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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

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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_2O_2. Heme *b* (Fe protoporphyrin IX or protoheme) 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_5 route (4, 20, 32, 36). The key C_5 enzyme glutamyl-tRNA reductase converts charged glutamyl-$tRNA^{Glu}$ 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 $tRNA^{Glu}$ 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 $tRNA^{Glu}$ 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

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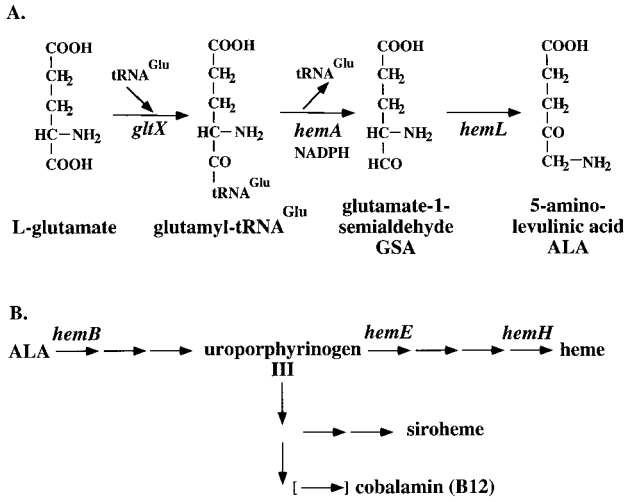

FIG. 1. The heme biosynthetic pathway. (A) Reactions leading from glutamate to ALA. Charged glutamyl-tRNA^{Glu} is the substrate for the first committed enzyme of the heme biosynthetic pathway, glutamyl-tRNA reductase (HemA), which acts sequentially with glutamate-1-semialdehyde aminotransferase (HemL) to synthesize ALA. (B) Outline of the steps between ALA and heme, including the branch point intermediate (uroporphyrinogen III) and minor products of the pathway. Mutants defective in the *hemB*, *hemE*, and *hemH* genes were used in starvation experiments. Arrows denote individual enzymes; the bracketed arrow indicates a large number of steps in vitamin B_{12} biosynthesis. Not all cofactors are shown.

(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 strain construction (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 TE3739 is at the *Nhe*I site at codon 161 of *hemA* (18). 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 *hemB*, *hemE*, 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 1:50 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 1. Bacterial strains used in this study

Strain	Genotype	Source or reference
S. typhimurium		
LT-2	Wild type	Lab collection
TE472	DEL [*zae-1868**Mud-J**hemL332*]	TT12006 (21)
TE719	*hemA60*	TT11991 (21)
TE1303	*hemE1 env-53*	From SAST40 (17a, 21)
TE2504	*hemE509*::Mud-J *env-53 zde-1858*::Tn*10d*-Tet *hemA*$^+$	49
TE2695	*hemB479*::Mud-J *env-53 zde-1858*::Tn*10d*-Tet *hemA*$^+$	49
TE2698	*hemH465*::Mud-J *env-53 zde-1858*::Tn*10d*-Tet *hemA*$^+$	49
TE2701	*hemB479*::Mud-J *env-53 zde-1858*::Tn*10d*-Tet *hemA60*	49
TE3726	LT-2/pTE367	18
TE3739	*hemA702*::Kan/pTE367	18
E. coli		
DH5α	K-12 F$^-$ λ$^-$ *endA1 hsdR17* (r_K^- m_K^+) *supE44 thi-1 recA1 gyrA96* (Nal^r) *relA1* Δ(*lacZYA-argF*)*U169* (ϕ80*dlacZ*Δ*M15*)	P. Higgins
MC4100	K-12 F$^-$ λ$^-$ *araD139* Δ(*lacIpoZYA*, *argF*)*U169 flb-5301 relA1 rpsL150 deoC1 ptsF25 rbsR*	J. S. Parkinson
TE5814	MC4100 *hemA41*	15
TE6160	MC4100 *hemL*::Kan (*Eco*RI)	This study
MG1655	K-12 F$^-$ λ$^-$ prototroph	D. Biek
TE4288	MG1655 *hemL*::Kan (*Eco*RI)	This study
BL21(DE3)	B F$^-$ *hsdS gal* (λ*lacI*$^+$ *lacp*$_{uv5}$-T7 gene 1)	F. W. Studier

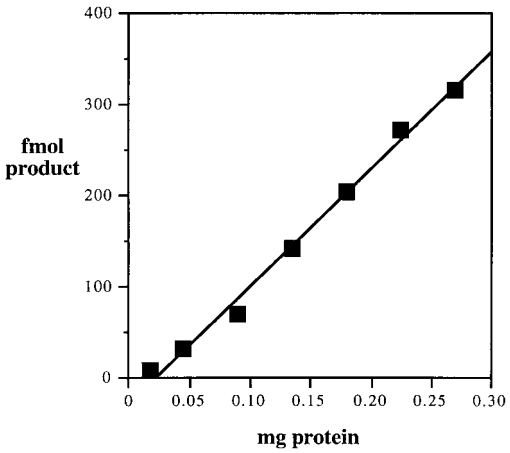

FIG. 2. HemA enzyme assay. Product formation is linearly proportional to the amount of protein. A crude extract of a *hemB* mutant (TE2695) was prepared and assayed as described in the text.

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 $MgCl_2$, 1 mM dithiothreitol, 20 μM 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 × *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 the Bradford reagent (Bio-Rad) and 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 [^{3}H]glutamate by the method of Schneegurt et al. (41). Ten absorbance units (260 nm) of tRNA (≈750 μg according to the manufacturer) were dissolved in 100 μl of 10 mM Tris-HCl (pH 7.5)–10 mM $MgCl_2$–100 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 $MgCl_2$, and, in addition, 15 μl (1.5 absorbance units) of $tRNA^{Glu}$, 25 μCi of [2,3,4-^{3}H]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 glutamate was 22,000 cpm/pmol, assuming a counting efficiency of 40%. The synthetase was prepared from *E. coli* HB101 overexpressing *E. coli* glutamyl-tRNA synthetase (pLQ7611Δ*Nru*I) (10) and centrifuged at 150,000 × *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 [^{3}H]glutamate into cold trichloroacetic acid-precipitable material was ≈180 pmol (4×10^6 cpm). Reactions were terminated by addition of 2 volumes of 0.1 M morpholineethanesulfonic acid (MES; pH 5.8)–10 mM $MgCl_2$–10 mM glutamate. The products were extracted with phenol and then with chloroform–isoamyl 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 acetoacetate 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 Scinti-Verse 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 hemA* gene which carries a *Bam*HI linker upstream of codon 24 was inserted into the GST fusion vector pGSTag (39). The insert is bounded on the downstream side by an *Eco*RI site placed just beyond the *hemA* 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 hypoxanthine-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 hybridoma wells were subcloned by limiting dilution to establish monoclones. 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 blots 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 $NaPO_4$ [pH 7.0], 10 mM KCl, 1 mM $MgSO_4$) 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 (ΔOD_{420} 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 tetrapyrrole(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 precursor accumulation become greater as the exogenous 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) to added heme 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 [^{3}H]glutamate as the substrate. After the reaction, the product is purified by ion-exchange chromatography, and a pyrrole derivative is formed

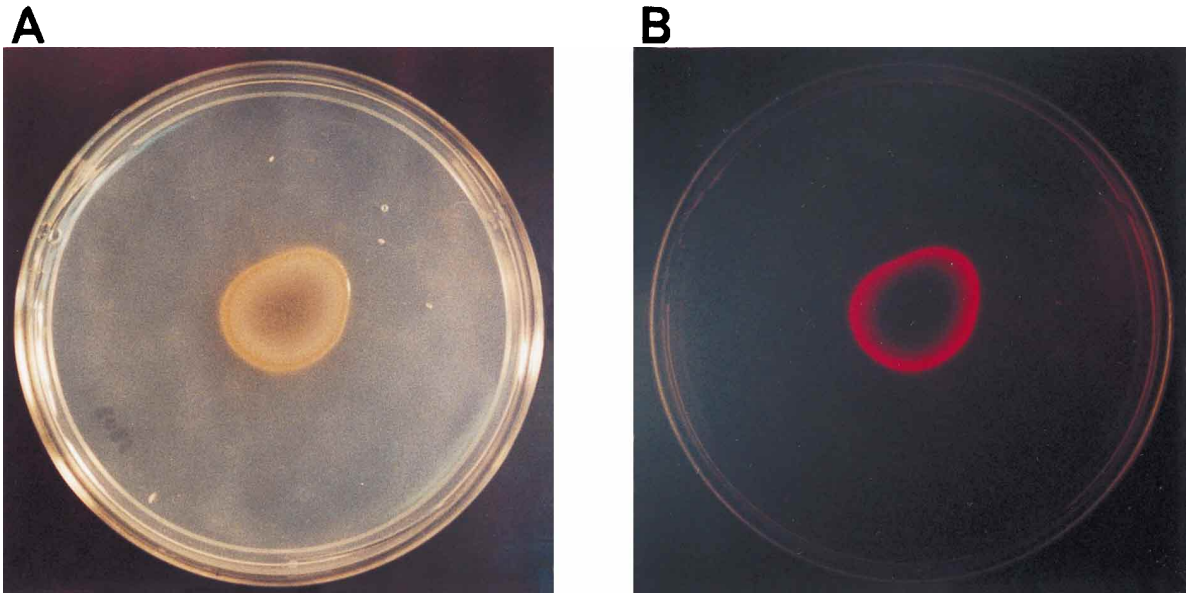

FIG. 3. Porphyrin accumulation revealed by UV fluorescence. About 10^8 cells of a *hemE* mutant strain (TE1303 *hemE1 env-53*) were spread on minimal glycerol cystine agar selective for Hem^+. A 10-μl portion of heme (4 mg/ml) was spotted in the center of the plate. After 48 h of aerobic incubation at 37°C, the plate was photographed under visible (A) or UV (B) light.

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 μM 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.

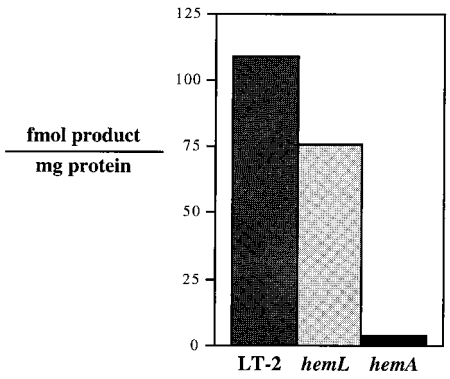

FIG. 4. Assay of *hemA* and *hemL* mutants. Cultures of a wild-type strain (LT-2), a *hemL* mutant (TE472), and a *hemA* mutant (TE719) were grown in minimal glycerol medium containing 2 μM ALA. Extracts were prepared and assayed for HemA activity as described in the text.

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

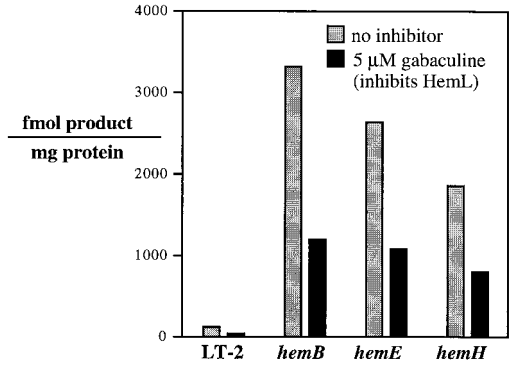

FIG. 5. Starvation for heme induces HemA activity. Cultures of the wild-type strain LT-2 or the *hem* mutants *hemB* (TE2695), *hemE* (TE2504), and *hemH* (TE2698) were grown in LB medium. HemA activity was assayed as described in the text; duplicate assays were performed in the absence and presence of gabaculine (5 μM), an inhibitor of HemL enzyme.

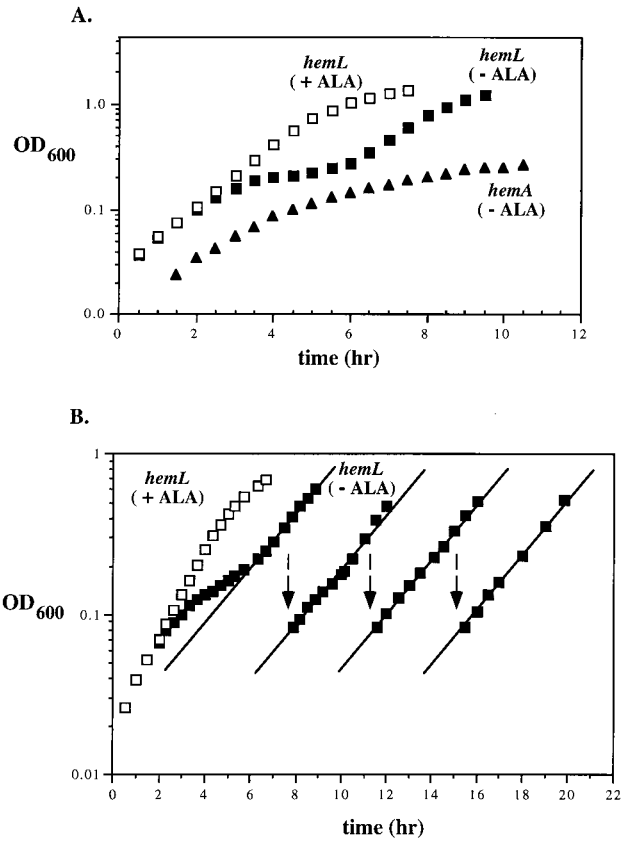

FIG. 6. Adaptation of a *hemL* mutant to growth without ALA. (A) A deletion mutant of *hemL* (TE472) and a *hemA* mutant (TE719) were grown in minimal glycerol medium in the presence of 2 μM ALA, washed, and diluted into the same medium either containing 2 μM ALA or without ALA. Growth was monitored by measuring the OD_{600} 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.

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 nonenzymatic 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 proportion of high-activity extract. Thus, this experiment shows no evidence for a diffusible activator or inhibitor. Experiments of the same design were carried out with starved *hemB* mutant and wild-type extracts with similar results (data not shown).

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

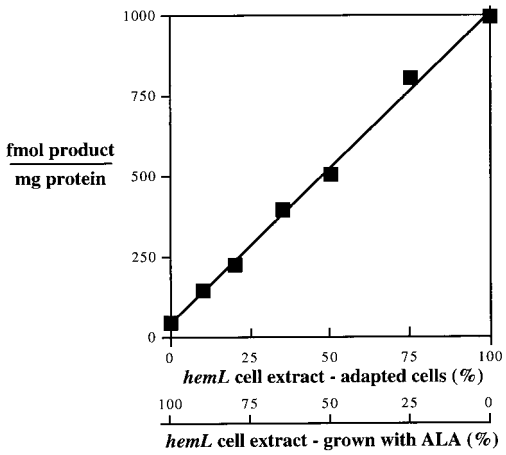

FIG. 7. Mixing experiment rules out a diffusible inhibitor of HemA. The *hemL* mutant strain TE472 was grown in minimal glycerol medium, either in the presence of 2 μM ALA or after adaptation to growth without ALA (as shown in Fig. 6 and described in the text). Extracts of both cultures were prepared and adjusted to equal protein concentration. Mixtures of the two extracts containing the indicated proportions of each component were assayed for total HemA enzyme activity.

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_{tac} 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

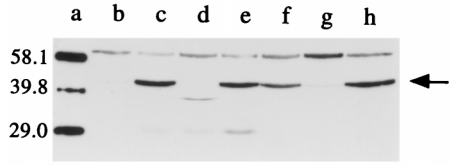

FIG. 8. Western blot analysis of HemA protein abundance. Extracts were prepared, and equal amounts of protein were analyzed by Western blotting with anti-HemA monoclonal antibody H23 as described in Materials and Methods. The arrow indicates the position of the native HemA protein. Strains analyzed and the relative amounts of HemA protein as determined by laser densitometry (arbitrary units) are as follows: lane b, wild type LT-2 (<0.5 U); lane c, TE2695 *hemB*::Mud-J (16 U); lane d, TE2701 *hemB*::Mud-J *hemA60* (1.6 U in the truncated HemA peptide); lane e, TE2504 *hemE*::Mud-J (13 U); lane f, TE2698 *hemH*::Mud-J (8 U); lane g, TE472 Δ*hemL* grown with 2 μM ALA (1 U); lane h, TE472 adapted to growth without ALA supplementation (21 U). All strains were grown under the same conditions as described for the experiments in which the HemA enzyme activity was determined. Lane a contains molecular weight standards with sizes (in kilodaltons) indicated on the left.

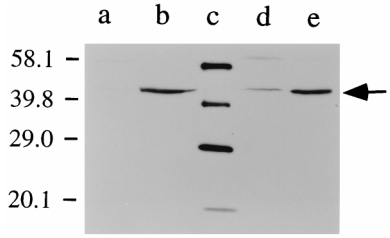

FIG. 9. HemA levels change in response to added heme. Extracts were prepared, and equal amounts of protein were analyzed by Western blotting with anti-HemA monoclonal antibody H6 as described in Materials and Methods. The arrow indicates the position of native HemA protein. Strains analyzed: lane a, extract of the wild-type strain LT-2 analyzed in Fig. 8, lane b; lane b, *hemB* mutant sample analyzed in Fig. 8, lane c; lane c, molecular weight standards; lane d, TE2695 *hemB*::Mud-J grown in LB medium with 10 μg of heme per ml; lane e, TE2695 starved for heme.

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*,

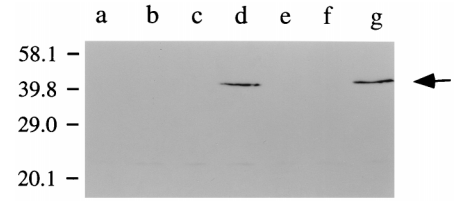

FIG. 10. HemA induction in adapted *hemL* mutant strains of *E. coli*. Extracts were prepared, and equal amounts of protein were analyzed by Western blotting with anti-HemA monoclonal antibody H6 as described in Materials and Methods. The arrow indicates the position of native HemA protein. Strains analyzed: lane a, TE5814 (MC4100 *hemA*) grown with ALA; lane b, MC4100 grown with ALA; lanes c and d, TE6160 (MC4100 *hemL*) grown with ALA (lane c) or adapted to growth without ALA (lane d); lane e, MG1655 grown with ALA; lanes f and g, TE4288 (MG1655 *hemL*) grown with ALA (f) or adapted to growth without ALA (g).

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 *hemA60* mutant is present at an intermediate level, higher than that of native HemA in an unstarved 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 unstarved cells and their overproduction does not lead to tetrapyrrole 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.

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