayed. Each strain carries a mutation in a different hem gene (hemB, hemE, or hemH), but all are hemA^+. Cultures of these strains were grown to exponential phase (OD_{600} = 0.5) in LB medium containing 10 μg of heme per ml, washed, and diluted into LB medium without heme. Slow starvation is characteristic of hemA mutants, as well as strains blocked later in the heme pathway between ALA and heme (50), due to the catalytic function of heme-containing cytochromes in energy production. After starvation for heme and cessation of growth (3 h), the cells were harvested and extracts were prepared and assayed.

For each mutant, HemA activity was dramatically increased compared to that in an extract of the wild type (Fig. 5). The hemB and hemE mutants had HemA activity 20- to 25-fold greater than that observed in wild type LT-2, while the hemH mutant was induced about 15-fold. For each extract, gabaculine treatment gave the same fractional inhibition as seen in the wild type, showing that the increase is specifically in HemA activity. Furthermore, no activity was observed in the starved
hemB mutant if the strain also carried the hemA60 allele, confirming that hemA function was required (46a).

This experiment strongly suggested that heme starvation increases HemA enzyme activity. A limitation of the experiment is its reliance on nongrowing cultures. We sought a condition in which exponentially growing cultures could be subjected to limitation for heme. The finding that hemL activity is not required for the assay provided a simple way to do this.

The "leaky" phenotype of hemL mutants. As described above (Fig. 1A), a simple linear pathway for the early steps in heme synthesis leading to ALA invokes the sequential action of HemA and HemL. Since GSA, the product of HemA and substrate of HemL, has no other known source or function, we should expect hemA and hemL mutants to have the same growth characteristics. However, these two mutant types are quite different. A strain carrying a null mutation in the hemL gene exhibits a "leaky" or pseudo-wild-type phenotype which is not seen with hemA mutants or with mutants blocked later in the heme pathway (19a, 20, 21, 46). This phenotype suggests that hemL mutants can transform GSA to ALA at a reduced rate and is consistent with the known enzymatic conversion of GSA to ALA.

To explore the leaky phenotype of hemL mutants further, we studied growth in liquid medium. Cultures of hemA and hemL mutants growing exponentially in minimal glycerol medium containing 2 μM ALA were centrifuged and resuspended in the same medium either with or without ALA. In the absence of ALA, the hemA mutant continued to grow at a steadily decreasing rate (with linear kinetics) until growth finally ceased after about three generations (Fig. 6A). Growth of the heml mutant also slowed and stopped in the absence of ALA; however, it resumed following a lag period of approximately 2 h. This behavior is in striking contrast to that of the hemA mutant(s) blocked later in the pathway (data not shown). After the lag, growth was exponential with a growth rate approximately 70% of that seen in the presence of ALA. Growth of a heml mutant without added ALA can be sustained at this rate for at least 10 generations, achieved by repeated fivefold dilutions (Fig. 6B). We refer to this process as the adaptation of hemL mutants to growth without ALA. Analysis of colonies grown from adapted hemL cultures shows no evidence for a genetic alteration affecting the Hem phenotype.

Adapted (heme-limited) hemL cells contain elevated HemA activity. Extracts were prepared from cultures of adapted hemL cells as well as hemL cells grown in the presence of 2 μM ALA. We observed that the activity of HemA enzyme was 10- to 20-fold elevated in adapted cells. (Compare values for ALA-grown cells on the far-left axis of Fig. 7 to those for adapted cells on the far-right axis.) This observation confirms that heme limitation can elicit an increase in the HemA enzyme activity of exponentially growing cells, similar to that seen in the starvation experiments described above. The lag period observed during adaptation may be related to the speed at which HemA enzyme can be accumulated.

We tested for the presence of diffusible inhibitors or activators of HemA activity by mixing low-activity and high-activity extracts. The results of one such experiment are shown in Fig. 7. Extracts of adapted and ALA-supplemented hemL mutant cultures were adjusted to have an equal concentration of protein, mixed in various proportions, and assayed for total HemA activity. The activity was found to be a linear function of the protein, mixed in various proportions, and assayed for total HemA activity. Analysis of colonies grown from adapted hemL cultures shows no evidence for a genetic alteration affecting the Hem phenotype.

Immunological detection of HemA protein by Western blots. We have been able to overproduce segments of HemA al-

FIG. 5. Starvation for heme induces HemA activity. Cultures of the wild-type strain LT-2 or the hemL mutants hemB (TE2603), hemE (TE2504), and hemL (TE2608) were grown in LB medium. HemA activity was assayed as described in the text; duplicate assays were performed in the absence and presence of gaba-
culine (5 μM), an inhibitor of HemL enzyme.

FIG. 6. Adaptation of a hemL mutant to growth without ALA. (A) A deletion mutant of hemL (TE472) and a hemA mutant (TE719) were grown in minimal glycerol medium in the presence of 2 μM ALA, washed, and diluted into the same medium either containing 2 μM ALA or without ALA. Growth was monitored by measuring the OD₆₅₀ of the cultures. The growth curve for the hemA mutant is shifted to the right for clarity. Growth of both hemA and hemL mutants in the presence of ALA was identical to that of the wild type (data not shown). (B) The hemL mutant was adapted to growth without ALA as in panel A, and exponential growth was maintained by repeated fivefold dilutions at the times indicated by the vertical arrows.
though not the native protein. One construct joins GST as an N-terminal segment to a deletion lacking the first 23 amino acids of hemA (see Materials and Methods for details). This construct produces massive amounts of a GST-HemA fusion protein under the control of the P
lac promoter (data not shown), and the fusion protein was used to immunize mice for generation of monoclonal antibodies.

We tested the same extracts assayed for enzyme activity (see above) to determine the abundance of HemA protein by Western blotting (Fig. 8). As seen in lanes c, e, and f, the starved hem mutant strains showed a large increase in the abundance of HemA protein compared to the wild-type strain (lane b). Native HemA was not detectable in extracts of a starved hemB mutant when the strain also carried the hemA60 allele (lane d). The hemA60 allele is an unsequenced mutation which maps to the C-terminal segment of hemA (18) and is apparently a nonsense mutation because it shows a new band of ~37 kDa. The appearance of a nonsense fragment reactive with the anti-HemA antibody further confirms the specificity of the monoclonal antibodies. In a separate experiment, we compared the level of HemA protein in the hemB mutant strain either grown with heme supplementation or starved for heme (Fig. 9). Growth in the presence of heme results in sharply reduced HemA levels.

Induction of HemA protein was also observed when extracts of adapted and ALA-grown hemL mutant cells were compared (Fig. 8, lanes g and h), and the increase was approximately equivalent to that seen with heme-starved cells. Again, high activity of HemA in the enzyme assay correlates with high levels of HemA protein detected immunologically. At least four different monoclonal antibodies from the panel react well with full-length HemA protein in extracts of S. typhimurium by Western blotting; all give identical results in experiments of the type shown in Fig. 8, except that some antibodies do not react with the hemA60 gene product. Densitometry was used to quantitate the increase in the amount of HemA protein (described in the legend to Fig. 8) in these experiments; induction ratios of 10- to 20-fold were obtained in both the heme starvation and hemL adaptation protocols.

We also asked whether HemA induction can be observed in E. coli by testing the adaptation response of E. coli hemL mutants. Adaptation to growth without ALA was observed in both the MG1655 and MC4100 backgrounds (data not shown), very similar to that shown for S. typhimurium in Fig. 6. Adapted cells of the hemL mutants of both E. coli strains contained elevated levels of HemA protein, whereas HemA was barely detectable in the wild-type strains or in hemL mutants grown with ALA supplementation (Fig. 10).

Lack of transcriptional control of HemA. In our earlier study, a small effect of starvation for heme on the expression of a hemA-lac operon fusion, in both E. coli and S. typhimurium,
was noted (15, 19). Consistent with these and other unpublished experiments, we find only a 1.5- to 2-fold increase in hemA-lac expression after starvation for heme in a hemB mutant, or during heme limitation in adapted hemL mutants, when the standard method for these experiments is used (46a). These results were obtained with a lac operon fusion to codon 181 of the hemA gene (15). Expression of another fusion to codon 416 was not changed at all in response to heme starvation. From these results, we conclude that HemA regulation is mediated either at a step after transcription initiation or through modulation of protein stability.

**DISCUSSION**

The results described here provide the first direct evidence for regulation of the heme biosynthetic pathway in enteric bacteria. The activity of the HemA enzyme (glutamyl-tRNA reductase) was substantially elevated after limitation for heme in *S. typhimurium*. The increase was 10- to 25-fold depending on the strain and the method used to impose starvation. Induction of HemA activity was observed in mutants blocked at three different places in the heme pathway: in hemB, hemE, and hemH mutants (Fig. 1B). The induction was highest in a hemB mutant and somewhat lower in the other mutants (Fig. 5); we do not know if these differences are significant. The results suggest that, to a first approximation, intermediates in the biosynthetic pathway do not significantly affect regulation. The actual effector could be protoheme itself, the immediate product of the pathway. Alternatively, regulation might be responsive to a modified heme, a heme-containing protein, or a heme-dependent process such as respiration.

HemA enzyme activity is increased by a change in the abundance of the HemA protein as determined by Western blotting. Within the limits of the methods used, the increase in the amount of protein accounts for the entire change in enzymatic activity. Operon fusions of lac to two sites in hemA result in either no increase or only a twofold increase in hemA transcription during heme limitation. We conclude that the observed regulatory response does not act on transcription initiation but either increases synthesis at a later step or decreases protein turnover. Western blot analysis showed that HemA induction can also be observed during adaptation of hemL mutants of *E. coli*, suggesting that heme synthesis is regulated similarly in these two enteric species.

We note further that the truncated HemA protein produced by the hemA660 mutant is present at an intermediate level, higher than that of native HemA in an unstressed wild-type strain but lower than the induced HemA levels seen in the starved strains (Fig. 8). Since nonsense fragments are often subject to rapid turnover by cellular proteases, it is possible that this truncated protein is actually produced at levels comparable to those of HemA, implying that its regulation could be normal. This would be compatible with regulation of either synthesis or degradation.

The enzyme assay and monoclonal antibodies developed for this study will be used to ask several other questions including whether regulation of HemA occurs in unstressed cells as a function of growth rate or nutrient composition of the medium. Preliminary experiments do not show a significant effect of excess heme on HemA levels. By analogy to the histidine and other biosynthetic pathways, two separate mechanisms could respond in alternate ways to the stress of starvation or an excess of end product. Control of HemA abundance might respond only to starvation, while HemA enzyme activity could be regulated by some type of feedback mechanism (28). Although wild-type cells are not permeable to heme, transport systems for heme are known in related species, and ALA is transported into enteric bacteria (19a). In fact, the function of the ALA transporter encoded by *dpp* is required for the adaptation of hemL mutants shown in Fig. 6A (46a).

Although the activities of the HemL and HemB enzymes are high in unstressed cells and their overproduction does not lead to tetrapture accumulation, it should be tested directly whether they are coregulated with HemA. Finally, labeling and immunoprecipitation experiments will indicate whether control of HemA abundance occurs via synthesis or turnover and provide tools for establishing the details of the mechanism.

**ACKNOWLEDGMENTS**

This work was supported by Public Health Service grant GM40403. We are grateful to Sam Beale and Pat Higgins for advice and criticism during the course of this work.

**REFERENCES**


