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Regulation of heme biosynthesis targets the key enzyme HemA by a mechanism of protein stabilization in Salmonella typhimurium

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Regulation of heme biosynthesis targets the key enzyme HemA by a mechanism of protein stabilization in *Salmonella typhimurium*.

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DISSERTATION

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ABSTRACT

Heme serves as a cofactor of cytochromes and catalases. It is essential for energy generation and in defense against toxic hydrogen peroxide in nearly all cells including *Salmonella typhimurium* and *Escherichia coli*. Indirect evidence has suggested that heme synthesis is a regulated process. Little is known about how heme synthesis is regulated in enteric bacteria even though the heme synthetic pathway is genetically well-defined. This research represents the first report that heme synthetic regulation affects the first committed heme pathway enzyme, glutamyl-tRNA reductase (HemA), by an unusual mechanism.

HemA, encoded by *hemA* gene, catalyzes the rate-limiting step of heme biosynthesis. This project demonstrated that when these bacteria are starved for heme, HemA enzyme activity and protein abundance increase 10-25 fold, while gene expression is not affected much (less than 2-fold induction). These results provide the first direct evidence that heme synthetic regulation targets HemA and suggest that the HemA regulation occurs at the post-transcriptional level.

The results of this project revealed a unique mechanism of HemA regulation by a conditional stability of the HemA protein. The half-life of HemA is about 20 min in unrestricted cells, but increases to > 300 min in heme-limited cells. The ATP-dependent proteases responsible for HemA turnover were discovered by testing *E. coli*

mutants. HemA turnover is completely blocked in a *lon clpP* double mutant, but not in either single mutant, indicating that both Lon and ClpP are involved in HemA proteolysis. ClpA, but not ClpX was further determined to have a role in HemA degradation as the chaperone of ClpP.

The amino acids of HemA that signal degradation were determined in this project. A hybrid HemA-lacZ protein containing the first 18 amino acids of the HemA N-terminal region, is also stabilized in a *lon clpP* mutant. Insertion of two lysines after the second N-terminal amino acid of HemA completely stabilizes this protein while not impairing enzyme function. This finding confirms the hypothesis that HemA degradation tag lies in the N-terminus. Several models are discussed in this dissertation for the signals and regulatory components of the HemA regulation pathway.

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Heme

Bioenergy generation in bacteria including *S. typhimurium and E. coli*

Enteric bacteria such as *S. typhimurium* and *E. coli* are extremely versatile organisms which can grow in a wide diversity of ecologic niches with extremes in certain available nutrients, temperature, pH, and oxygen tension (Joklik *et.al*. 1992a). *S. typhimurium* and *E. coli* occupy subepithelial tissue in the small intestine where there is almost no oxygen unless the bacteria move to or near the intestinal epithelium. They are facultative anaerobes with the ability to use respiratory metabolism in the presence of oxygen and fermentation when oxygen is absent. Both aerobic and anaerobic respiratory systems allow them to oxidize a wide variety of organic substrates (*e.g.*, NADH, succinate, lactate) and pass the electrons ultimately to any of a number of oxidants (*e.g.*, oxygen, nitrate, dimethyl sulfoxide) (Gennis and Stewart, 1996). With the participation of a respiratory system, they can use one of many different nonfermentable compounds as a sole carbon and energy source (Gutnick *et al*., 1969). Therefore, these bacteria can adapt to different growth conditions and environmental challenges in order to survive. Switching between aerobic or anaerobic respiration and fermentation not only provides a wide selection of conditions in which the bacterium can survive, but also is an important strategy for its pathogenesis. For example, the organism grown in an atmosphere of 0-1% oxygen are almost 70% more adherent and invasive than those grown in 20% oxygen (Joklik *et al*., 1992b). The ability of the bacteria to exist under such circumstances reflects their adaptability to the required nutrients and their capacity to respond successfully to the

stimulus, such as oxygen tension. These strategies require accurate regulation in which heme biosynthetic regulation is an important aspect.

The bioenergetic principles are the same for both the aerobic and various anaerobic respiratory systems of these bacteria and no different than the principles considered in understanding mitochondria function in eukaryotes (Gennis and Stewart, 1996). Bacterial respiratory chains are composed of a variety of electron transport constituents, such as flavoproteins, iron-sulfur proteins, quinones, and cytochromes. The cytochromes are electron transfer proteins that carry heme as a prosthetic group. Their redox function is intimately related to the valence change of heme iron (Thony-Meyer, 1997). The differential transport of electrons and protons through the cytoplasmic membrane leads to the formation of a proton gradient across the membrane that can be used to drive ATP formation. In facultative anaerobes organisms, like *S. typhimurium* and *E. coli*, a wide variety of terminal electron acceptors other than oxygen can be used by the same electron transport systems which are coupled to these acceptors (Joklik *et al.*, 1992b). The respiratory system has a modular design to facilitate fine tuning to meet the physiological needs of the organism. The advantage of this adaptation is that only minimal substitutions or alterations are needed for different energy sources and environmental conditions (Gennis and Strewart, 1996).

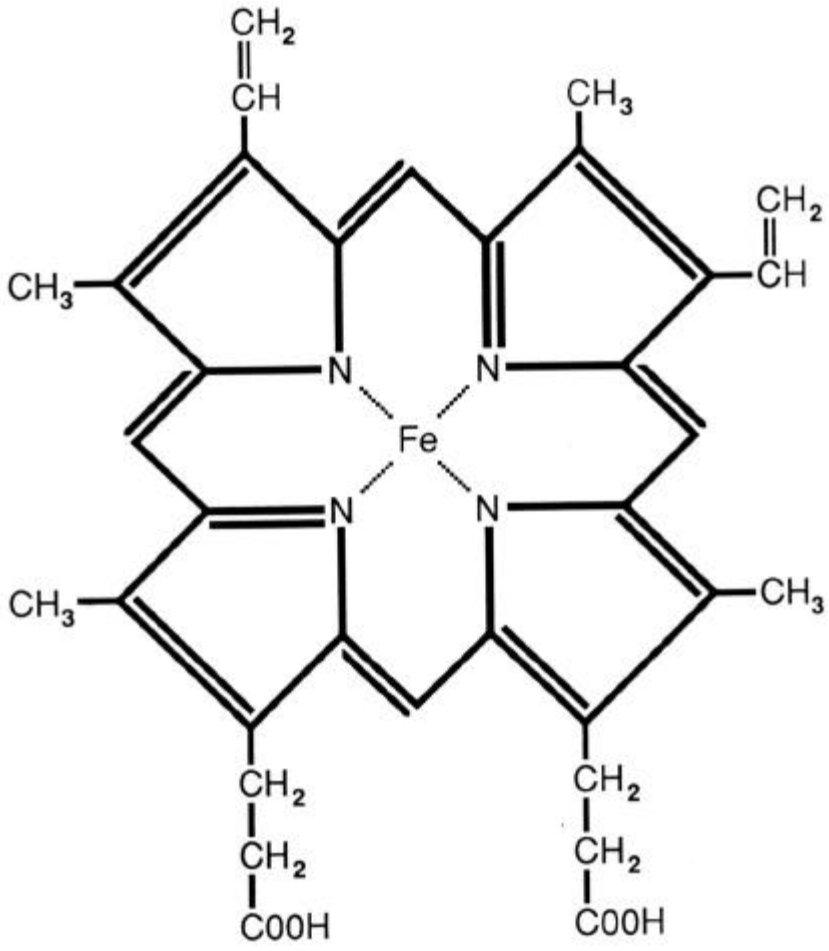
Many of the respiratory enzymes, like cytochromes, are heme proteins (Beale, 1996). Although *S. typhimurium* and *E. coli* can grow without hemes in broth or in minimal media that contain fermentable sugars by using a set of glycolytic enzymes that produce

ATP independently of respiration, mutants that are unable to produce hemes show several characteristic changes. For example, they stop growing at a certain cell density (10^7-10^8 cells/ml in LB medium) and are no longer motile; they form tiny colonies, known as dwarf colonies, with diminished respiratory activity (Sasarman and Horodniceanu, 1967; Nakayashiki *et al*., 1995; Nakayashiki and Inokuchi, 1997). These changes indicate that the fermentative metabolism does not supplement for the failure of the respiratory system under aerobic conditions. Obviously, heme is an essential part of the respiratory enzymes during energy conservation. The rate of heme synthesis in these bacteria varies according to their respiration need (Beale, 1996; Elliott and Roth, 1989). Knowledge about how heme synthesis is regulated in the enteric bacteria will give a better understanding of bio-energy generation. This project focuses on the molecular basis and genetic mechanism of this regulation.

Heme structure and function:

Hemes are members of the tetrapyrrole (porphyrin) family of biomolecules (Beale, 1996; Nakayashiki and Inokuchi, 1997). In all cells, porphyrin is used for the production of hemes. The structure of the porphyrins consists of four pyrrole rings, as shown on the next page.

In *S. typhimurium* and *E. coli*, heme plays an important role in maintaining normal physiological functions. Heme *b* (Fe protoporphyrin IX) and various modified hemes are cofactors of a number of cytochromes including the *cyo*-encoded cytochrome *o* complex and the *cyd*-encoded cytochrome *d* complex which are required for the respiratory



heme

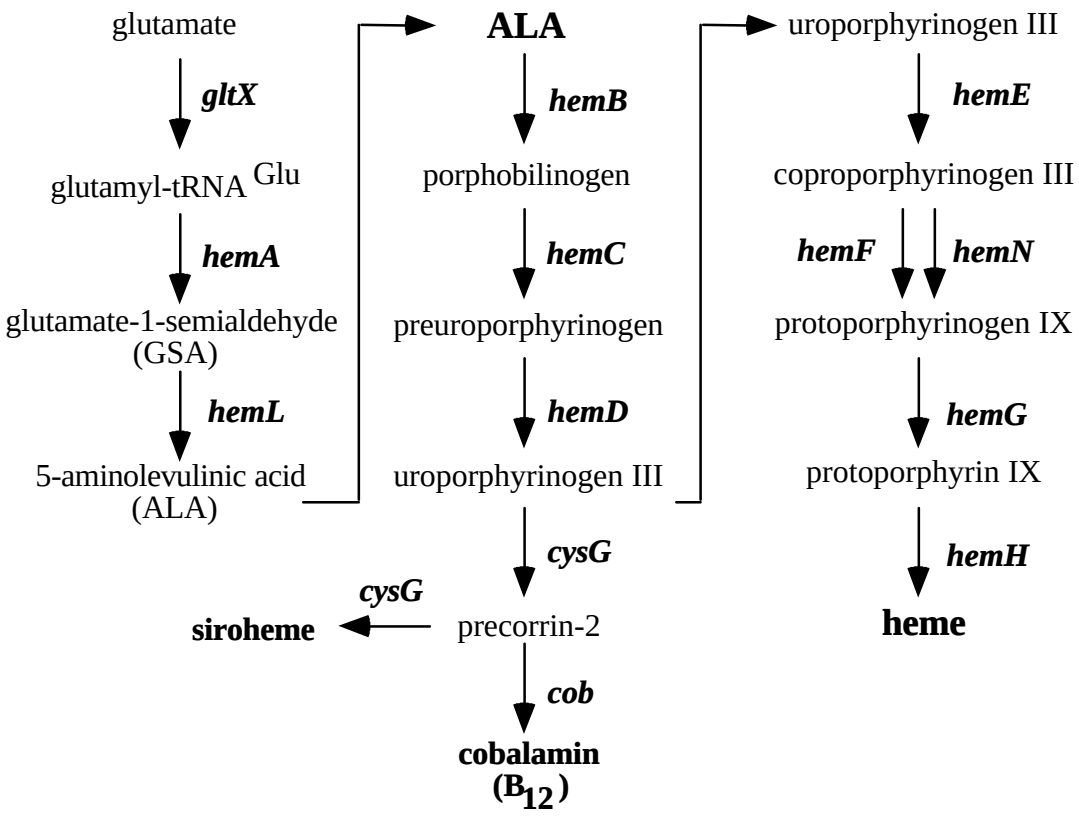
metabolism (Ingledew and Poole, 1984). Hemes are also cofactors of two catalases which detoxify hydrogen peroxide (H_2O_2) during aerobic respiration (Anraku and Gennis, 1987; Chiu *et al*., 1989; Mogi *et al*., 1994). Mutants that do not produce heme cannot grow on nonfermentable carbon sources such as glycerol. The growth can be restored by supplementing the medium with heme with an *env* mutation. Because *Salmonella* is impermeable to heme, the *env* mutation alters the porins in the outer membrane therefore allowing the diffusion of heme into the periplasmic space of the bacterium (Janzer *et al.*, 1981).

Two minor products also branch from this pathway, siroheme and cobalamin (vitamin B12). Siroheme is a cofactor for nitrite and sulfite reductase and is therefore required for synthesis of cysteine from sulfate during growth on minimal medium (Goldman and Roth, 1993). *S. typhimurium* synthesizes cobalamin only under anaerobic or low oxygen growth conditions (Jeter *et al*., 1984). Cobalamin serves as a cofactor for at least four other enzymes (O'Toole *et al*., 1996): (1) homocysteine methyltransferase (*metH*), which can function in methionine synthesis as an alternative to a B12-independent enzyme (*metE*), (2) ethanolamine ammonia-lyase (*eut*), which, catalyzes the first step in ethanolamine catabolism, (3) 1,2-PDL dehydratase (*pdu*), which catalyzes the reaction to utilize 1,2-propanediol as a carbon and energy source (Jeter, 1990), and (4) queuosine synthetase, which involves the formation of queuosine, a hypermodified nucleotide found in some tRNAs (Frey *et al*.,1988). Thus, the products of the heme biosynthetic pathway, especially the main product heme, are required for essential metabolic functions related to oxygen, respiration, and electron transfer.

The heme biosynthetic pathway in enteric bacteria.

All biological tetrapyrroles can be produced from a single, branched biosynthetic pathway. The pathway is highly conserved among many organisms (Beale, 1996; Dailey, 1990; Jordan, 1990). The currently accepted model for the heme synthetic pathway in bacteria consists of 10 reactions which convert glutamate to heme (see next page). The pathway can be viewed as having three segments: (1) Formation of the first universal tetrapyrrole precursor 5-aminolevulinic acid (ALA) (detail see below). (2) Eight molecules of ALA are combined to produce the first tetrapyrrole, uroporphyrinogen III. At this point, the pathway branches to form siroheme and vitamin B_{12} *via* the multi-functional CysG enzyme. (3) In the heme-specific branch of the pathway, uroporphyringen III is modified, oxidized, and iron is added to form heme (Beale. 1996; Xu *et al.,* 1992).

The heme synthetic intermediates and reactions in the pathway are very similar or identical in all heme-making organisms. The main difference is the route of biosynthesis of the ALA (Avissar, 1989; Jahn *et al*., 1992). Most bacteria, including *S. typhimurium* and *E. coli*, use the C5 route (Avissar and Beale, 1989; Elliott *et al*., 1990; Li *et al*., 1989; O'Neill *et al*., 1989). In this pathway, glutamate is transformed to ALA by three enzyme-catalyzed reactions. First, glutamate is activated by ligation to $tRNA^{Glu}$, in the presence of ATP and Mg^{2+}, a reaction catalyzed by glutamyl-tRNA synthase (encoded by *gltX*). The $tRNA^{Glu}$ and glutamyl-tRNA synthase are the same as used to charge $tRNA^{Glu}$ for protein synthesis (Bruyant and Kannangara, 1987; Jahn *et al*., 1992). In the next step, glutamyl-$tRNA^{Glu}$ is reduced by glutamyl-tRNA reductase (HemA, encoded by *hemA*) in a



The heme biosynthetic pathway in *S. typhimurium*. It can be viewed as having three segments marked by key intermediates ALA and uroporphyrinogen III.

NADPH-dependent reaction to yield glutamate-1-semialdehyde (GSA), a hydrated hemiacetal form of GSA, which may convert to a cyclized form of GSA. Because only a small portion of glutamyl-tRNA enters the heme pathway, and there are no known *gltX* mutations that specifically affect heme synthesis, HemA therefore is considered the first committed heme enzyme in the pathway. Finally, GSA aminotransferase (encoded by *hemL*) transfers the amino group from the C4 carbon to the C5 carbon of GSA to form ALA. This step is also observed in vitro by a nonenzymatic pH-dependent conversion (Hoober *et al.,* 1988). The C4 pathway exists in animals, fungi, and some of the purple bacteria. Different from the C5 pathway, glycine and succinyl CoA are condensed to form ALA by a single enzyme, ALA synthase.

hemA and HemA, *hemL,* and other heme genes

All heme genes have been mapped and sequenced. They are scattered on the chromosome of *S. typhimurium* and *E. coli*, except for the *hemCD* operon (Beale, 1996; Elliott and Roth, 1989). The *hemA* gene is at 35 min on the *S. typhimurium* chromosome and in the same operon as the downstream *prfA* gene which encodes an essential protein, polypeptide release factor (RF-1) (Elliott, 1989; Elliott and Wang, 1991). Several *hemA* genes have been cloned and sequenced from a number of bacteria and higher plants. The sequence of the *S. typhimurium hemA* gene (at least) shows remarkable homology with that of the *E. coli hemA* gene (Elliott, 1989; Elliott and Roth, 1989). *hemA* encodes glutamyl-tRNA reductase in species that use the C5-route ALA biosynthetic pathway (Avisser and Beale, 1989; Majumdar *et al*.,1991). Cell extracts obtained from an *E coli hemA* mutant lost the capability of catalyzing the conversion of glutamate or glutamyl-

tRNA to ALA. The *E. coli hemA* sequence was reported to bear no homology to ALA synthase genes from organisms which utilize the C4 route for ALA formation (McClung *et al.,* 1987).

The HemA enzyme encoded by the *hemA* gene is a 46 kDa protein in *S. typhimurium.* In *E. coli*, both the 46-kDa HemA protein and another glutamyl-tRNA reductase of 85-kDa have been purified, but the origin and metabolic role of the latter protein is unknown (Jahn *et al*., 1991). Glutamyl-tRNA reductases have been purified and studied from different organisms, *e. g., Chlamydomonas reinhardtii* (Chen *et al*., 1990), *E. coli* (Jahn *et al.,* 1991) *Synechocystis* (Rieble and Beale, 1991), and barley (Pontoppidan and Kannangara, 1994). These glutamyl-tRNA reductases appeared to be significantly different with respect to their specific catalytic activity, subunit composition and molecular mass. This protein is present in low abundance in the cell and is unstable. The glutamyl-tRNA reductase is a rare enzyme which catalyzes a unique reaction. It has the unusual property of requiring tRNA as "cofactor" (Chen *et al.,* 1990). The HemA protein purified from green alga *Chlamydomonas* was shown forming a stable complex with $tRNA^{Glu}$. Barley glutamyl-tRNA reductase recognizes seven specific nucleotides in the $tRNA^{Glu}$ molecule (Willows *et al*., 1995). Glutamyl-tRNA reductase is able to discriminate between different glutamate tRNA species. For example, it was reported that *E. coli* glutamyl-tRNA reductase utilizes only the homologous *E. coli* substrate (Jahn *et al.,* 1991). There was the observation that *hemA* mutants transformed by plasmids carrying the ALA synthase gene from other organisms regained the ability to grow on the media without added ALA (Leong *et al*., 1982; Schoenhaut and Dailey, 1993; Woodard and Dailey, 1995).

The *prfA* gene encoding polypeptide release factor 1 (RF-1) forms an operon with the *hemA* gene. This arrangement may suggest a relationship between the synthesis of proteins and that of heme. Nakayashiki *et al* (1995a) obtained preliminary data which suggested that overexpression of the *prfA* gene activates the synthesis of porphyrin. Thus, they proposed that there is a competition between protein synthesis and porphyrin synthesis *in vivo*, and which may be important for the balance between cell growth and energy generation under energy-starved conditions.

A gene located downstream of the *prfA* gene was demonstrated by nucleotide sequence to be co-transcribed from the promoter of *hemA* (Elliott, 1989). It was designated as *hemK* (Nakayashiki *et al.,* 1995b). Although the *hemK* gene and its product (225 amino acid protein) showed no significant homology to any known gene or protein at the nucleotide or amino acid level, the *hemK* gene product was shown to be involved in the oxidation of protoporphyrinogen to protoporphyrin IX (Nakayashiki *et al.,* 1995b). All *hemA-prfA-hemK* genes therefore may be related in the biosynthesis of heme, as they are in one operon.

A large number of *S. typhimurium* mutants auxotrophic for ALA were analyzed by Elliott and Roth in 1989, which confirm and extend earlier results indicating that *hemA* and *hemL* are two genes required for ALA synthesis in this bacterium. Mutants in *hemA* and *hemL* are defective for aerobic and anaerobic respiration, and appear to be oxygen sensitive. The *hemA* mutants are severe auxotrophs requiring ALA for growth on minimal media (Fig.6., page 52). The measurement of heme in *hemA* mutants has shown the expected results of a drastic reduction of the heme content in these cells (Rompf *et al*., 1998; Haddock and Schairer, 1973).

The *S. typhimurium hemL* gene maps at 5 min on the genetic map, and has been cloned and sequenced (Elliott, 1989; Elliott *et al.,* 1990). The *hemL* mutants exhibit a "leaky" phenotype: in liquid minimal glycerol medium supplemented with ALA, the growth curve of *hemL* mutant closely resembles that of a wild type strain (Fig.6., page 52,53). Without ALA, *hemL* mutants grow for about three generations and then starve for ALA, but after about two hours "adaptation", they resume exponential growth with a slightly slower rate (95-min doubling time) than during unlimited growth with ALA supplementation (68-min doubling time). This mutant provides a simple way to obtain heme limitation during exponential growth. It is especially useful for studies of HemA protein synthesis and degradation in pulse-labeling experiments.

The *hemD* (uroporphyrinogen III synthase) mutant, or any mutant defective in a subsequent *hem* gene, will accumulate tetrapyrrole(s) before the block (Xu *et al.,* 1992). These accumulated intermediates are fluorescent red under UV light. This red fluorescence can also be seen as a sign of increased flux of heme synthetic pathway, like addition of exogenous ALA to the cell growth medium. *hemB, hemCD, hemE, hemG,* and *hemH* mutants were studied in our laboratory and described by Xu and Elliott (Xu *et al*., 1992; Xu and Elliott, 1993; Xu and Elliott, 1994). The *hemB, hemE* and *hemH* mutants were used in my project, which are all auxotrophic for heme and which are similar to *hemA* mutants starved for ALA.

The Heme biosynthetic regulation

Indirect evidence strongly suggests that the heme biosynthetic pathway in enteric bacteria is a regulated process. First, the levels of heme found in the membrane vary

depending on the mode of growth (Hino and Ishida, 1973; Richmond and Maakoe, 1962). Second, in *E. coli,* it was found that the amount of heme and in particular glutamyl tRNA reductase activity can be increased dramatically by treatment with certain thiols and this increase is blocked by chloramphenicol (Javor and Febre, 1992). Third, it is commonly observed that *E. coli* strains carrying multi-copy plasmids encoding heme proteins (catalase, cytochrome or hemoglobin) are visibly red and may overproduce heme as much as 10- to 20-fold (Hino and Ishida, 1973; Woodard and Dailey, 1995). Fourth, *E. coli* cells that are grown in the presence of ALA accumulate heme (Philip-D and Doss, 1975), suggesting that ALA formation is the rate-limiting step of heme synthesis.

The hypothesis that heme itself, functions as an end product-feedback regulator to regulate the heme synthesis is very attractive. This model was tested by several different methods which resulted heme limition *in vivo*. One such condition was created by introducing and expressing exogenous apoprotein of cytochrome b_5, as a “heme sink”. This heme protein readily binds any available free heme present in the cell, therefore reducing the heme concentration. Heme synthetic pathway intermediates, especially ALA production, were found to be activated by this cellular heme content change (Woodard and Dailey, 1995). Another study described the regulation of porphyrin synthesis by using a heme-permeable, *hemH* deletion mutant (Nakayashiki and Inokuchi, 1997). This mutant utilizes only exogenous hemin and accumulates porphyrins since the last step on heme synthesis is blocked. By measuring the accumulation of porphyrins, the rate of synthesis of heme can be examined, and was found to be dependent on the availability of heme. The lower concentration of heme added in the medium, the more porphyrins that accumulated.

This stimulation mainly occurred at the step of synthesis of ALA. These results suggest that an interruption of heme formation regulates heme precursor biosynthesis and that heme is the effector of feedback regulation. The purified barley HemA activity was shown to be inhibited by hemin with the concentration of 4 μM (Pontoppidan and Kannangara, 1994). Some other studies also support this model for feedback regulation of glutamyl-tRNA reductase (HemA) by heme (Gough and Kannangara, 1979; Weinstein and Beale, 1985; Huang and Wang, 1986; Rieble and Beale, 1991; Javor and Febre, 1992). However, the molecular basis and the mechanism of this regulation is not clear. There is also a study showing that heme did not inhibit HemA enzyme activity even at high concentrations (Jahn *et al.,* 1991).

Oxygen was also considered as a factor to regulate heme biosynthesis since heme concentration varies according to aerobic or anaerobic conditions (Page and Guerinot, 1995). But there is no evidence that oxygen tension has a direct effect on inducing the formation of heme or ALA. The explanation for this may be “free heme pool” regulation: oxygen may affect the induction of various apocytochromes which bind heme molecules. The subsequent depletion of the free heme stimulates additional synthesis of heme (Darie and Gunsalus, 1994; O’Neill *et al*., 1989; Woodard and Dailey, 1995).

A gene that maps directly upstream from the *Escherichia coli hemA* gene was named *hemM* (Ikemi, 1992). It was reported that more ALA was produced by strains harboring a plasmid with both *hemA* and *hemM* than by those with *hemA* alone (Chen *et al*., 1994). However, in contrast to the suggested role of *hemM* in ALA synthesis in the cytosol, the

hemM gene encodes a novel outer membrane lipoprotein (LolB) (Matsuyama *et al.*, 1997), and unrelated to any enzyme known to play a role in ALA synthesis (Ikemi *et al.*, 1992). Verderber *et al* (1997) also reported that increasing the copy number of *hemM* had no effect on ALA pools.

lac fusions to all genes in the heme pathway were constructed in this laboratory in the past (Elliott, 1992; Xu and Elliott, 1992, 1993, and 1994). Starvation for heme did not lead to appreciable induction of transcription for any of the fusions which were examined. Recently, an*E. coli hemA* mutant with undetectable level of heme, was studied for gene expression under heme-deficiency (Rompf. *et. al*. 1998). The induction of tryptophanase (*trpA*), citrate synthase (*gltA*), aldehyde dehydrogenase (*adlA*), and the repression of enolase (*eno*) and phosphoglycerate kinase (*pgk*) were observed, but no obvious heme-associated proteins were found. The results showed just indirect effects of heme depletion on gene expression in *E. coli*. All induced genes are under the control of the catabolite repressor protein Crp, suggesting a stress signal derived from heme-limitation, which forces the cell to develop strategies for the utilization of alternative carbon sources.

The studies reviewed above do not give a clear picture of the regulation of heme synthesis in bacteria. The challenge for the further investigation of heme biosynthetic regulation is: there is almost nothing known about the target(s) directly induced by differences of the intracellular heme concentration and the physiological consequences resulting from the stress of heme depletion; further, what is the molecular basis of the

mechanism of this regulation? However, *hemA* was believed to be an important gene for the heme synthetic regulation as reviewed below.

HemA in heme biosynthetic regulation

It is well accepted that ALA formation is the rate-limiting step in the heme biosynthetic pathway and HemA is the key enzyme for ALA formation. The role of HemA, ALA and heme in *E. coli* heme pathway regulation has been investigated by several groups. ALA-feed studies supported that heme synthesis is limited by the rate at which ALA is formed. For example, addition of exogenous ALA to the growth medium or strains carrying cloned *hemA* genes of various species have a fluorescent red phenotype due to an overproduction of tetrapyrrol intermediates (Doss and Philipp-Dormston, 1973; Chen, 1994). This phenotype is not observed when either HemL or HemB is overproduced (our unpublished results). Verderber *et al* (1997) performed studies of the role of *hemA*, ALA, and heme in *E. coli* cells by introducing multiple copies of a heme sink, recombinant human hemoglobin in *E. coli*. The cellular regulatory heme content would be decreased when this heme sink was expressed. ALA synthesis was stimulated in this situation and inhibited by heme or ALA. Their results confirmed that HemA catalyzes a rate limiting step in the heme synthetic pathway. The *hemA* gene and HemA enzyme, therefore, are considered to be potential targets for heme synthetic regulation. Another hint is that eukeroytic ALA synthase, which functions similar to the HemA in bacteria, catalyzing the formation of ALA in the C4 route, is regulated both by gene expression and enzyme activity (Drew and Ades, 1989; Houston *et al.*,1994; Smith and Cox, 1997).

There are two promoters in the *hemA* operon in *S. typhimurium* (Choi *et al*., 1996). P1 plays a primary role in *hemA* transcription. The expression of a *hemA-lac* operon fusion is virtually unchanged during starvation for ALA. Primer extension analysis shows no increase in the abundance of RNA from either promoter during heme starvation. These studies suggest that HemA regulation does not occur at the level of transcription initiation. Study of HemA regulation at the post-transcriptional level therefore became necessary. It was a difficult tack, since the HemA enzyme exists at an extremely low concentration, which is difficult to detect. Several groups failed to overproduce the native HemA protein for biochemical studies. A panel of anti-HemA monoclonal antibodies generated recently in our laboratory provides a very useful tool to measure HemA protein abundance by Western immunoblot and for HemA immunoprecipitation (Study I, II, and III).

Gene regulation at the protein level

General information

The availability of protein is often controlled by gene transcription or translational regulation of synthesis (Gottesman, 1996). The activity of a protein can be regulated by a myriad of well-studied mechanisms: reversible covalent modification, changes in localization, interactions with other proteins and small molecule effectors, and proteolytic processing. Instability of full-length proteins in exponential phase is rare in enteric bacteria, and regulated stability is very rare. The only known examples are the sigma factors RpoH and RpoS, the repressor LexA, (Gottesman, 1996), UmuD (Gonzalez *et al.*, 1998), and possibly the chromosomal addiction system antidote MazE (Aizenman *et al.*,

1996). Giving RpoS as an example: in *E. coli*, the concentration of a secondary sigma factor σ^{32} (RpoS, encoded by *rpoS* gene) increases in stationary phase. This increase involves regulation at the transcriptional, translational, and post-translational levels (Schweder *et al*., 1996). In the post-translational mechanism, it was shown that the RpoS turnover in exponential phase requires the ClpXP protease. The stability of RpoS protein increased markedly in stationary phase with no decrease in ClpXP levels in wild-type cells. Thus, RpoS is regulated to be resistant to this protease in stationary phase. Further studies tested the stability of translational fusions containing different lengths of RpoS coding region. The amino acid residues 173-188 of RpoS are critical for its degradation, which suggested that these residues may directly or indirectly serve as at least part of the target for ClpXP protease.

Studies in recent years have revealed the importance of rapid degradation as an essential element of the regulatory circuitry in both prokaryotes and eukaryotes. The complex energy-dependent proteases are involved in this degradation (Gottesman and Maurizi, 1992).

Energy-dependent proteases

Most proteins are stable in growing cells in *E.coli* (Miller, 1996). Degradation of unstable proteins in growing cells has two major functions: to get rid of abnormal proteins and to regulate the level of some regulatory proteins. This process is tightly controlled and highly regulated. There are at least 40 proteases in *E. coli*. Two distinct classes of compartmentalized cytoplasmic proteases are presented as ATP-dependent and

ATP-independent. A major portion of protein turnover is carried out by energy dependent proteases (Larsen and Finley, 1997; Gottesman *et al*., 1997). It needs to be emphasized that rather than energy-dependent proteolysis, ATP-dependent proteases carry out energy-dependent scanning and energy-dependent presentation of substrates. This provides an additional control on the selectivity of the proteases, and provides a mechanism for completely degrading a folded protein with a single site of protease recognition (Gottesman and Maurizi, 1992). There are three families of ATP-dependent proteases in bacteria: Lon, Clp and FtsH. Lon and Clp are soluble proteins, while FtsH is anchored in the cytoplasmic membrane. Both Lon and Clp are not essential for viability in *E. coli.* Mutants lacking both Lon and Clp can grow under standard growth conditions (Suzuki *et al*., 1997), but grow very poorly in *S. typhimrium* (obtained in this laboratory).

Lon, encoded by *lon* gene, plays two roles in the cell: it degrades a special class of proteins that are designed to be unstable, and abnormal proteins (Miller, 1996; Gottesman and Maurizi, 1992). The *lon* mutation has a pleiotrophic phenotype: UV sensitivity, mucoidy, deficiency for lysogenization by bacteriophage λ and P1, and lower efficiency in the degradation of abnormal proteins (Gottesman and Maurizi, 1992; Miller, 1996). Lon protease is a 87-KDa protein that functions as a homo-oligomer of four subunits. The activity of the peptide bond-hydrolyzing site of Lon is regulated by two allosteric sites, one of which binds ATP and ATP analogs and the other of which binds proteins which are Lon substrates.

ATP functions as an allosteric effector, maintaining the peptide bond-hydrolyzing site in an open, active conformation (Gottesman and Maurizi, 1992). ATP hydrolysis is necessary for degradation of high molecular-weight proteins. ADP binds to Lon with a higher affinity than does ATP (Menon and Goldberg, 1987), promoting a "closed", inactive state. Protein substrates, but not smaller peptide substrates, promote the release of tightly bound ADP from Lon. A substrate protein acts as an allosteric effector altering both the ATP site and the peptide hydrolysis site. The substrate protein appears to remain attached to Lon during the degradation process. The major determinant of susceptibility to Lon cleavage is not the presence of a particular amino acid sequence at the cleavage site itself but, rather, the presence in the substrate protein of some signal that Lon recognizes. There is no evidence that regulation of Lon synthesis is important for regulating degradation of its natural substrates (Gottesman, 1996). Dervyn *et al*. (1990) reported that Lon can be saturated by its overproduced substrate SulA, thus protecting the other substrates from degradation. Deletion of the C-terminal end of SulA affects its activity but not its susceptibility to Lon. The N-terminal 113 amino acids of SulA are recognized by Lon (Dervyn *et al.*, 1990).

Extracts from an *E. coli lon* null mutant still catalyze the ATP-dependent degradation of casein. Clp protease was found to mediate most of this residual activity. Clp protease contains two dissimilar subunits: ClpP which is responsible for peptide bond hydrolysis, and ClpA (or ClpX) which associates with ClpP to form an active protease, ClpAP or ClpXP, for protein substrates (Miller, 1996).

ClpP alone can rapidly cleave short (3- to 6-amino acid) peptides and longer unstructured polypeptides at 2% of the rate seen with ClpA and ClpP combined. Degradation of large proteins, however, requires ClpAP or ClpXP formation as well as ATP hydrolysis. The ATPase component, ClpA or ClpX, plays an important role in the Clp protease-dependent proteolysis. ClpA or ClpX has a basal ATPase activity that is activated in the presence of ATP and appropriate protein substrates . ClpA or ClpX acts as a chaperone which mediates the ATP-dependent disassembly of a multimeric substrate and releases it as active monomers. When associated to ClpP, ClpA or ClpX presents the substrate to ClpP and targets it for ATP-dependent degradation by ClpP. Thus, ClpA and ClpX not only have catalytic functions but also are responsible for the substrate selection. Determined by the selection of ClpA and ClpX, CplAP and ClpXP are involved in the degradation of distinct classes of protein.

ATP plays several roles in Clp-proteolysis. ClpA or ClpX, as a chaperone, promote the folding or unfolding of substrate polypeptides or proteins in an ATP-dependent manner; ClpA or ClpX interacts with ClpP only in the presence of ATP, but ATP hydrolysis is not required for association between them. ClpP requires hydrolysis of ATP for proteolytic activity (Gottesman and Maurizi, 1992).

Recognition of signal for degradation

The proteolytic systems can distinguish appropriate substrate proteins and avoid damage to other cellular proteins. This control, especially in the cytoplasm, is likely to be critical for maintaining the proper protein availability. Appropriate targets for proteolysis include both naturally unstable proteins and abnormal proteins. These proteins must contain degradation signals which either are buried in the native protein or formed or assembled from elements in the damaged or denatured protein (Gottesman, 1992). These

signals are recognized and targeted by the degradation machinery, which could be by the protease directly or through participation of a partner (Gottesman, 1996).

Although the target elements for the proteases are not well defined yet, comparisons between the sequences of substrates known to be degraded by the same protease has provided some hints. The clearest case is the N-end rule in both eukaryotes (not reviewed here) and prokaryotes (Tobias *et al*., 1991; Varshavsky, 1996). Proteins carrying certain amino acids at the N terminus are rapidly degraded. Because all translation begins with a methionine, the second amino acid of the N-terminus of a protein is usually critical for this process. The residues located near the N terminus can also act as the tag for protease(s). For example, the UmuD degradation signal for Lon-mediated proteolysis is localized between residues 15 and 18 (FPLF) of the N-terminus of this protein (Gonzalez *et al*., 1998). A "alanine-stretch" mutagenesis on these residues stabilized the protein, and transfer of the amino terminus of UmuD (residues 1-40) to an otherwise stable protein imparts Lon-mediated proteolysis. This result indicates that the N-terminus of UmuD is sufficient for Lon recognition and the ensuing degradation of the protein. The amino acids causing instability are not normally found at the N terminus. Proteases that carry out the initial cleavage of proteins might specifically cut bonds resulting in products with destabilizing amino acids at the amino termini. For example, Lon and Clp both tend to cleave hydrophobic regions of proteins, often (but not always) yielding products with hydrophobic amino-terminal amino acids. There are also some reports implicating the carboxy terminus in protease recognition. For example, the degradation rate of a cloned amino-terminal fragment of λ*c*I repressor is affected by the

composition of five amino acids at its C-terminus. The presence of hydrophobic amino acids in the last five positions resulted in a highly unstable protein. Replacement of any of these five amino acids with hydrophilic amino acids led to slower degradation *in vivo* (Parsell, *et al.*, 1990; Keiler *et al.*, 1995). Other examples also suggest that proteases recognize a sequence of amino acids in a variety of 5 amino acids or longer, which occurs infrequently in proteins (Gottesman and Maurizi, 1992). There are also some cases that the degradation target is located anywhere in the protein other than in the N or C terminus, *e.g.*, the amino acid residues 173-188 of RpoS directly or indirectly serve as at least part of the target for ClpXP protease (Schweder *et al.*, 1996). Interaction with other proteins or effectors is also important in determining the degradation rate of a protein (Gottesman, 1996).

The previous works as reviewed above suggest that the heme synthesis is a regulated process. HemA enzyme is a potential target for this regulation, but *hemA* transcription is not affected by heme-limitation. This project will extend this finding by examining post-transcriptional level of HemA regulation during heme-limitation. The challenge of this study is that there are only few examples of gene regulation by proteolysis and is almost no knowledge about heme synthetic regulation at the protein level. The unknown mechanism of this regulation therefore leaves an important question, and this project is going to get the answer.

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Regulation of heme biosynthesis in *Salmonella typhimurium* : activity of glutamyl-tRNA reductase (HemA) is greatly increased during heme limitation by a mechanism which increases abundance of the protein

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Abstract

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 (immunoblot) analysis using these antibodies reveals that the increase in HemA enzyme activity during heme limitation is mediated by an increase in 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 minimal (less than 2-fold) increase in expression of *hemA-lac* operon fusions; thus HemA regulation is mediated either at a post-transcriptional step or through modulation of protein stability.

Introduction

In *Salmonella typhimurium* and *Escherichia coli*, heme is essential both for respiration and in defense against the toxic oxygen metabolite H_20_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 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 refs. 7 and 29); a non-enzymatic pH-dependent conversion of GSA to ALA is also observed *in vitro* (25). Since only a small fraction of the cell's charged $tRNA^{Glu}$ is used to make heme, the reductase reaction is considered to be the first committed step in heme and tetrapyrrole biosynthesis.

Heterologous expression in yeast (45) and $tRNA^{Glu}$ substrate specificity studies (5) showed that the *hemA* gene encodes glutamyl-tRNA reductase. Null mutants in *hemA* exhibit 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, 85-kDa glutamyl-tRNA reductase have been purified from *E. coli* cells; the latter enzyme's origin and metabolic role are unknown (26).

Indirect evidence has strongly suggested that 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 (e.g. see ref. 21,24,38). Second, in *E. coli* it was found that the amount of heme and in particular, glutamyl tRNA reductase activity, can be increased dramatically by treatment with certain thiols and this increase is blocked by chloramphenicol (28). Third, it is commonly observed that *E. coli* strains carrying multi-copy plasmids encoding heme proteins (whether a catalase, cytochrome or hemoglobin) are visibly red in color and may overproduce heme as much as 10- to 20-fold (e.g. reference 23,29a,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 flux through the pathway, but additional HemL or HemB do not. In organisms other than the enteric bacteria, ALA production is known to be regulated at the levels of both gene expression and enzyme activity (e.g. refs. 31a,51). In previous work, we examined 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 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 GST-HemA fusion protein containing all but the N-terminal 23 amino acids of HemA was overproduced, and this protein was used to elicit a panel of monoclonal antibodies that react specifically with HemA. Western (immunoblot) analysis confirms 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 markers. Details of strain construction and properties are given in the references listed in Table 1. The *hemL* mutant strain (TE472) is a deletion 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 the 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 Luria-Bertani (LB) medium (43) or in minimal MOPS (morpholinepropanesulfonic acid) medium (35) as modified (9) containing 0.2% glycerol as the carbon source. Plates were prepared using nutrient agar (Difco) with 5 g of NaCl per liter or using NCE medium (8) with 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 with heme to OD_{600} = 0.5. The cells were collected by centrifugation, washed with LB medium, and resuspended in 250 ml of LB medium pre-warmed to 37°C.

Table 1. Bacterial Strains.

Strain	Genotype	Source
Salmonella 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)
Escherichia coli		
DH5α	K-12 F$^-$ λ$^-$ *endA1 hsdR17* ($r_K^-m_K^+$) *supE44 thi-1 recA1 gyrA96* (Nal^r) *relA1 D(lacZYA-argF)U169* (ϕ80*dlacZDM15*)	P. Higgins
MC4100	K-12 F$^-$ λ$^-$ *araD139* Δ(*lacIpoZYA, argF*)*U169 flb5301 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. Studie

Growth was continued for 3 hr 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 OD_{600} = 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 minimal MOPS glycerol medium, 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 hr 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 wild type) from a 1:100 dilution of an overnight culture which was grown to a final OD_{600} = 0.4. Cultures were chilled, 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 DTT, 20 μM pyridoxal phosphate) also containing 200 μM phenylmethylsulfonyl flouride (PMSF). 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 PMSF, and stored in aliquots at -70°C. Protein concentrations were 1-3 mg/ml as determined by assay with the Bradford reagent (BioRad) using bovine serum albumin as the standard.

Preparation of substrate. Purified *E. coli* $tRNA^{Glu}$ was obtained from Sigma (R-6591) and charged with ^{3}H-glutamate according to Schneegurt *et al* (41). Ten A_{260} units

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 DTT and stored frozen at -20°C. (Buffers for charging reactions were prepared in DEPC-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 A_{260} units) of $tRNA^{Glu}$, 25 μCi of [2,3,4-^{3}H]-glutamate (Amersham, 49 Ci/mmol, 1 μCi/μl), 5 μM unlableled 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 over-expressing *E. coli* glutamyl-tRNA synthetase (pLQ7611Δ*Nru*I; 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 Dr. J. LaPointe). In either case, the total incorporation of ^{3}H-glutamate into cold TCA-precipitable material was 180 pmol (4 x 10^6 cpm). Reactions were terminated by addition of 2 vols. of 0.1M MES, pH 5.8, 10 mM $MgCl_2$, 10 mM glutamate, extracted with phenol and then with chloroform/isoamyl alcohol (24:1). Aliquots were ethanol precipitated after addition of 1/10 vol 3 M Na 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 direct assay of HemA. The charged tRNA substrate was recovered by centrifugation, dried briefly, and resuspended in assay buffer. Each reaction contained 100,000 cpm of substrate and in addition, 2 mM NADPH, 5 mM levulinic acid (to inhibit HemB), 2 μl RNasin (Promega) and 50-150 μl of extract containing 50 to 450 μg of protein in a final volume of 250 μl. Incubation was for 60 min at 37°C. Reactions were terminated by the addition of 50 μl 1 M citric acid, 250 μl 10% SDS and 20 μl 1 mM unlabeled ALA, heated at 95°C for 2 min, cooled, and microfuged. ALA and GSA product in the supernatant was purified by ion-exchange chromatography on Dowex

50W-X8 (Na^+), the eluate was derivatized with ethylacetoacetate and extracted into ether exactly as described (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 (genetic requirements for activity are described in Results). Fig 2 shows that formation of product was linearly dependent on the amount of extract added, over the range assayed (up to 1 mg/ml total protein). 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 amount of extract was obtained with extracts of low activity from wild type cells (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 IPTG. Crude extracts were prepared by disruption in the 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% NP-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 intramuscular and subcutaneous routes, followed by four boosts of antigen without the adjuvant, at four day intervals. Cells harvested from inguinal and popliteal lymph nodes and spleens the day after the final

boost were hybridized with the non-secreting myeloma partner P3X63.Ag8.653 as previously described (30), and placed in HAT-containing medium. Supernatants from wells containing growth were screened three weeks later for binding to the GST-HemA extract in an ELISA. 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. Specificity of antibodies was further confirmed by Western blot as well as immunoprecipitation analysis. Screening was done initially employing 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 using a panel of biotin-conjugated isotype-specific antibodies (Southern Biotechnology).

Immunological detection of proteins. Techniques for Western (immuno)-blots have been described in detail (11). The primary antibody was a mouse monoclonal anti-HemA antibody of 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 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 $MgSO_4$), then permeabilized by treatment with SDS and chloroform (33). Assays were performed in Z-buffer containing 50 mM β-mercaptoethanol by a kinetic method using a plate reader as described (11). Activities (ΔOD_{420} per min) are normalized to 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, becomes 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-colored 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 Methods for details). The assay employs *E. coli* $tRNA^{Glu}$ charged with ^{3}H-glutamate as the substrate. After the reaction, the product is purified by ion exchange chromatography, 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. Fig 4 shows that the activity of an extract of the wild type strain LT-2 was about 100 fmol/mg protein in this assay, whereas activity was very low in an extract of a *hemA* mutant (*hemA60*), and a *hemA*::Kan insertion mutant had no

detectable activity (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 employed does not distinguish whether the product was GSA or ALA (Fig 1A; see discussion in ref. 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, non-enzymatic 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 activity is mostly independent of *hemL* function (Fig 4). We explain the slight decrease in 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 HemL enzyme is about 10^4-fold higher than that observed in this assay and should not be rate-limiting (20). To show that 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 reactions 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/ml heme, washed, and diluted into LB medium without heme. Slow starvation is characteristic of *hemA* mutants and 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 hr), cells were harvested and extracts were prepared and assayed.

For each mutant, HemA activity was dramatically increased compared to 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 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 non-growing 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 non-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 3 generations (Fig 6A). Growth of the *hemL* mutant also slowed and stopped in the absence of ALA, however, growth resumed following a lag period of approximately 2 hours. This behavior is in striking contrast to that of the *hemA* mutant or mutants blocked later in the pathway (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 5-fold 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. One such experiment is shown in Fig 7. Extracts of adapted and ALA-supplemented *hemL* mutant cultures were adjusted to have

an equal concentration of protein, then 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 using starved *hemB* mutant and wild type extracts with similar results (not shown).

Immunological detection of HemA protein by Western blots (immunoblots). We have been able to overproduce segments of HemA although not the native protein. One construct joins glutathione-*S*-transferase (GST) as an N-terminal segment to a deletion lacking the first 23 amino acids of *hemA* (see Methods for details) This construct produces massive amounts of a GST-HemA fusion protein under the control of the P_{tac} promoter (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 (above), to determine the abundance of HemA protein by Western blot (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 nonsense because it shows a new band of 37 kDa. The appearance of a nonsense fragment reactive with the anti-HemA antibody further confirms 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, confirming that HemA responds to the level of extracellular heme.

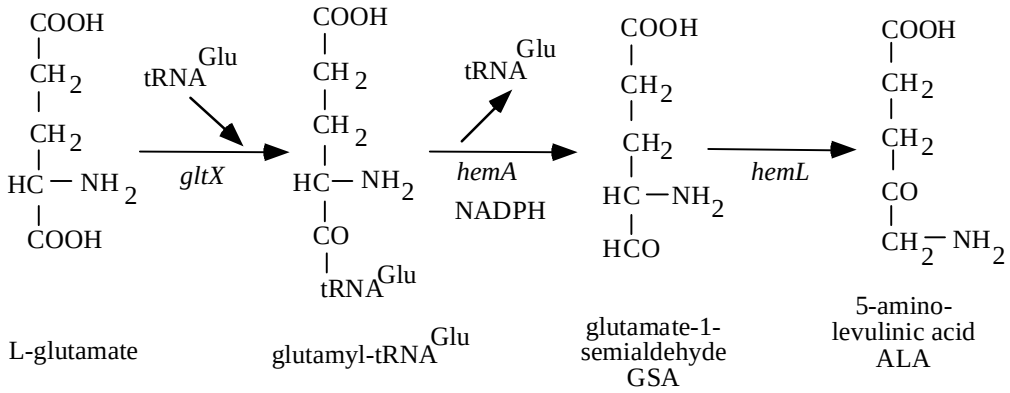
Induction of HemA protein was also observed when extracts of adapted and ALA-grown *hemL* mutant cells were compared (Fig 8, lanes g and h), and the increase was approximately equivalent to that seen with heme-starved cells. Again, high activity of HemA in the enzyme assay correlates with high levels of HemA protein detected immunologically. At least 4 different monoclonal antibodies from the panel react well with full-length HemA protein in extracts of *S. typhimurium* by Western blot; 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 HemA protein in these experiments: induction ratios of 10- to 20-fold were obtained in both the heme starvation and *hemL* adaptation protocols (Fig 8 legend).

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 both in the MG1655 and MC4100 backgrounds (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 was noted on expression of a *hemA-lac* operon fusion, both in *E. coli* and *S. typhimurium* (15). 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 protocol of these experiments is followed (46a). These results were obtained with a *lac* operon fusion to codon 181 of the *hemA* gene (15). Another fusion to codon 416 showed no change at all in response to heme starvation. Although we have constructed *hemA-lac* protein fusions, their instability to proteolysis precludes use for this type of experiment

(3). From these results, we conclude that HemA regulation is mediated either at a post-transcriptional step or through modulation of protein stability.

A.



B.

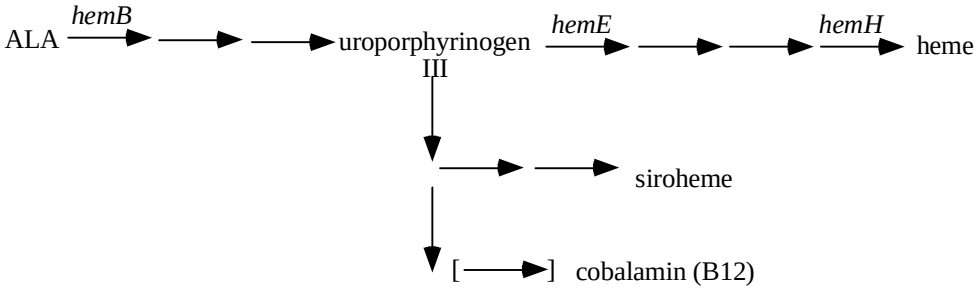


Fig. 1. The heme biosynthetic pathway. (A). Reactions leading from glutamate to ALA. Charged glutamyl-tRNAGlu is the substrate for the first committed enzyme of the heme biosynthetic pathway, glutamyl-tRNA reductase (HemA), which acts sequentially with glutamate-1-semialdehyde aminotransferase (HemL) 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 steps in B12 biosynthesis.

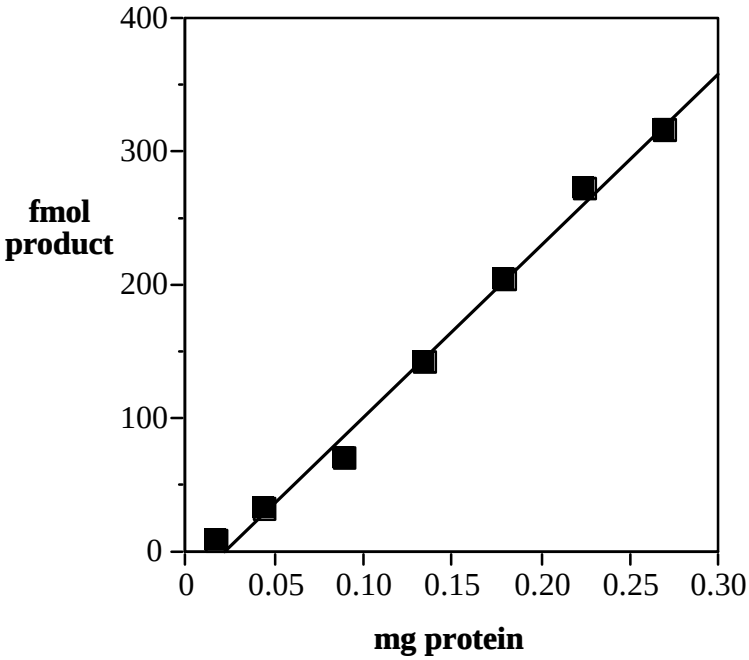


Fig. 2. HemA enzyme assay: product formation is linearly proportional to the amount of protein. A crude extract of a *hemB* mutant (TE2695) was prepared and assayed as described in the Methods and discussed in the text.

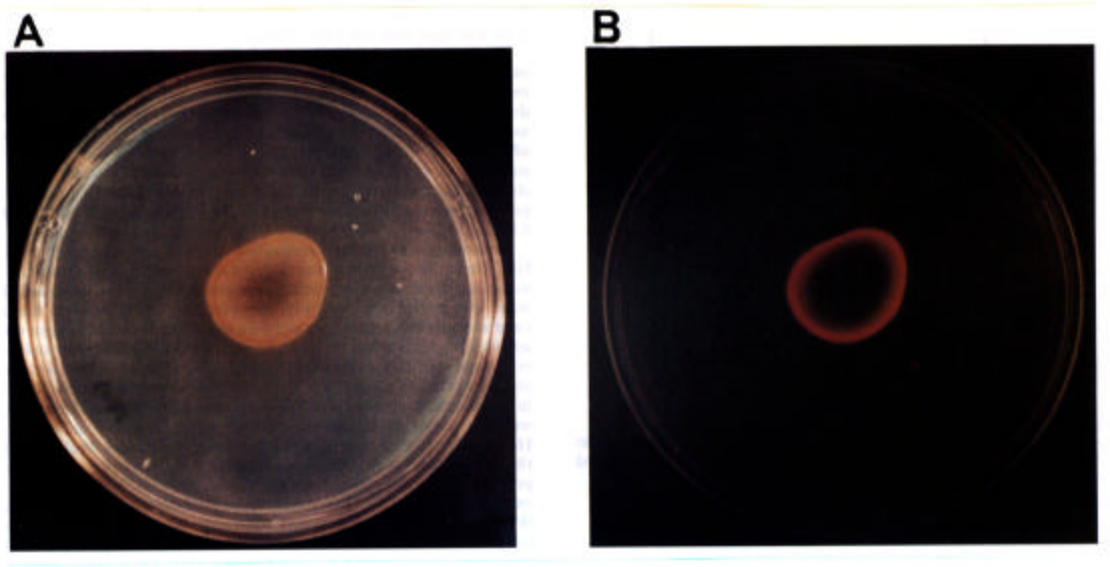


Fig. 3. **Porphyrin accumulation revealed by UV fluorescence.** About 10^8 cells of a *hemE* mutant strain (TE1303 *hemE1 env-53*) were spread on minimal glycerol cystine agar selective for Hem^+. Ten μl of heme (4 mg/ml) was spotted in the center of the plate. After 48 hr of aerobic incubation at 37°C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

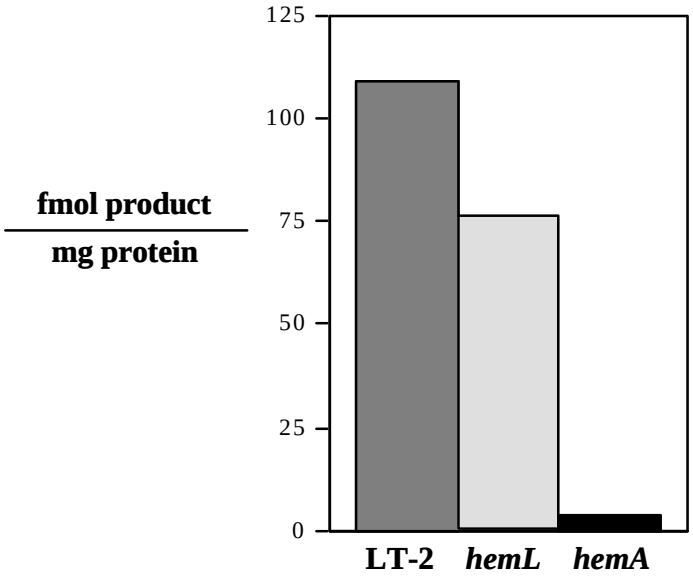


Fig. 4. Assay of *hemA* and *hemL* mutants. Cultures of a wild type strain (LT-2), a *hemL* mutant (TE472) and a *hemA* mutant (TE719) were grown in minimal glycerol medium containing 2 μM ALA. Extracts were prepared and assayed for HemA activity as described in the text.

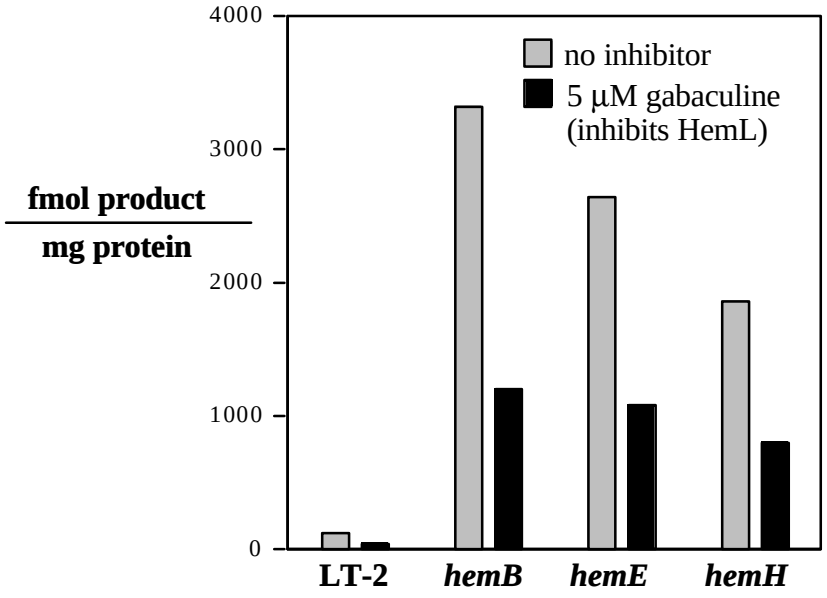


Fig. 5. Starvation for heme induces HemA activity. Cultures were grown of the wild type strain LT-2, or the following hem mutants: *hemB* (TE2695), *hemE* (TE2504) and *hemH* (TE2698). All cultures were grown in LB medium. HemA activity was assayed as described in the text; duplicate assays were performed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

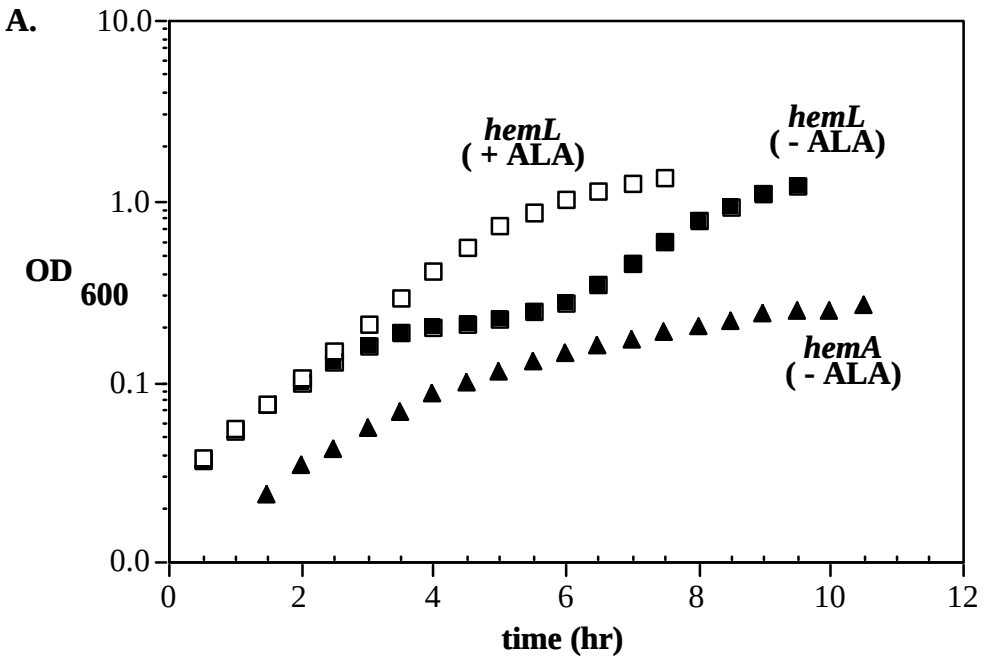


Fig. 6. (A). Adaptation of a *hemL* mutant to growth without ALA. A deletion mutant of *hemL* (TE472), and a *hemA* mutant (TE719) were grown in minimal glycerol medium in the presence of 2 μM ALA, then washed and diluted into the same medium either containing 2 μM ALA or without ALA. Growth was monitored 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 wild type (not shown).

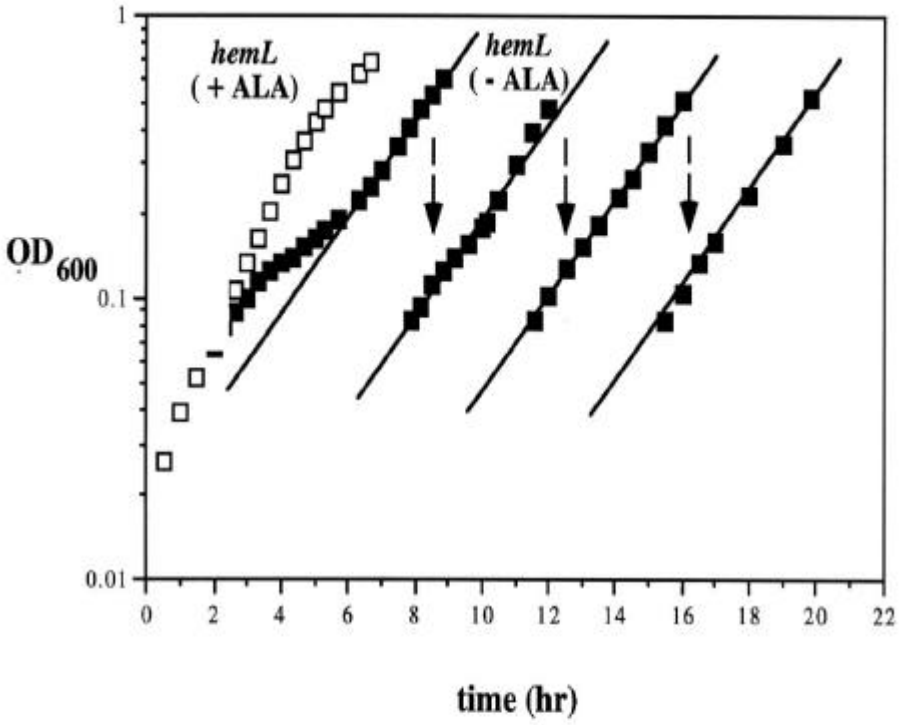


Fig. 6. (B). The *hemL* mutant was adapted to growth without ALA as in Fig 6A, and exponential growth was maintained by repeated 5-fold dilutions at the times indicated by the vertical arrows.

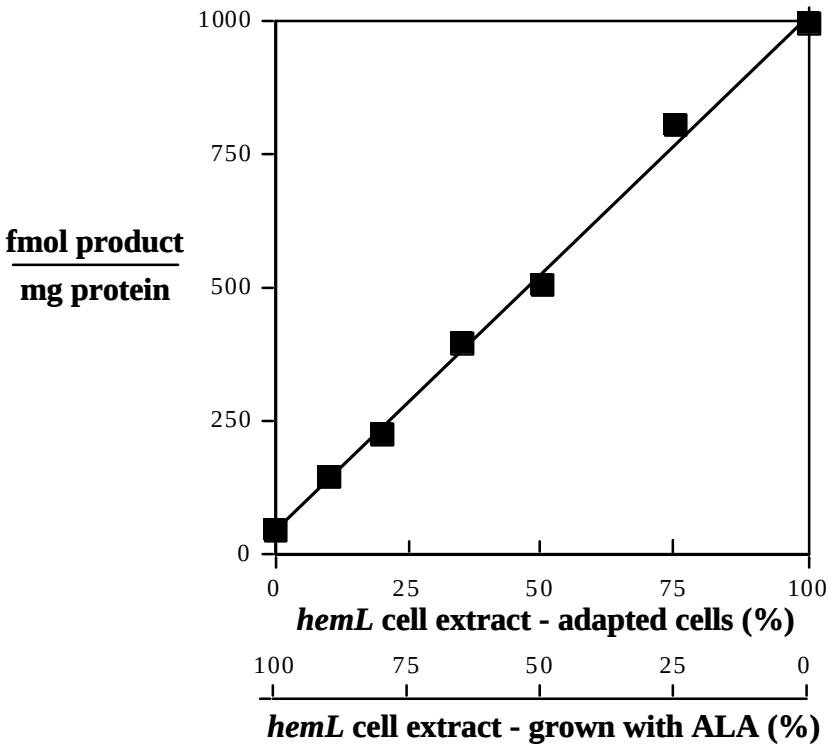


Fig. 7. Mixing experiment rules out a diffusible inhibitor of HemA. The *hemL* mutant strain TE472 was grown in minimal glycerol medium, either in the presence of 2 μM ALA, or after adaptation to growth without ALA (as shown in Fig 6 and described in the text). Extracts of both cultures were prepared 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.

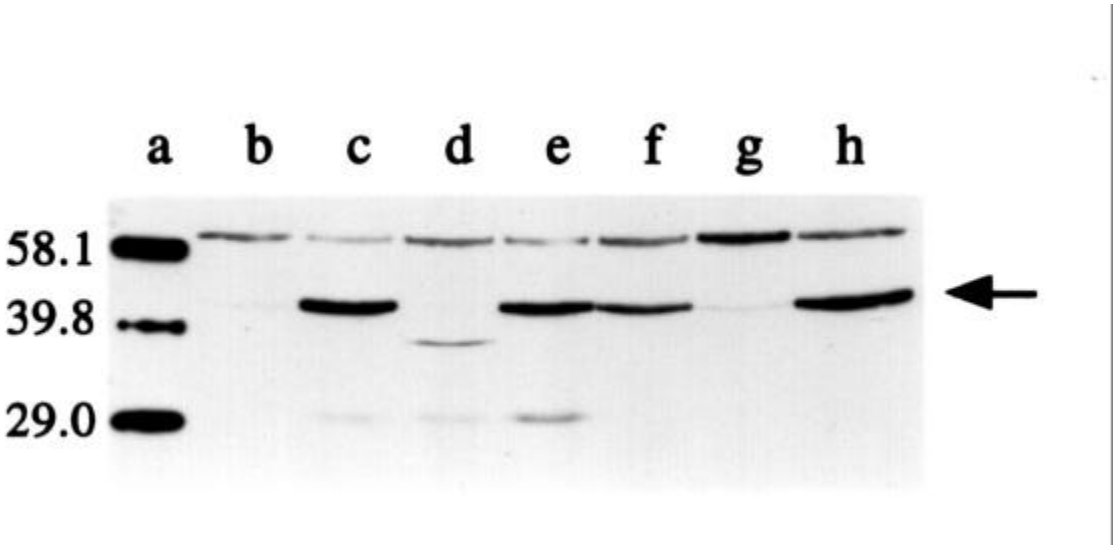


Fig. 8. Western blot (immunoblot) analysis of HemA protein abundance. Extracts were prepared and equal amounts of protein were analyzed by Western blot with anti-HemA monoclonal antibody H23 as described in Methods. The arrow indicates the position of native HemA protein. Strains analyzed and the relative amounts of HemA protein as determined by laser densitometry (arbitrary units): (b) wild type LT-2 (< 0.5 U); (c) TE2695 *hemB*::Mud-J (16 U); (d) TE2701 *hemB*::Mud-J *hemA60* (1.6 U in the truncated HemA peptide); (e) TE2504 *hemE*::Mud-J (13 U); (f) TE2698 *hemH*::Mud-J (8 U); (g) TE472 Δ*hemL* grown with 2 μM ALA (1 U); (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 HemA enzyme activity was determined. Lane (a) contains molecular weight standards with sizes indicated at the left.

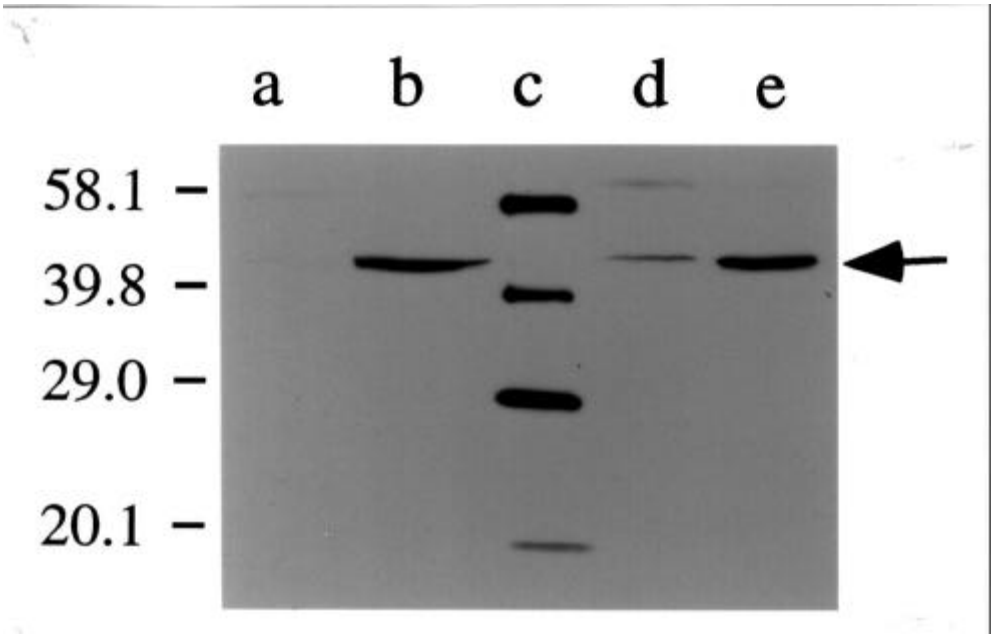


Fig. 9. HemA levels change in response to added heme. Extracts were prepared and equal amounts of protein were analyzed by Western blot with anti-HemA monoclonal antibody H6 as described in Methods. The arrow indicates the position of native HemA protein. Strains analyzed: (a) extract of the wild type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

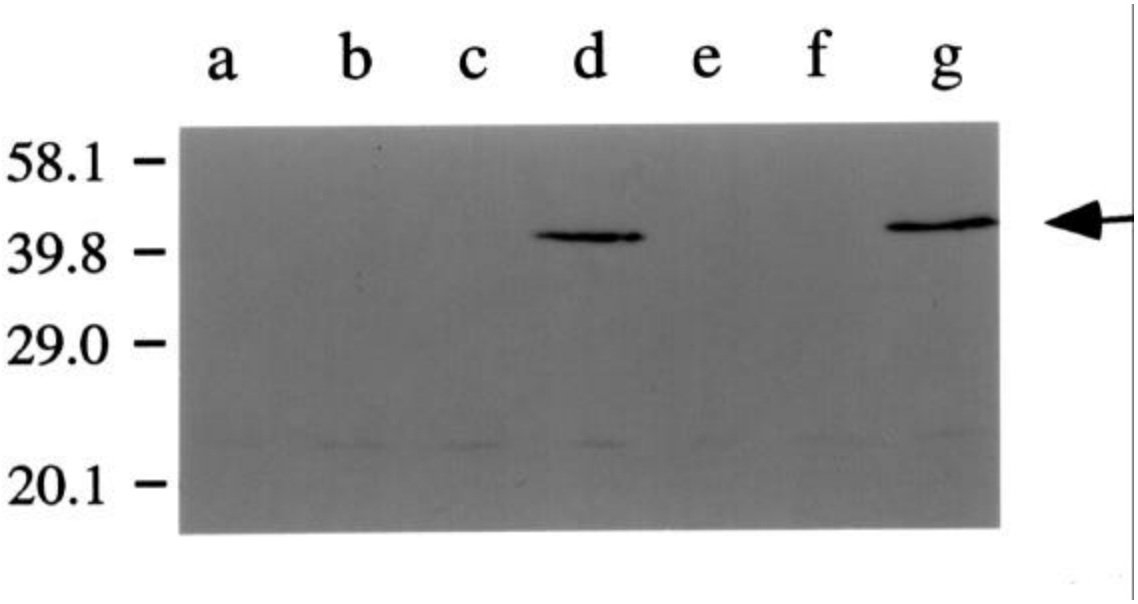


Fig. 10. HemA induction in adapted *hemL* mutant strains of *E. coli*. Extracts were prepared and equal amounts of protein were analyzed by Western blot with anti-HemA monoclonal antibody H6 as described in Methods. The arrow indicates the position of native HemA protein. Strains analyzed: (a) TE5814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

Discussion

The results described here provide the first direct evidence for regulation of the heme biosynthetic pathway in enteric bacteria. Activity of the HemA enzyme (glutamyl-tRNA reductase) was found to be substantially elevated after limitation for heme in *S. typhimurium*. The increase was 10- to 25-fold depending on the strain and the protocol used to impose starvation. Induction of HemA activity was observed in mutants blocked at three different places in the heme pathway: in a *hemB*, a *hemE* and a *hemH* mutant (Fig 1B). The induction was highest in a *hemB* mutant and somewhat less in the other mutants (Fig. 5); we do not know yet 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 (immuno) blots. Within the limits of the methods used, the increase in protein accounts for the entire change in enzymatic activity. Operon fusions of *lac* to two sites in *hemA* report either no increase or only a 2-fold 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 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 HemA, implying that its regulation could be normal. If true, such a result 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 possible to test directly whether they are co-regulated 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.

ACKNOWLEDGEMENT

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Conditional stability of the HemA protein (glutamyl-tRNA reductase) regulates heme biosynthesis in *Salmonella typhimurium*

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Abstract

In many bacteria including the enteric species *Salmonella typhimurium* and *Escherichia coli*, heme is synthesized starting from glutamate by a pathway in which the first committed step is catalyzed by the *hemA* gene product, glutamyl-tRNA reductase (HemA). We have demonstrated previously that when heme limitation is imposed on cultures of *S. typhimurium*, HemA enzyme activity is increased 10- to 25-fold. Western (immunoblot) analysis using monoclonal antibodies reactive with HemA revealed that heme limitation results in a corresponding increase in the abundance of the enzyme. Similar regulation was also observed in *E. coli*. The near absence of regulation of *hemA-lac* operon fusions suggested a post-transcriptional control. We report here the results of pulse-labeling and immunoprecipitation studies of this regulation. The principal mechanism that contributes to elevated HemA abundance is protein stabilization. The half-life of HemA protein is 20 min in unrestricted cells but increases to > 300 min in heme-limited cells. Similar regulation was observed for a HemA-LacZ hybrid protein containing almost all of the HemA protein (416 residues). Sodium azide prevents HemA turnover *in vivo*, suggesting a role for energy-dependent proteolysis. This was confirmed by the finding that HemA turnover is completely blocked in a *lon clpP* double mutant of *E. coli*, but each single mutant shows only a small effect. The ClpA chaperone, but not ClpX, is required for ClpP-dependent HemA turnover. A hybrid HemA-LacZ protein containing just 18 amino acids from HemA is also stabilized in the *lon clpP* double mutant, but this shorter fusion protein is not correctly regulated by heme limitation. We suggest that the 18 N-terminal amino acids of HemA may constitute a degradation tag, whose function is conditional and modified by the remainder of the protein in a heme-dependent way. Several models are discussed to explain why the turnover of HemA is promoted by Lon/ClpAP proteolysis only when sufficient heme is available.

Introduction

Salmonella typhimurium and the other enteric bacteria including *Escherichia coli* are nutritionally versatile organisms. For example, *S. typhimurium* can use any one of at least 73 different compounds as a sole carbon and energy source (20). Many of these carbon sources are known or predicted to be non-fermentable: they are metabolized by oxidative pathways that utilize a terminal electron acceptor and require the participation of respiratory chains with heme-containing cytochromes. At the same time, heme can be dispensable for growth. Null mutants completely defective in heme biosynthesis grow normally under anaerobic conditions using a fermentable carbon source such as glucose, so long as cysteine is provided (30). The level of heme is accordingly high during aerobic growth, especially on non-fermentable carbon sources, and low during fermentative growth. An important unsolved problem is to understand how heme synthesis is regulated in the enteric bacteria.

The first segment of the heme pathway involves the formation of 5-aminolevulinic acid (ALA). In the enterics, this occurs by a C5 mechanism. Glutamate, which has first been activated by esterification to $tRNA^{Glu}$, is reduced by the *hemA*-encoded glutamyl-tRNA reductase (HemA) to form glutamate-1-semialdehyde, which is then converted to ALA by the *hemL*-encoded enzyme, glutamate-semialdehyde aminotransferase (HemL).

Previous work (reviewed in references 5, 8, 29) has provided suggestive evidence regarding modes of heme regulation including the following possibilities: (i) that the formation of ALA is either mainly or partially rate-limiting; (ii) that HemA activity might be feedback inhibited by heme; and (iii) that late oxidative enzymes in the pathway (HemF, HemN and HemG in Fig 1) might control heme synthesis by virtue of the coupling of their activity to respiratory capacity. Transcriptional control is conspicuously

absent from proposed models. No evidence has been found for substantial control of expression of the *hem* genes, which are scattered on the genetic map (8, 30).

Our recent development of a panel of monoclonal antibodies reactive with HemA, together with use of a specific enzyme assay, led to the first direct demonstration of regulation of heme biosynthesis in the enteric bacteria (29). In that study, the level of HemA enzyme and protein were shown to rise in concert by 10- to 25-fold after limitation of growing *S. typhimurium* and *E. coli* cultures for heme. One method by which this was accomplished was to adapt *hemL* mutants, which are leaky ALA and heme auxotrophs (bradytrophs), to growth in the absence of any supplementation. Here we explore the mechanism of this regulation further. We show that the main way in which HemA is regulated by heme limitation is through conditional proteolysis. This proteolysis, which is active in normally growing but not in heme-limited cells, depends on the Lon and ClpAP proteases *in vivo*. Models for the molecular mechanisms that might regulate HemA turnover are presented in the Discussion.

Materials and Methods

Bacterial strains. The bacterial strains used in this study are listed in Table 1. All *S. typhimurium* strains are otherwise isogenic with the wild type strain LT-2 except for the indicated markers; similarly, except for the indicated markers all *E. coli* strains are isogenic with either the wild type strain MG1655, or with the standard *lac* deletion strain MC4100 (SG20250 in Table 1). The *S. typhimurium hemL* mutant strain TE472 is a deletion lacking nearly all of the *hemL* gene; reference 11 contains a deletion map of *hemL* showing the extent of this and the *hemL376* deletion, also used in this work. The *hemA60* mutant strain TE719 carries a point mutation that maps to the C-terminus of *hemA*. Strain TE3739 carries a DNA fragment encoding Kan^R inserted at the *Nhe*I site at codon 161 of *hemA* (9); this insertion is polar on *prfA* , an essential gene. The *hemA*::Kan insertion strain also carries the plasmid pTE367 to provide *prfA* function (12). Fusions of *hemA* to *lac* were constructed and placed in single copy in the *S. typhimurium* chromosome, using a method described previously (10). These constructs are present at the *put* locus. Details of the *lac* fusion to codon 18 of *hemA* (TE2685 and its derivatives) have been described (4,10). The *lac* fusion at codon 416 of *hemA* was constructed in exactly the same way as the *hemA-prfA-lac* fusion described in ref 10. Fusions were transferred to F' plasmids (10) and introduced to *E. coli* by conjugation by using the intermediate strain HMS174 as shown in Table 1. Because the F' *hemA-lac* plasmids and the *clpX* and *clpA* mutant *E. coli* strains all carry Kan^Ras the selective marker, a Cam^R was added to the F plasmid in strain TE7137 and its derivatives.

We constructed a Kan^R insertion mutant in *E. coli clpX* for this work, because we were unable to construct certain strains with the existing mutation for unknown reasons.

Table 1. Bacterial Strains.

Strain	Genotype	Source
Salmonella typhimurium		
LT-2	wild type	Lab collection
TE299-1	Δ*hemL376*	TT12009 (11)
TE472	DEL854 [*zae-1868**Mud-J**hemL332*]	TT12006 (11)
TE565	*proAB47* / F^+ *zzf-1854*::Tn*10d*-Cam	Lab collection
TE719	*hemA60*	TT11991 (11)
TE2470	*araC1* DUP[(*hemA702*::Kan cob-4)*Tn10*(zdd-1852)]	(9)
TE2685	*putPA1303*::Kan^R-*hemA-lac* [pr] (codon 18)	(4)
TE2713	Δ*hemL376 putPA1303*::Kan^R-*hemA-lac* [pr] (codon 18)	P22.TE2685 x TE299-1
TE3413	*putPA1303*::Kan^R-*hemA-lac* [pr] (codon 416)	this study
TE3726	LT-2 / pTE367 (Amp^R, *E. coli prfA*$^+$)	(12)
TE3739	*hemA702*::Kan / pTE367 (Amp^R, *E. coli prfA*$^+$)	P22.TE2470 x TE3726
TE4351	*pyrD121* Δ*put(PA)521* / F^+ *zzf-6807*::Tn*10d-putA1302*::Cam	(10)
TE4377	*pyrD121* Δ*put(PA)521* / F^+ *zzf-6807*::Tn*10d-putPA1303*::Kan^R-*hemA-lac* [pr] (codon 18)	P22.TE2685 x TE4351
TE6595	Δ*hemL376 putPA1303*::Kan^R-*hemA-lac* [pr] (codon 416)	P22.TE3413 x TE299-1
TE6920	*pyrD121* Δ*put(PA)521* / F^+ *zzf-6807*::Tn*10d-putPA1303*::Kan^R-*hemA-lac* [pr] (codon 416)	P22.TE3413 x TE4351
TE7137	*pyrD121* Δ*put(PA)521* /F^+ *zzf-6807*::Tn*10d-putPA1303*::Kan^R-*hemA-lac* [pr] (codon 416) *zzf-1854*::Tn*10d*-Cam	P22.TE565 x TE6920
TE7160	Δ*hemL376 his*::Tn*10d*-Cam (*hisB* or *hisH*)	this study

Table 1. Bacterial Strains. (continued)

Strain	Genotype	Source
Escherichia coli		
MG1655	K-12 F^- λ^- prototroph	D. Biek
HMS174	K-12 F^- *hsdR recA* Rif^R	W. F. Studier
SG12047	C600 *lon-146*::ΔTn*10*	S. Gottesman (15)
SG22007	MC4100 *clpP1*::Cam	S. Gottesman (24)
SG20250	MC4100 = K-12 F^- λ^- *araD139* Δ(*lacIpoZYA, argF*)	
	U169 flb5301 relA1 rpsL150 deoC1 ptsF25 rbsR	S. Gottesman
SG22099	MC4100 *clpA*::Kan	S. Gottesman (23)
TE5301	MG1655 Δ*lacX74*	(3)
TE6905	MG1655 *lon-146*::ΔTn*10*	P1.SG12047 x MG1655
TE6906	MG1655 *clpP1*::Cam	P1.SG22007 x MG1655
TE6907	MG1655 *clpP1*::Cam *lon-146*::ΔTn*10*	P1.SG12047 x TE6906
TE7023	MC4100 *clpP1*::Cam *lon-146*::ΔTn*10*	P1. SG12047 x SG22007
TE7028	MC4100 *clpP1*::Cam *lon-146*::ΔTn*10* / F^+ *zzf-6807*:: Tn*10d-putPA1303*::Kan^R-*hemA-lac* [pr] (codon 18)	TE4377 c HMS174 c TE7023
TE7029	MC4100 *clpP1*::Cam *lon-146*::ΔTn*10* / F^+ *zzf-6807*:: Tn*10d-putPA1303*::Kan^R-*hemA-lac* [pr] (codon 416)	TE6920 c HMS174 c TE7023
TE7031	MC4100 *clpP1*::Cam/ F^+ *zzf-6807*::Tn*10d-putPA1303*::Kan^R-*hemA-lac* [pr] (codon 416)	TE6920 c HMS174 c SG22007

Table 1. Bacterial Strains. (continued)

Strain	Genotype	Source
Escherichia coli		
TE7033	MC4100 *lon-146*::ΔTn*10* / F^+ *zzf-6807*::Tn*10d-putPA1303*::Kan^R-*hemA-lac* [pr] (codon 416)	TE6920 c HMS174 c TE7034
TE7034	MC4100 *lon-146*::ΔTn*10*	P1.SG12047 x SG22007
TE7091	MC4100 / F^+ *zzf-6807*::Tn*10d-putPA1303*::Kan^R-*hemA-lac* [pr] (codon 416)	TE6920 c HMS174 c SG20250
TE7121	MC4100 *clpA*::Kan *lon-146*::ΔTn*10*	P1.SG12047 x SG22099
TE7151	MC4100 *clpA*::Kan *lon-146*::ΔTn*10* / F^+ *zzf-6807*::Tn*10d- putPA1303*::Kan^R *hemA-lac* [pr] (codon 416) *zzf-1854*::Tn*10d*-Cam	TE7137 c HMS174 c TE7121
TE7254	MG1655 *recD1903*::mini-Tet *clpX*::Kan	this study
TE7276	MG1655 Δ*lacX74 clpX*::Kan	P1.TE7254 x TE5301
TE7282	MG1655 Δ*lacX74 clpX*::Kan *lon-146*::ΔTn*10*	P1.SG12047 x TE7276
TE7290	MG1655 Δ*lacX74 clpX*::Kan *lon-146*::ΔTn*10* / F^+ *zzf-6807*::Tn*10d- putPA1303*::Kan^R-*hemA-lac* [pr] (codon 416) *zzf-1854*::Tn*10d*-Cam	TE7137 c HMS174 c TE7282
TE7315	MG1655 Δ*lacX74 lon-146*::ΔTn*10*	P1.SG12047 x TE7276
TE7319	MG1655 Δ*lacX74 lon-146*::ΔTn*10* / F^+ *zzf-6807*::Tn*10d- putPA1303*::Kan^R-*hemA-lac* [pr] (codon 416) *zzf-1854*::Tn*10d*-Cam	TE7137 c HMS174 c TE7315

To make this construct, plasmid pWPC9 (*clpP*$^+$, *clpX*$^+$) was digested with *Bgl*II and a *Bam*HI fragment from pUC4K encoding Kan^R was inserted, disrupting *clpX* at codon 294. Digestion with *Bam*HI and linear transformation of a *recD* mutant of MG1655 (10) gave TE7254. After backcross to MG1655 Δ*lacX74*, the mutation showed >95% linkage in transduction using P1 donor phage grown on SG12047 (*lon-146*::ΔTn*10*). We were unable to transfer this mutation into SG20250, hence tests of *clpX* function were carried out in the MG1655 background.

Growth of cultures. All cultures were grown at 37°C in either Luria-Bertani (LB) medium (27) or in minimal MOPS (morpholinepropanesulfonic acid) medium (25) as modified (7) containing 0.2% glycerol as the carbon source. Plates were prepared using nutrient agar (Difco) with 5 g of NaCl per liter or using NCE medium (6) with 0.2% glycerol as the carbon source. ALA was used at 2 μM in minimal medium (11). Antibiotics were added to rich medium to final concentrations as follows: 100 μg/ml sodium ampicillin, 20 μg/ml chloramphenicol, 50 μg/ml kanamycin sulfate, 20 μg/ml tetracycline hydrochloride, and 200 μg/ml streptomycin sulfate. For strains with F' plasmids grown in minimal medium, final antibiotic concentrations were: 10 μg/ml chloramphenicol and 100 μg/ml kanamycin sulfate.

Adaptation of *hemL* mutant strains of *S. typhimurium* and *E. coli* was carried out according to reference 29. Cells were first grown overnight in minimal MOPS glycerol medium with 2 μM ALA, then diluted 1:50 into the same medium and grown to OD_{600} = 0.4 before growth was stopped by rapidly chilling the flask in ice-water. A 2.5 ml aliquot of the culture was centrifuged and resuspended in 10 volumes of minimal MOPS glycerol medium and grown to OD_{600} = 0.4 (adaptation). This culture was also chilled and held overnight.. Each culture was diluted 1/10 into the appropriate medium and grown to OD_{600} = 0.4 for labeling.

For testing the specificity of HemA induction, strain TE7160 (Δ*hemL his*::Tn*10d*-Cam) was grown in minimal MOPS glycerol medium under the following conditions: (a) unlimited growth in medium with 10 mM NH_4Cl and containing 2 μM ALA and 0.1 mM L-histidine; (b) heme-limited growth in medium containing NH_4Cl and L-histidine but with adaptation to lack of ALA as described above; (c) histidine-limited growth in medium containing 50 μg/ml L-histidinol as the source of histidine, containing ALA and with NH_4Cl as the nitrogen source; (d) nitrogen-limited growth in medium containing ALA, with 0.1 mM L-histidine and 5 mM L-arginine as sources of nitrogen.

Labeling and immunoprecipitation. The rates of synthesis and turnover of native HemA and HemA-LacZ hybrid proteins were examined by labeling or pulse-chase protocols using immunoprecipitation with anti-HemA monoclonal antibody H17 of the γ1 isotype (29) and/or anti LacZ (β-galactosidase) antibody (Promega). Strains were grown to OD_{600} = 0.4 in minimal MOPS medium containing 0.2% glycerol, with or without 2 μM ALA, and with antibiotics as necessary. Trans-label ([^{35}S]-L-methionine and [^{35}S]-L-cysteine; ICN) was added to a 1 ml sample of each culture at 100 μCi/ml, and after 5 min unlabeled L-methionine and L-cystine were added to final concentrations of 1.3 mM and 0.6 mM respectively. For the pulse-chase protocol, all amounts were scaled up to provide 1 ml of labeled culture corresponding to each sampling point. TCA precipitation, immunoprecipitation and adsorption onto protein A-Sepharose and subsequent processing were all exactly as described and referenced (4, 9, 21). After processing, samples totaled 35 μl, of which 15 μl were analyzed by SDS/PAGE. For the anti-HemA mAb, a secondary antibody was used (monoclonal anti-mouse γ1^a of the IgG2a isotype; ATCC).

Detection of proteins by Western blot. Techniques for Western (immuno)-blots have been described in detail (29). The primary antibody was a mouse monoclonal anti-HemA antibody of γ1 isotype (H23), which was detected by sequential application of biotin-conjugated goat anti-mouse IgG1, followed by streptavidin-conjugated horseradish

peroxidase (Southern Biotechnology), and finally visualized by enhanced chemiluminescence (Amersham).

Results

Pulse-labeling and immunoprecipitation of HemA protein. In order to establish the mechanism by which HemA abundance is regulated during heme-limited growth, we compared the rates of synthesis and turnover of the HemA protein in an adapted (heme-limited) *S. typhimurium* culture, compared to cells grown in medium supplemented with ALA and thus not limited for heme. To do this, a monoclonal antibody reactive with HemA was employed to immunoprecipitate the protein from cultures that had been pulse-labeled for 5 min with a mixture of ^{35}S-labeled methionine and cysteine. In a preliminary experiment to establish the specificity of the antibody (Fig 2), a band of the correct size to be HemA (46 kDa) was observed in immunoprecipitates of labeled wild type (lane c) and *hemL* mutant cells (lanes a and b), but the HemA band was not seen in a *hemA*::Kan insertion mutant (lane d). A minor species migrating slower than HemA can be seen in Fig 2 (more prominent in lane b) and in subsequent Figures; it is a gel artifact caused by the large amount of unlabeled IgG heavy chain (data not shown), and its intensity depends on the amount of labeled HemA protein on the gel. Although HemA protein synthesis was apparently somewhat greater in heme-limited cells (compare lanes a and b in Fig 2), this difference cannot account for the 20-fold induction observed by Western blot analysis (29).

Proteolysis regulates HemA abundance. These initial observations suggested that a change in the rate of protein turnover might be the primary means by which HemA abundance is increased during heme limitation. A pulse-chase analysis confirmed that this inference was correct (Fig 3). In a time-course comparison of the amount of HemA protein seen in adapted *hemL* mutant cells (heme-limited, bottom left panel) versus cells grown with ALA supplementation (top left panel), HemA protein was much more stable in heme-limited cells. Identical gels were also analyzed by Phosphorimager analysis

(right panel). The amount of HemA protein remaining after various times of the chase was quantitated and each data point is plotted as a percentage of the initial amount of labeled HemA present in cells not limited for heme. Heme limitation results in only a small increase in the rate of HemA synthesis (2-fold or less ; compare values at zero time). In contrast, the half-life of HemA was calculated to be 20 min in unlimited *hemL* mutant cells and in wild type *S. typhimurium* (data not shown), while the half-life was more than 10 times longer (> 300 min) in heme limited cells. HemA turnover is therefore conditional, rapid in normally growing cells but inhibited in heme-limited cells, thereby resulting in an elevated level of the enzyme.

HemA turnover by energy-dependent proteases. We expect HemA to be a cytoplasmic enzyme based on the lack of a signal sequence and its use of glutamyl-tRNA as substrate and NADPH as a cofactor. Cytoplasmic proteolysis is almost entirely due to energy-dependent proteases (16,17). A standard test of energy-dependence is to measure the rate of protein turnover after cultures have been treated with sodium azide (reviewed in 13); this treatment poisons respiration and ATP generation among other processes (26). Addition of sodium azide to pulse-labeled cultures of *S. typhimurium* prevented the turnover of HemA protein (Fig 4).

We wished to determine which proteases are responsible for HemA proteolysis. To do this we analyzed HemA turnover in *E. coli*, because of the existence of a large set of mutants defective in energy-dependent proteases (reviewed in 16). Also, a *lon clpP* double mutant of *S. typhimurium* grows very poorly, a phenotype which is not seen with *E. coli* mutants. We were encouraged to use *E. coli* because Western blot analysis had shown similar regulation of HemA by heme limitation in *E. coli* as in *S. typhimurium* (29). This study confirms and extends that result (see below).

We tested mutations affecting the proteases Lon, ClpP and ClpQ, as well as the ClpP chaperones ClpA and ClpX (Table 1; the mutant strains were generously provided by Dr. S. Gottesman). Pulse-chase and immunoprecipitation experiments established that HemA protein is completely stabilized in a *lon clpP* double mutant (Fig 5). Either a *lon* or *clpP* single mutation, by itself, stabilized HemA by only a small amount (2- to 3-fold increase in half-life; data not shown). The stability of HemA protein was not further enhanced in a *clpQ lon* double mutant compared to the otherwise isogenic *lon* mutant. These results indicate that both Lon and ClpP have roughly equal ability to degrade HemA and that contributions from other enzymes are probably minimal.

Heme limitation also regulates a full-length HemA-LacZ hybrid protein. The experiments described above were extended by determining the stability of two HemA-LacZ hybrid proteins in a pulse-chase protocol followed by immunoprecipitation. Results with the full length fusion protein ($HemA_{1-416}$-LacZ) recapitulate those found with native HemA. This large protein also gives a stronger signal, especially in *E. coli*, and confirms the specificity of the antibodies used. We first determined that $HemA_{1-416}$-LacZ is correctly regulated by heme limitation in an *S. typhimurium hemL* deletion mutant (Fig 6). In this strain, the half-life of $HemA_{1-416}$-LacZ was increased more than 15-fold by heme-limitation. In contrast, a fusion protein including only the first 18 amino acids of HemA ($HemA_{1-18}$-LacZ) was unstable, but its short half life was not conditional on heme-limitation (Fig 7). For both $HemA_{1-18}$-LacZ and $HemA_{1-416}$-LacZ, turnover was blocked in a *lon clpP* double mutant of *E. coli* (data for $HemA_{1-416}$-LacZ shown in Fig 8; for $HemA_{1-18}$-LacZ the data are not shown). Because the same two proteases are needed for turnover of both HemA-LacZ fusion proteins as well as native HemA (see also reference 4), the N-terminal 18 amino acids or a subset of them may constitute a degradation tag which confers sensitivity to proteolysis (see the Discussion).

ClpA chaperone but not ClpX chaperone is required for ClpP-directed HemA turnover. Using $HemA_{1-416}$-LacZ as a model substrate we examined the contribution of the two known ClpP chaperones, ClpA and ClpX, to ClpP-directed turnover of HemA *in vivo*. These two proteins are jointly required with ClpP for all ClpP-dependent proteolysis in *E. coli*. We found that $HemA_{1-416}$-LacZ was significantly more stable in an *E. coli lon clpA* double mutant than in the *lon* single mutant (Fig 9), whereas in a similar experiment the addition of a *clpX* allele to the *lon* mutant did not further increase the stability of HemA protein (Fig 10). These results indicate that Lon and ClpAP, but not ClpXP or any other energy-dependent protease, are the main enzymes responsible for HemA turnover under the conditions examined (37°C and minimal glycerol medium).

HemA induction is not a general consequence of growth limitation. We used Western (immunoblot) analysis to determine the specificity of HemA induction by heme limitation (Fig 11). We compared the amount of HemA protein observed using a single strain (Δ*hemL his*::Tn*10d*-Cam) grown under conditions where the growth rate was: (i) limited by available nitrogen (280 min doubling time), (ii) limited by available histidine, using histidinol as the source of histidine (154 min doubling time, reference 2); (iii) limited by available heme (95 min doubling time); or unlimited growth (68 min doubling time). The only condition in which HemA abundance was elevated was growth under heme-limitation. Other experiments indicate that the abundance of HemA is not markedly different in cultures grown with glucose, pyruvate or acetate rather than glycerol as the sole carbon and energy source (unpublished observations). Together, these findings suggest that the induction of HemA by heme limitation is a specific response, rather than a result of a lower growth rate.

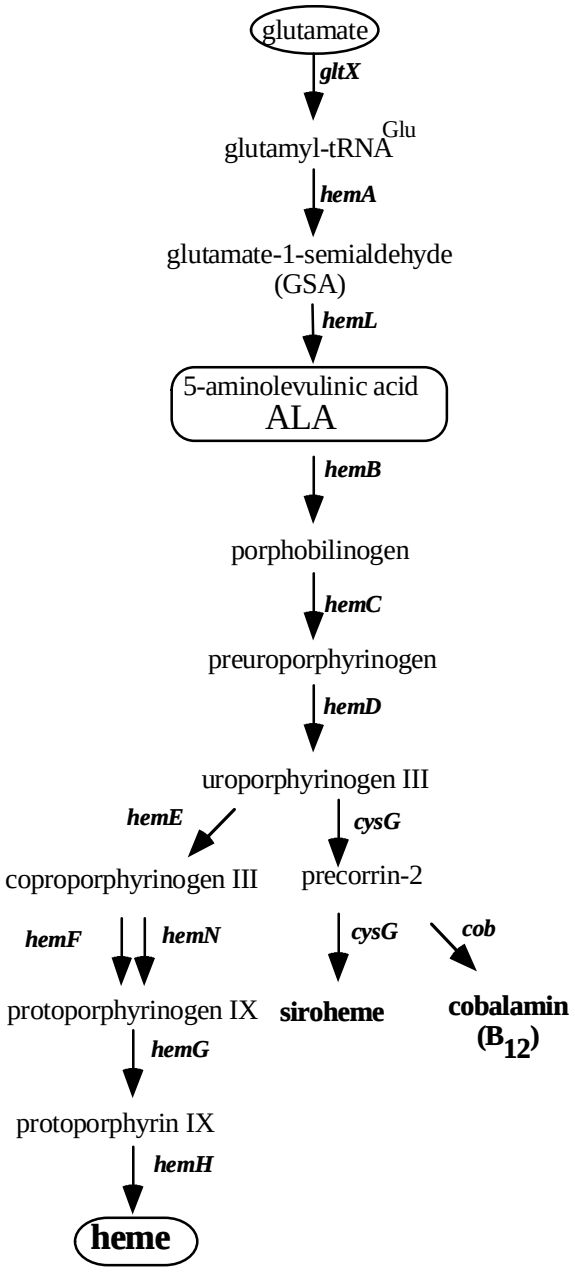

Fig. 1. Heme biosynthesis. The heme biosynthetic pathway consists of ten reactions by which glutamate is converted to heme; minor branches lead to siroheme and cobalamin. Glutamyl-tRNA reductase (HemA) is considered the first committed enzyme in the heme pathway since the vast majority (>99%) of charged $tRNA^{Glu}$ is used for protein synthesis. Mutants defective in either *hemA* or *hemL* require either ALA or both heme plus cysteine supplementation for wild type growth. In contrast to *hemA* mutants, *hemL* strains are leaky auxotrophs and can adapt to growth in the absence of supplementation, as described previously (29).

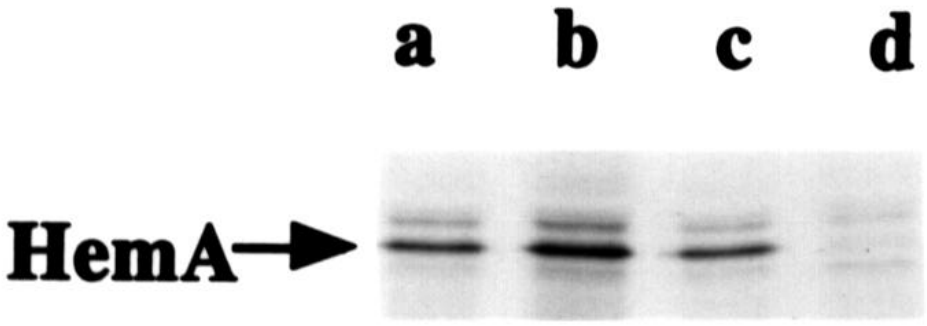

Fig. 2. Pulse-labeling and immunoprecipitation of HemA. HemA protein was analyzed by pulse-labeling of a *hemL* deletion mutant of *S. typhimurium* (TE2713) grown in MOPS glycerol medium in the presence of 2 μM ALA (lane a) or adapted to growth in the same medium without ALA (lane b). Also analyzed were the wild type strain LT-2 (lane c) and a *hemA*::Kan insertion mutant (lane d), both grown in MOPS glycerol medium in the presence of ALA; these two strains also carried plasmid pTE367 which provides the essential function of *prfA* to the *hemA*::Kan insertion mutant (9, 12). One ml of each culture was pulse-labeled (OD_{600} = 0.4) with 100 μCi of ^{35}S Trans-label for 5 min, then chased with unlabeled L-methionine (1.3 mM) and L-cystine (0.6 mM) for 2 min. Protein extracts were prepared, immunoprecipitated with anti-HemA mAb H17 and analyzed by SDS/PAGE. The position of the HemA protein is indicated by an arrow.

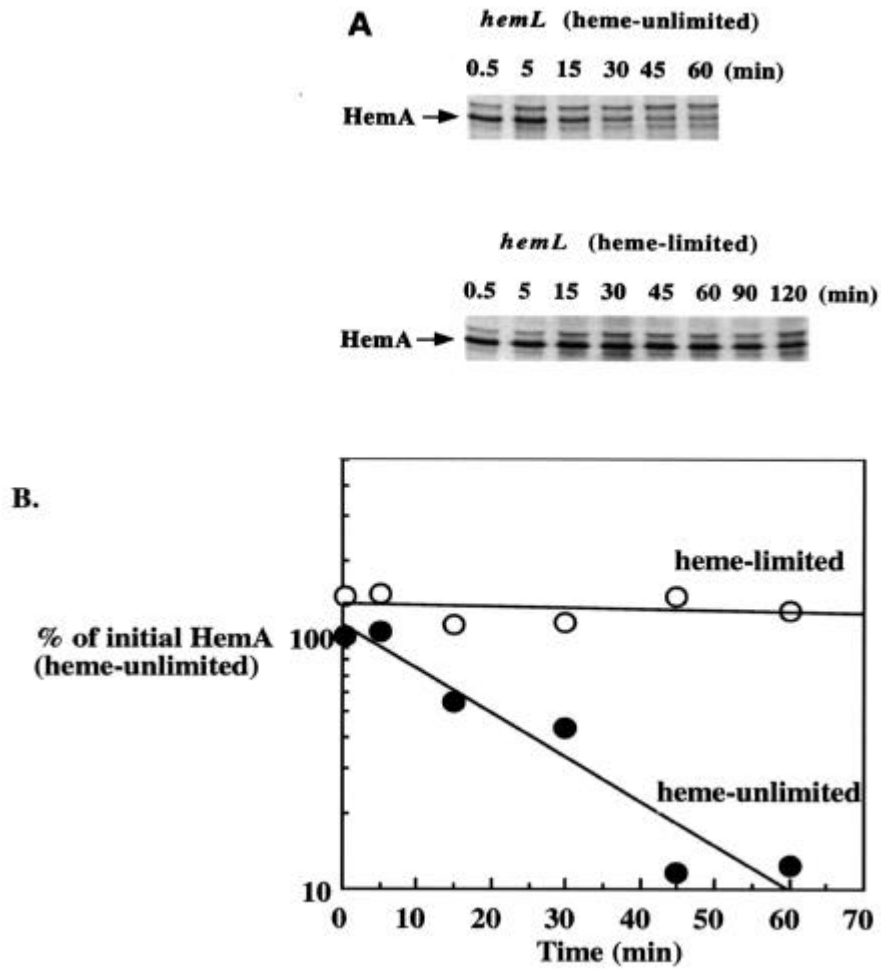

Fig. 3. Pulse-chase analysis of HemA turnover in adapted *hemL* mutant cells. A *hemL* deletion mutant of *S. typhimurium* (TE472) was grown in MOPS glycerol medium to OD600 = 0.4 in the presence of 2 μM ALA (top panel in panel A; heme unlimited) or adapted to growth in the same medium but without ALA and grown to OD600 = 0.4 (bottom panel in panel A; heme-limited). Cultures were labeled and analyzed as described in the legend to Fig. 2, except that the chase with unlabeled methionine and cystine was extended as shown above each lane. Identical gels (not treated with fluor) were analyzed by using a Phosphorimager and its ImageQuant software to produce the data plotted (B). The calculated half-life of HemA protein in unlimited cells is 20 min (closed circles), as compared with a half-life estimated to be in excess of 300 min in adapted, heme-limited cells (open circles).

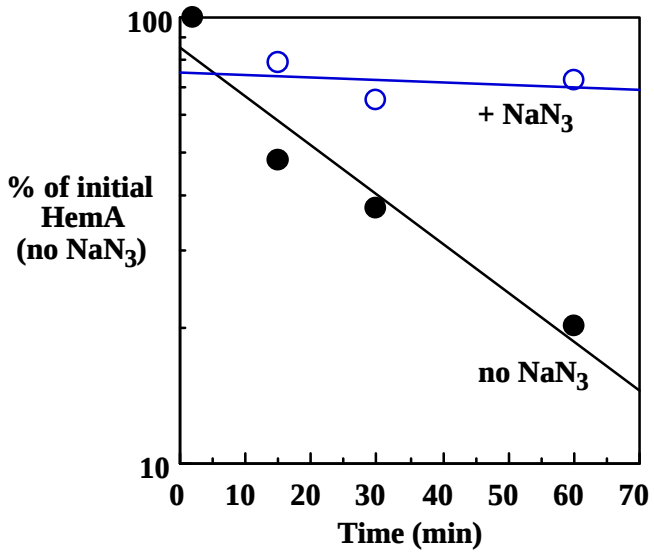

Fig. 4. HemA turnover is sensitive to azide. The wild type *S. typhimurium* strain LT-2 was grown in MOPS glycerol medium to OD_{600} = 0.4, duplicate samples were then pulse-labeled with ^{35}S Trans-label, chased for various times and analyzed by immunoprecipitation with anti-HemA mAb H17. One sample (open circles) received 5 mM NaN3 at 2 min after the addition of unlabeled amino acids; the second sample was untreated (closed circles). Data were obtained by using a Phosphorimager and ImageQuant software.

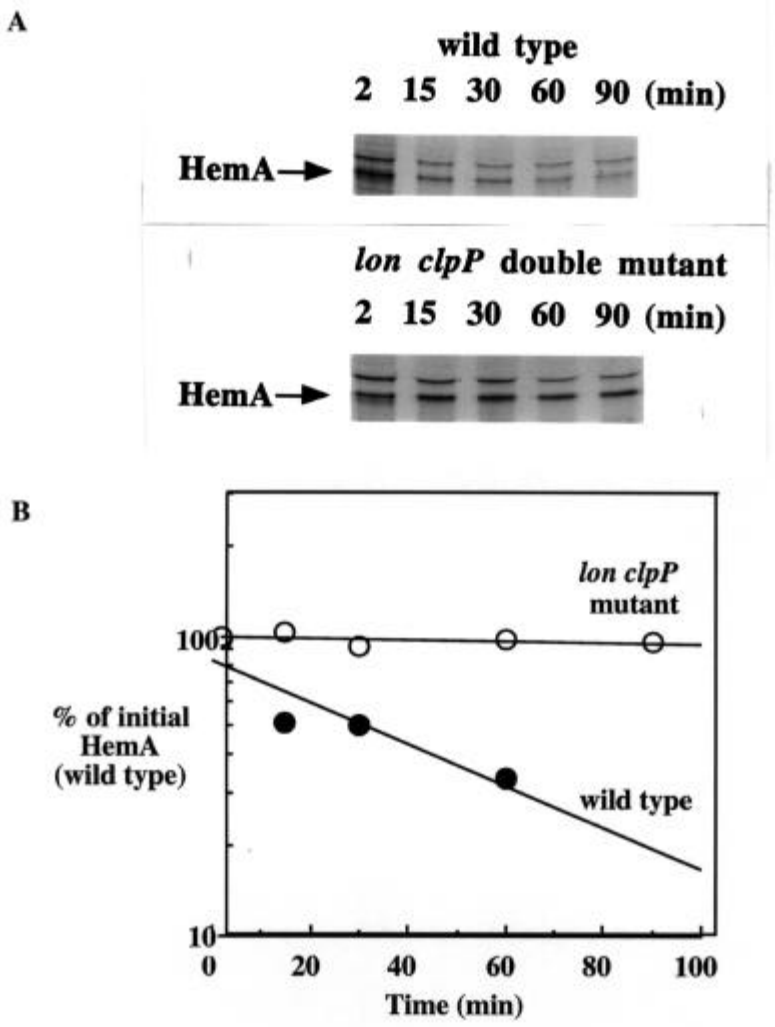

Fig. 5. Proteases Lon and ClpP are both involved in HemA turnover. The genetic requirements for proteolysis of HemA in vivo were determined in *E. coli* because of the poor growth of *lon clpP* double mutants of S. typhimurium. The wild type *E. coli* strain MG1655 and its *lon*::Tn*10 clpP*::Cam double mutant derivative (TE6907) were analyzed for HemA turnover by the same methods as used for previous experiments. (A) Top, pulse-chase analysis of wild type; bottom, pulse-chase analysis of the double mutant; (B) data obtained from Phosphorimager analysis of duplicate gels. HemA was unstable in the wild type strain (half-life ≈ 30 min), while it was stable (half-life > 300 min) in the *lon clpP* double mutant.

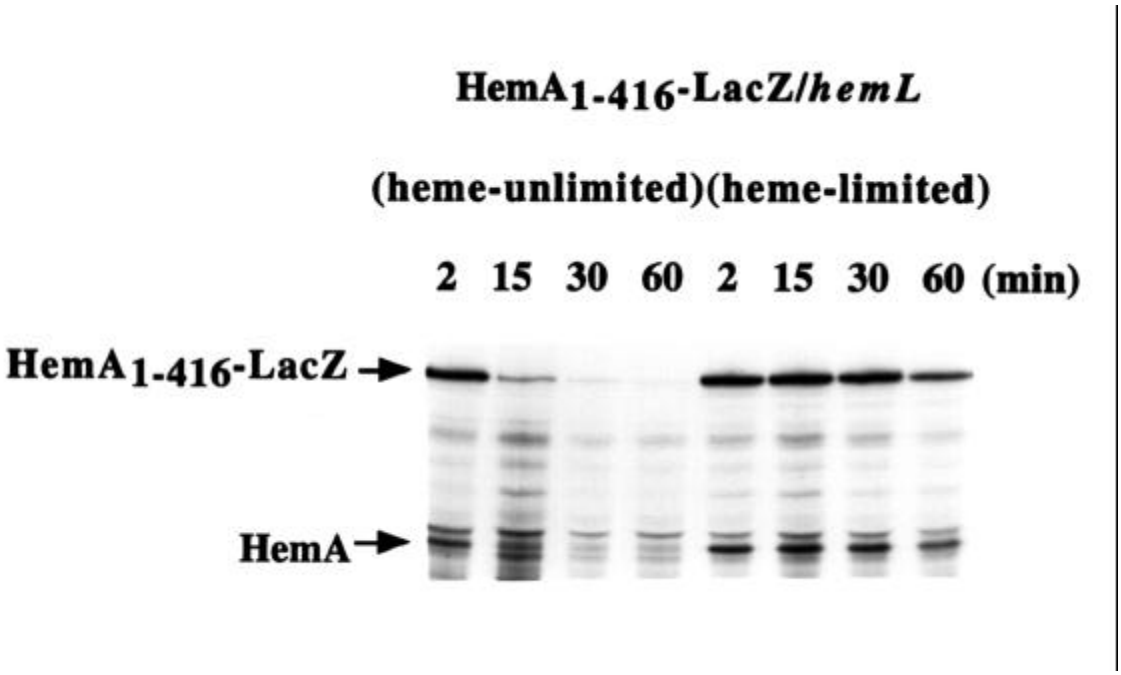

Fig. 6. A. Turnover of a HemA-LacZ hybrid protein is correctly regulated by heme limitation. A fusion construct that expresses the $HemA_{1-416}$-LacZ hybrid protein was introduced into an *S. typhimurium hemL* mutant background (TE6595). Two cultures of this strain (either adapted to heme limitation or not heme limited) were grown and analyzed as described in the legend to Fig 3, except that a mixture of anti-HemA mAb and anti-LacZ mAb (Promega) was used for the immunoprecipitation. Both native HemA and $HemA_{1-416}$-LacZ were detected and are indicated by arrows (A).

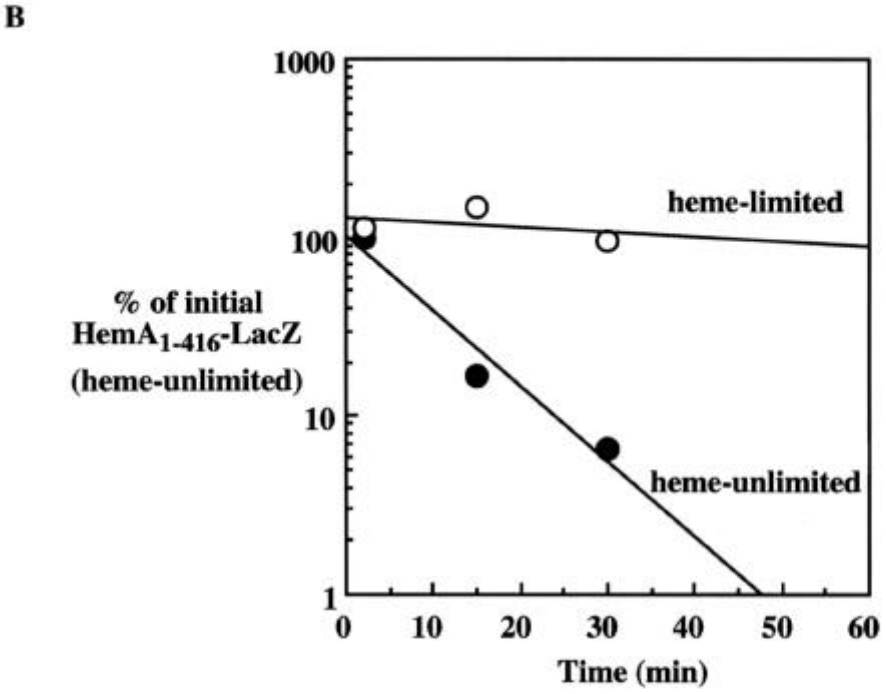

Fig. 6. (B). The half-life of $HemA_{1-416}$-LacZ was increased more than 15-fold by heme-limitation (open circles) compared to growth in the presence of ALA (closed circles).

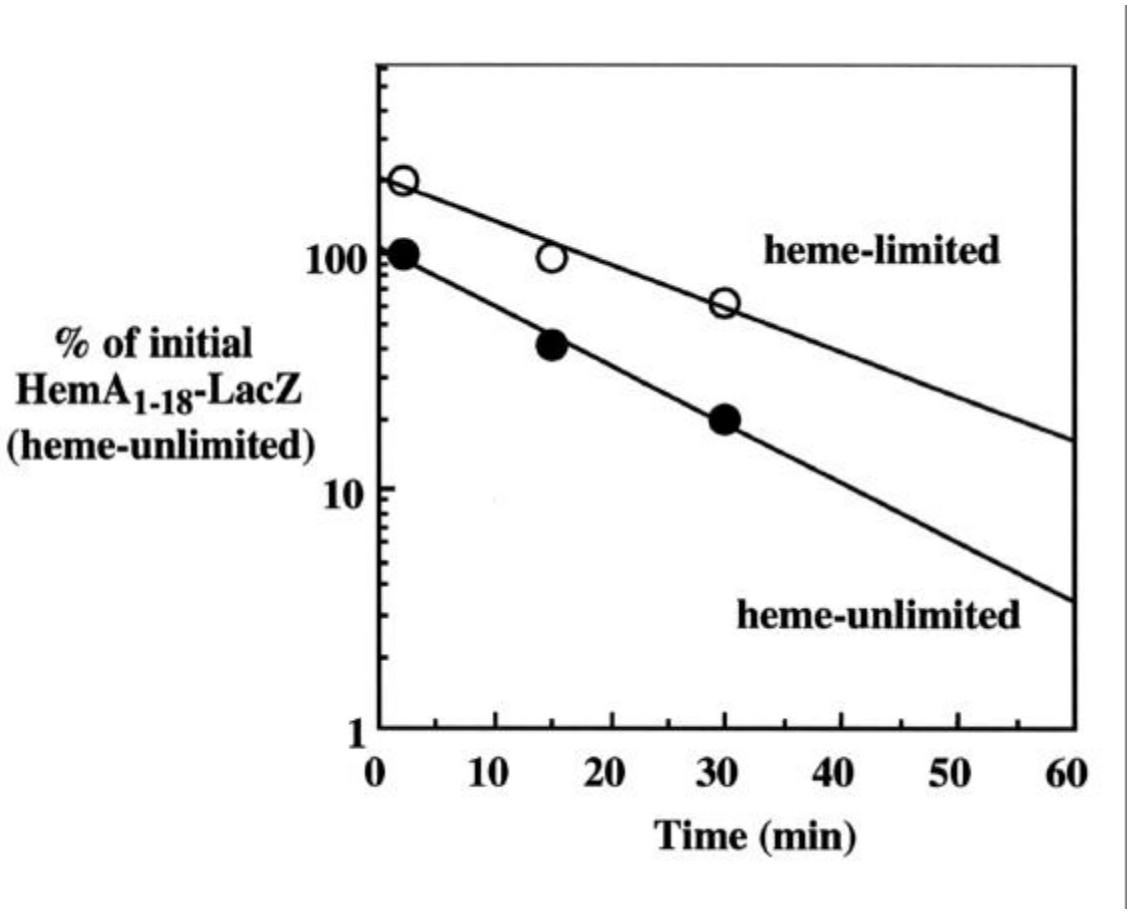

Fig. 7. Turnover of $HemA_{1-18}$-LacZ is not regulated by heme limitation. A fusion construct that expresses the $HemA_{1-18}$-LacZ hybrid protein was introduced into an *S. typhimurium hemL* mutant background (TE2713). Two cultures of this strain (either adapted to heme limitation or not heme limited) were grown and analyzed as described in the legend to Fig 3 and 6.

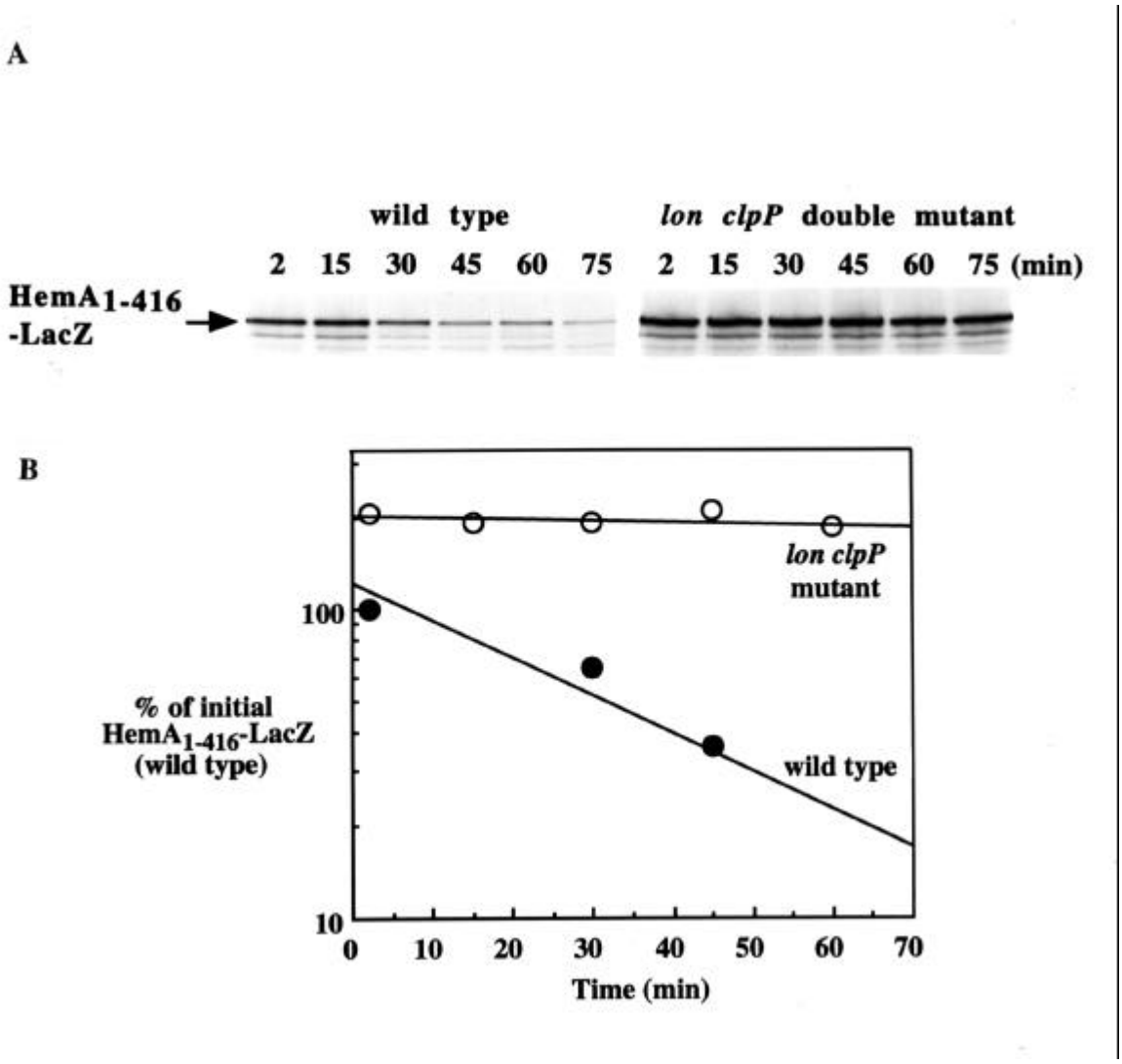

Fig 8. The $HemA_{1\text{-}416}$-LacZ hybrid protein is degraded by both Lon and ClpP proteases in *E. coli*. An F' plasmid encoding $HemA_{1\text{-}416}$-LacZ was introduced into *E. coli* MC4100 derivatives either wild type (TE7091) or a *lon clpP* double mutant (TE7029) and grown in MOPS glycerol medium with kanamycin to select for the plasmid. A pulse-chase protocol was employed, using the anti-HemA mAb H17 for immunoprecipitation (top panel). $HemA_{1\text{-}416}$-LacZ was > 10-fold more stable in the double mutant (open circles, bottom panel) compared to the wild type (closed circles). The half-life of $HemA_{1\text{-}416}$-LacZ in MC4100 was similar to that of native HemA.

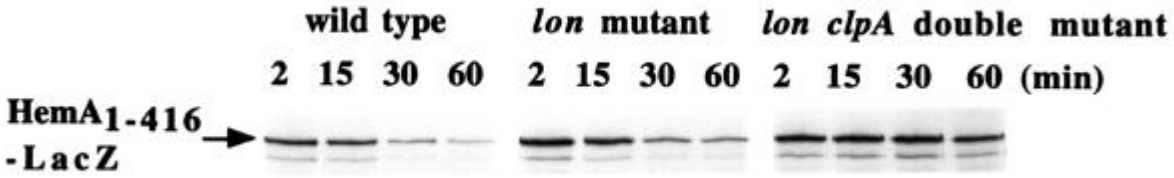

Fig. 9. Proteolysis of $HemA_{1\text{-}416}$-LacZ requires the ClpA chaperone. Stability of $HemA_{1\text{-}416}$-LacZ was examined in the wild type strain in an experiment like that shown in Fig 8, and is here compared with a *lon* single mutant and a *lon clpA* double mutant. The *lon* mutation does not alter $HemA_{1\text{-}416}$-LacZ stability very much by itself; in the *lon clpA* double mutant $HemA_{1\text{-}416}$-LacZ is nearly as stable as in the *lon clpP* double mutant (Fig 8).

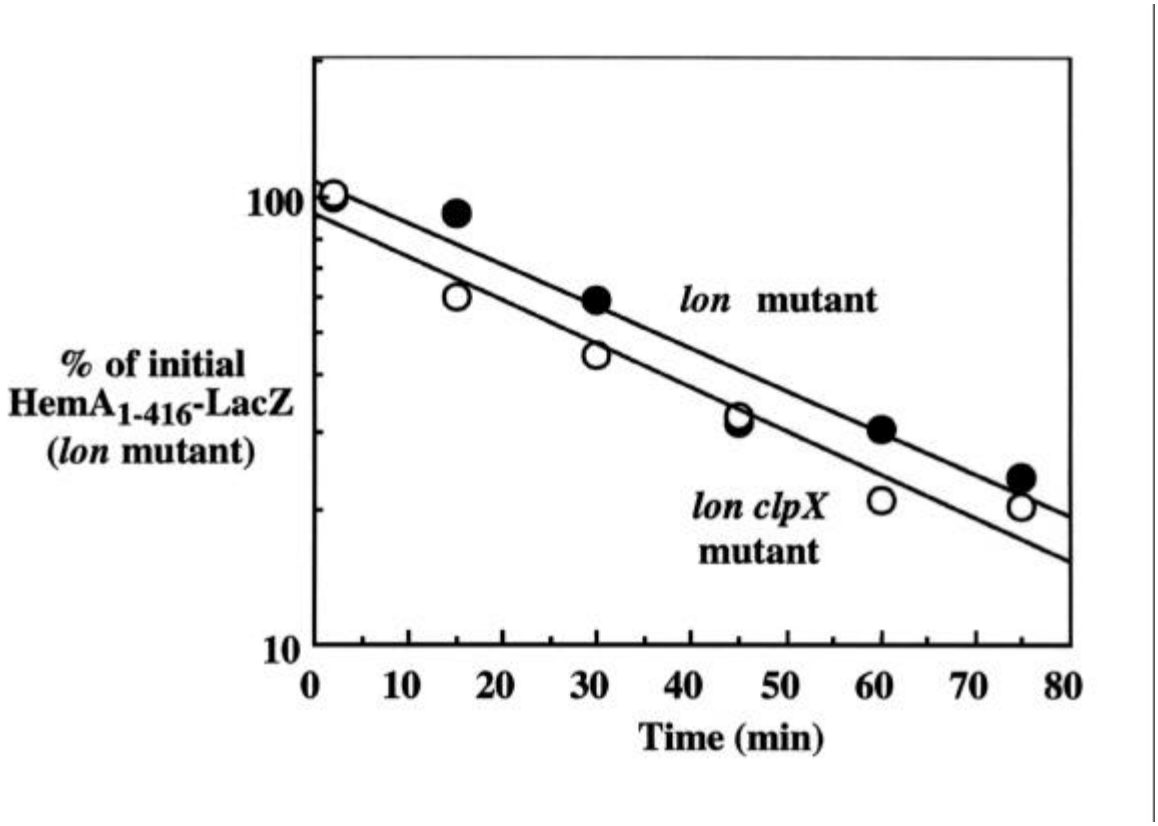

Fig. 10. Proteolysis of $HemA_{1\text{-}416}$-LacZ is not affected by lack of ClpX chaperone. Stability of $HemA_{1\text{-}416}$-LacZ was examined in a *lon clpX* strain in an experiment like that shown in Fig 8, and is here compared with a *lon* single mutant.

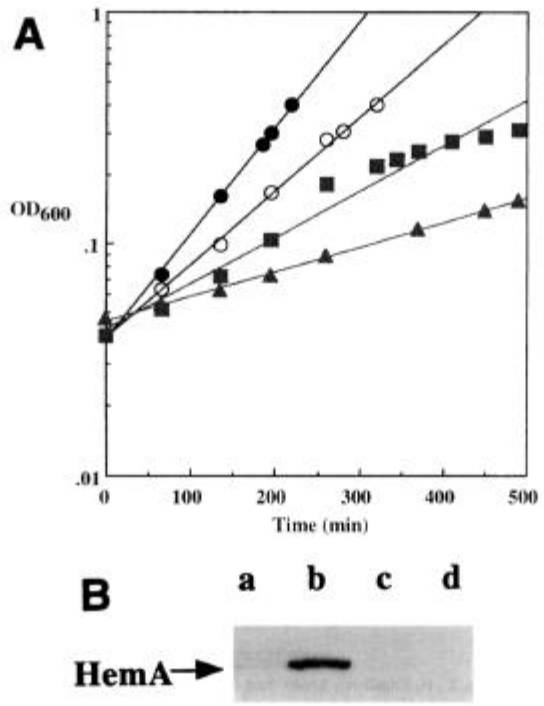

Fig. 11. Specificity of HemA induction by heme limitation. A *hisD hemL* double mutant of *S. typhimurium* (TE7160) was grown in MOPS glycerol medium, supplemented to achieve limitation for different nutrients as shown in the top panel: (a) unlimited growth in medium containing 2 μM ALA, 0.1 mM histidine, and NH_4 as the nitrogen source (closed circles); (b) heme-limited growth in medium containing histidine and NH_4 but without ALA (open circles); (c) histidine-limited growth in medium containing 50 μg/ml histidinol, with ALA and containing NH_4 as the nitrogen source (squares); (d) nitrogen-limited growth in medium containing ALA, with histidine and arginine as sources of nitrogen (triangles). Samples taken from these cultures were resuspended directly in protein gel sample buffer and analyzed for HemA protein level by Western blot using anti-HemA mAb H23, exactly as described previously (29); results are shown in the bottom panel.

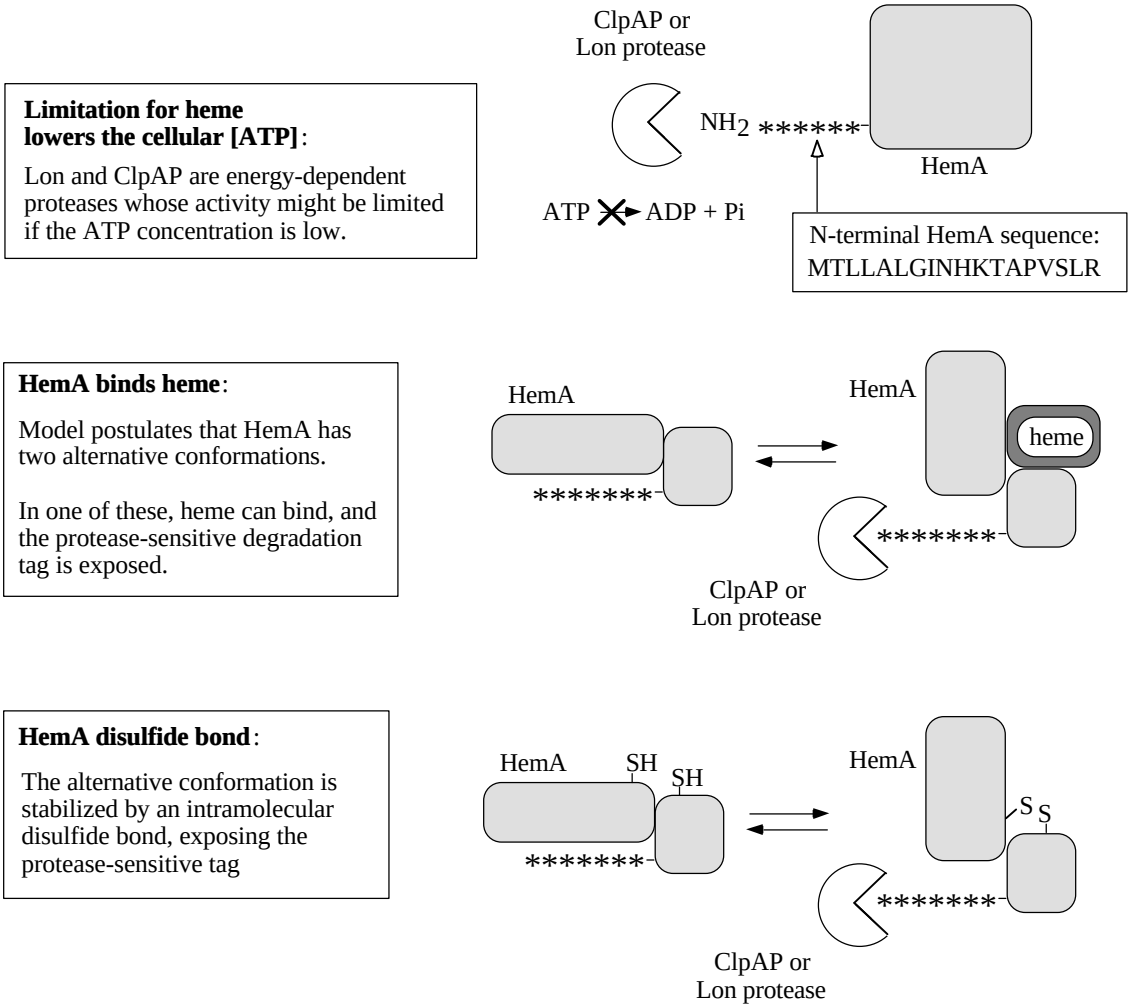

Fig. 12. Three models for regulation of HemA turnover by heme limitation. These models are discussed in the text: (a) control by ATP level; (b) control by direct binding of HemA to heme; and (c) control subsequent to formation of a disulfide bond in the HemA protein.

Discussion

HemA catalyzes the first committed step in the heme pathway (Fig 1), and is thus expected to be a target of regulation. In our previous work, analysis by Western blot (immunoblot) showed that the level of the HemA protein is elevated 10- to 20-fold during heme limitation, an increase that accounts for a similar rise in the enzyme activity as assayed *in vitro* (29). Pulse-chase and immunoprecipitation experiments reported here establish that regulation is achieved by an unusual mechanism: HemA is conditionally stable in a manner that is promoted by heme limitation. Instability of full-length proteins is rare in enteric bacteria and regulated stability is very rare. The only known examples are the sigma factors RpoH and RpoS, and the repressor LexA (reviewed in 17) and possibly the chromosomal "addiction" system antidote, MazE (1).

HemA turnover in *S. typhimurium* was found to be sensitive to azide, indicating the involvement of energy-dependent enzymes. This was confirmed by experiments with *E. coli* mutants. Single and double mutants of *lon, clpP*, *clpA*, *clpQ* and *clpX* were examined. HemA is completely stable in a *lon clpP* double mutant but only slightly stabilized by either a *lon* or *clpP* single mutation alone. We also tested the stability of two hybrid proteins: $HemA_{1-18}$-LacZ and $HemA_{1-416}$-LacZ. The nearly full-length fusion protein $HemA_{1-416}$-LacZ is regulated by heme in a manner similar to native HemA and is also stabilized in cells mutant for both Lon and ClpP. In contrast, turnover of the short fusion protein $HemA_{1-18}$-LacZ is insensitive to heme limitation, although it is stabilized in the same *lon clpP* double mutant. Extrapolating from results with $HemA_{1-416}$-LacZ, we infer that HemA is a substrate for ClpAP but not ClpXP. Instability of HemA may explain the difficulty several groups including our own have encountered in attempts to overproduce the enzyme for biochemical studies.

The amino acid sequence that marks HemA as a substrate for ClpAP and Lon, the "degradation tag", may be N-terminal since transplant of just the first 18 amino acids from HemA to LacZ makes HemA$_{1-18}$-LacZ a target for the Lon and ClpAP proteases. However, this tag does not confer correct regulation by heme. Because proteolysis by Lon and ClpP is processive, only one such "tag" or protease-sensitive site may be necessary.

Regulation of HemA during heme limitation is not a general property of media that restrict the growth rate of *S. typhimurium*: the level of HemA protein is not elevated during growth limited by a poor nitrogen source or when a low concentration of histidinol is used to satisfy a requirement for histidine (Fig. 11). Western blot analysis also showed no noticeable increase in HemA abundance during growth on carbon sources such as pyruvate or acetate which give a slower growth rate than does glycerol. This regulatory mechanism responds to an artificially limiting level of heme, achieved through a genetic defect, but in wild type cells there is no discrimination between growth in the presence or absence of excess ALA. In this respect, conditional stability of HemA is logically similar to the role played by the attenuator in histidine biosynthesis (for example), where control of enzyme level is only exerted during starvation for the end product. An unresolved question is the value of such a regulatory system to wild type cells, in which it is presumably selected. One possible use would be to respond transiently to starvation for the end product during a shift in growth conditions (as suggested in reference 14); alternatively, the mechanism may normally operate subtly, at much less than the maximum effect. Still other regulatory controls such as feedback inhibition of HemA, or regulation of later oxidative steps in the pathway, may also be important to vary the rate of heme synthesis during normal growth or in the presence of excess heme.

The molecule or process whose lack is ultimately sensed is not known. In principle, it might be heme or a modified derivative, or even a process affected by limited respiration or elevated H_2O_2 (29). Here, we consider three models for HemA regulation.

These very simple models do not invoke unknown proteins or new activities of known proteins.

In the first model, the ATP concentration *in vivo* is postulated to decrease during heme-limited growth to a point that ATP becomes limiting for energy-dependent proteolysis (or at least proteolysis of HemA). This general possibility has previously been judged unlikely because the ATP concentration measured in cells is much higher that the Km measured *in vitro* for those substrates examined so far (see reference 19). Several factors may be relevant in the case of HemA. First, ADP is a competititve inhibitor of ATP for the Lon protease (reviewed in 19, 24a); thus the energy charge rather than ATP level per se may be important. Second, when the Lon and ClpAP proteases act on HemA, the Km for ATP might be higher than with other substrates. It is thought that ATP hydrolysis by the chaperone subunit or domain facilitates unfolding of the substrate to allow access to the protease active site: perhaps HemA is particularly resistant to unfolding. If one or more ClpAP or Lon substrates were shown to be stabilized in parallel with HemA, the model would be supported. The fact that the short fusion protein, $HemA_{1-18}$-LacZ, is not regulated normally by heme limitation does not contradict the model, since HemA contributes only 18 residues to be unfolded in this protein, and also, it is not certain that this sequence is the one first recognized in the native HemA protein.

In the other two models, HemA is proposed to alternate between protease-sensitive and protease-resistant conformations (Fig 12). For example, the degradation tag may be sequestered in the resistant state but accessible in the sensitive state. In model 2, the protease-sensitive conformation is stabilized by direct binding of heme to the HemA protein. This model is suggested by the finding of heme in a partially purified preparation of a HemA homolog from barley (28) and the sensitivity of HemA to inhibition by heme in crude extracts of *E. coli* (22), as well as feedback inhibition of purified enzyme from other organisms. In model 3, the protease-sensitive conformation is stabilized by

formation of a disulfide bond, which is favored when the cell has excess oxidation capacity. The potential for disulfide-bond formation in HemA has not been tested yet, but the protein contains 3 cysteines, two of which are conserved in homologs from other organisms. Tests of these models are in progress.

ACKNOWLEDGEMENT

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N-terminal mutations which stabilize the HemA protein against heme-regulated protein turnover in *Salmonella typhimurium*

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Abstract

In *Salmonella typhimurium* and *Escherichia coli*, the *hemA* gene encodes the first committed heme enzyme, glutamyl-tRNA reductase (HemA). HemA catalyzes the rate-limiting step in heme biosynthesis, therefore it is a potential target for heme synthetic regulation. Our previous results have shown that HemA enzyme activity and its abundance are increased 10- to 25-fold during heme-limitation, while gene expression as measured by a *hemA-lac* operon fusion is not affected much. The principal mechanism that contributes to elevated HemA abundance is protein stabilization. The ATP-dependent proteases Lon and ClpAP are involved in HemA degradation. Turnover of HemA or a HemA-LacZ hybrid protein containing almost all of HemA (416 residues of 418 total) is totally blocked in a *lon clp* double mutant. A HemA-LacZ fusion protein containing the first 18 N-terminal amino acids of HemA is also stabilized in this mutant. This result suggests that HemA's degradation tag may lie in its N-terminal 18 amino acids. Here we report the finding that a mutation in the HemA N-terminal region completely stabilizes this protein while not impairing enzyme function. This result confirms the hypothesis that there is a degradation tag in the HemA N-terminus. The hydrophobicity of this tag seems to be critical for interaction between this protein and the protease(s). This finding gives important information in understanding the pathway of HemA regulation.

Introduction

In many bacteria, including *S. typhimurium* and *E. coli*, hemes are cofactors of a number of cytochromes and two catalases. These enzymes are important for the production of energy and cellular defenses against oxygen radicals (Beale, 1996). The first stable intermediate in the biosynthesis of heme is 5-aminolevulinic acid (ALA). Two pathways exist in nature for the formation of ALA (Avissar, 1989; Jahn *et al*., 1992). Most bacteria use the C5 pathway to convert glutamate to ALA by three enzyme catalyzed reactions. The key step in this pathway is catalyzed by glutamyl-tRNA reductase (HemA) (Doss and Pilipp-Dormston, 1973; Beale, 1996). In the C4 pathway, which is operative in yeast and mammalian cells, ALA is formed by the condensation of succinyl coenzyme A and glycine. Only a single enzyme, ALA synthase, is required for this formation. ALA formation is a rate-limited process in heme synthesis and is expected to be targeted by heme synthetic regulation. ALA synthase is regulated by transcription and enzyme activity (Drew and Ades, 1989; Houston et l, 1994). However, heme biosynthetic regulation in bacteria is not as well understood.

Our previous work has provided some new insights into heme synthetic regulation in *S. typhimurium* and *E. coli*. HemA protein is stabilized during heme limitation without being regulated at the transcriptional level. We also demonstrated that the energy dependent proteases Lon and ClpAP are involved in HemA proteolysis. A fusion of just the first 18 amino acids from HemA to LacZ ($HemA_{1-18}$-LacZ) is susceptible to the Lon and ClpAP proteases, while this hybrid protein is stabilized in a *lon clp* double mutant.

These results suggest that the degradation tag of HemA for ClpAP and Lon may lie in the N-terminal region of this protein.

The target elements for the specificity of the Lon and ClpAP proteases is not well defined, but there is some information which suggests that the N-terminus of proteins play a role in protein degradation (reviewed by Gottesman and Maurizi, 1992). Proteins carrying certain amino acids at the N terminus are rapidly degraded. It was reported that all the information necessary for Lon targeting for degradation can reside within a relatively short stretch of amino acids (Gottesman and Maurizi, 1992). The degradative role of one of the substrates of Lon protease, UmuD, was tested: the primary degradation signal is located between the N-terminal residues 15 and 18 of UmuD. The N-terminus of UmuD (1-40 residues) is sufficient for Lon recognition and the ensuing degradation of the protein (Gonzalez *et al*., 1998).

We wish to find the N-terminal degradation signal of HemA if it exists. Insertions or deletions at the N-terminal region of HemA were expected to disturb the signal. The mutant HemA protein's abundance, synthetic rate, and degradation were analyzed. Results of this study suggest that the N-terminal region is critical for the proteolysis of HemA.

Materials and Methods

Growth of cultures. The bacterial strains used in this study are listed in Table 1 (next page). All cultures were grown at 37°C in either Luria-Bertani (LB) medium (Silhavy) or in minimal MOPS (morpholinepropanesulfonic acid) medium (Neidhardt) as modified (Bochner) containing 0.2% glycerol as the carbon source. Plates were prepared using nutrient agar (Difco) with 5 g of NaCl per liter or using NCE medium (Berkowitz) with 0.2% glycerol as the carbon source. ALA was used at 2 μM in minimal medium (Elliott and Roth) and tryptophan was used to supplement Trp^- mutants at 0.002%. Antibiotics were added to rich medium to final concentrations as follows: 100 μg/ml sodium ampicillin, 20 μg/ml chloramphenicol, 50 μg/ml kanamycin sulfate, 20 μg/ml tetracycline hydrochloride, and 200 μg/ml streptomycin sulfate. For strains with F' plasmids grown in minimal medium, final antibiotic concentrations was 100 μg/ml kanamycin sulfate.

Construction of site-directed hemA mutations. The plasmid pTE644 carries the promoter region upstream of *hemA* (extending from bp 1 to bp 734 of the sequence in Genbank J04243). This segment is bounded by a naturally occuring $BamH_I$ site on the upstream side and extends to an engineered Nde_I site overlapping the *hemA* ATG initiation codon followed by an $EcoR_I$ site, inserted into pUC120 (with its Nde_I site filled-in) between the $BamH_I$ and $EcoR_I$ sites of pUC120. The Nde_I site was positioned by PCR (*Pfu* polymerase, Stratagene), and pTE644 has been sequenced to confirm the absence of

Table 1. Bacterial Strains.

Strain	Genotype	Source
Salmonella typhimurium		
LT-2	wild type	Lab collection
TE315	TR5877 = *hsdL6 hsdSA29* (*rLT- mLT+ rS- mS+*) *metA22 metE551 ilv-452 trpB2 xyl-404 rpsL120 (StrR) H1-b H2-e,n,x (Fels2-)nml*	B.A.D. Stocker
TE518	*hemA60 recA1*	Lab collection
TE2279	TE315 *zde-1858*::Tn*10d*-Tet *hemA427* (Am) *srl-203*::Tn*10d*-Cam *recA1*	Lab collection
TE3345	TE315 *putPA1303*::Kan^R-*hemA* (MluI fill-in)-*lac* [pr] (codon 416)	(this study)
TE3347	TE315 *putPA1303*::Kan^R-*hemA* (MluI fill-in)-*prfA-lac* [pr] (codon 6)	(this study)
TE4351	*pyrD121 D*(*putPA)521* / F$^+$ *zzf-6807*::[Tn*10d-putPA1302*::Cam]	(Elliott 92)
TE7590	*pyrD121 D*(*putPA)521* / F$^+$ *zzf-6807*::[*Tn10d-putPA1303*:: Kan^R-*hemA* (Mlu$_I$ fill-in)-*lac* [pr] (codon 416)]	P22.TE3345 x TE4351
TE7591	*pyrD121 D(putPA)521* / F$^+$ *zzf-6807*::[Tn*10d-putPA1303*:: Kan^R-*hemA* (Mlu$_I$ fill-in)-*prfA-lac* [pr] (codon 6)]	P22.TE3347 x TE4351
TE7619	*hemA60 recA1* / F$^+$ *zzf-6807*::[Tn*10d-putPA1303*:: Kan^R-*hemA* (Nde$_I$)-*lac* [pr] (codon 416)]	TE7610 c TE2279 c TE518
TE7620	*hemA60 recA1* / F$^+$ *zzf-6807*::[Tn*10d-putPA1303::* Kan^R-*hemA* (Nde$_I$)-*prfA-lac* [pr] (codon 6)]	TE7611 c TE2279 c TE518
TE7621	*hemA60 recA1* / F$^+$ *zzf-6807*::[Tn*10d-putPA1303*:: Kan^R-*hemA* (Nde$_I$, KK)-*lac* [pr] (codon 416)]	TE7612 c TE2279 c TE518
TE7622	*hemA60 recA1* / F$^+$ *zzf-6807*::[Tn*10d-putPA1303*:: Kan^R-*hemA* (Nde$_I$, KK)-*prfA-lac* [pr] (codon 6)]	TE7613 c TE2279 c TE518
Escherichia coli		
LMG194	*K-12 F$^-$ l$^-$ DlacX74 galE galK thi rpsL DphoA* (PvuII)	L. Guzman

Table 1. Bacterial Strains. (continued)

Strain	Genotype	Source
TE3057	*K-12 F⁻ l⁻ araD139 DlacX74 galU galK hsdR* (Str^R) *recD1903*::mini-Tet *hemA8 trpDC700::putPA1304::* [*'prsA-uorf-hemA-prfA-dorf-kdsA'*]	(Elliott & Wang)
TE2640	*K-12 F⁻ l⁻ IN (rrnD-rrnE) DlacX74 rpsL galK2 hemA8*	(Elliott & Wang)
TE6730	*K-12 F⁻ l⁻ araD139 DlacX74 galU galK hsdR* (Str^R) *recD1903*:: mini-Tet hemA8 trpDC700::putPA1304:: [*'prsA-uorf-hemA*::Kan (MluI)-*prfA-dorf-kdsA'*]	(this study)
TE7054	*K-12 F⁻ l⁻ IN (rrnD-rrnE) DlacX74 rpsL galK2 hemA8 leu::Tn10 Dara714*	P1.LMG194 x TE2640
TE7202	TE7054 / pTE570	(this study)
TE7207	TE7054 / pTE694 (PBAD-*hemA* [wild type])	(this study)
TE7420	TE7054 / pTE713 (PBAD-*hemA* [KK])	(this study)
TE7518	*K-12 F⁻ l⁻ araD139 DlacX74 galU galK hsdR* (Str^R) *recD1903*:: mini-Tet *hemA8 trpDC700::putPA1304*:: [*'prsA-uorf-hemA*(NdeI)-*prfA-dorf-kdsA'*]	(this study)
TE7519	*K-12 F⁻ l⁻ araD139 DlacX74 galU galK hsdR* (Str^R) *recD1903*:: mini-Tet *hemA8 trpDC700::putPA1304*:: [*'prsA-uorf-hemA*(NdeI, KK)-*prfA-dorf-kdsA'*]	(this study)
TE7610	TE2640 / / F^+ *zzf-6807*::[Tn*10d-putPA1303*:: Kan^R-*hemA* (NdeI)-*lac* [pr] (codon 416)]	(this study)
TE7611	TE2640 / / F^+ *zzf-6807*::[Tn*10d-putPA1303*:: Kan^R-*hemA* (NdeI)*prfA-lac* [pr] (codon 6)]	(this study)
TE7612	TE2640 / / F^+ *zzf-6807*::[Tn*10d-putPA1303*:: Kan^R-*hemA* (NdeI, KK)-*lac* [pr] (codon 416)]	(this study)
TE7613	TE2640 / / F^+ *zzf-6807*::[Tn*10d-putPA1303*:: Kan^R-*hemA* (NdeI, KK)*prfA-lac* [pr] (codon 6)]	(this study)

mutations in the 258 bp *hemA* promoter region between the Stu_I site and ATG initiation codon. The plasmid pTE647 is pTE644 with an additional Nde_I-$EcoR_I$ fragment carrying the N-terminal half of *hemA*. This fragment was produced by PCR (*Pfu* polymerase) and includes the sequence from bp 729 to bp 1285 of Genbank J04243. Thus, pTE647 carries 731 bp upstream and 551 bp downstream of the start of *hemA*, modified to include an Nde_I site overlapping the ATG initiation codon. The various mutations of *hemA* were constructed by PCR (*Pfu* polymerase) and eventually replaced the segment of *hemA* lying between the Nde_I and Mlu_I sites of pTE647. This region was sequenced for each derivative plasmid. In some cases the substitution was made directly, in other cases it proceeded through intermediate plasmids. All DNA fragments generated by PCR were subsequently sequenced to confirm the absence of mutations. Details of construction and primer sequences are available from the authors on request.

To test the function of these mutated *hemA* segments, each was substituted into a plasmid bearing the wild type *hemA* gene under the control of the P_{BAD} (arabinose-inducible) promoter (Guzman). Modification of the original plasmid pBAD18 to give pTE570, which contains a ribosome binding site and unique Nde_I site overlapping the ATG initiation codon, has been described (Brown 96). The plasmid pTE694 is pTE570 carrying *hemA* and the first 6 codons of *prfA* (bp 732 to bp 2048 of the sequence in Genbank J04243). This segment is bounded the Nde_I site overlapping the *hemA* ATG initiation codon and an $EcoR_I$ site on the downstream side. As before, the construction involved several steps, and all DNA fragments generated by PCR were subsequently

sequenced to confirm the absence of mutations. Mutated *hemA* segments were substituted into pTE594 as Nde_I-Nhe_I fragments and their identity confirmed by sequencing.

Transfer of *hemA* mutations to the *E. coli* chromosome. Three mutant alleles of *hemA* that retain the ability to complement a *hemA* mutant of *E. coli* when expressed from the P_{BAD} promoter, were next transferred to the bacterial chromosome by linear transformation. A wild type control containing the Nde_I site overlapping the ATG initiation codon was also transferred for comparison. The recipient strain for this transformation was constructed from *E. coli* strain TE3057, which has been described in detail previously (Elliott and Wang, 1991). TE3057 carries a 7.5 kb fragment of *S. typhimurium* DNA including the hemA gene, inserted into the *trp* operon of *E. coli*. It also carries a mutation in the *E. coli hemA* and so is dependent for *hemA* function on the *S. typhimurium* gene. A *hemA*::Kan disruption (at the Mlu_I site at codon 19 of the *S. typhimurium* gene) was introduced into TE3057 by linear transformation to give TE6730. Subsequently, *hemA* alleles were introduced from the pUC120-based plasmids described above that contain upstream flanking DNA from the *hemA* promoter and the N-terminal half of *hemA*. Plasmids were digested with Pst_I , or in some cases $BamH_I$, then added to $CaCl_2$ treated TE6730 cells, and plated on NB agar selecting Hem^+. Hem^+ transformants were first screened for a Kan^S Amp^S phenotype. DNA from candidate clones was then analyzed. To do this, 0.5 ml of overnight culture grown in LB medium was centrifuged and resuspended in 1/10 vol of 10 mM Tris, pH 8.0-0.1 mM EDTA. The cells were frozen at –70°C for 10 min, boiled for 10 min, microfuged for 10 min, and half the supernatant was retained. One µl of this preparation was added to a PCR reaction (*Taq* polymerase)

using *S. typhimurium* specific primers. PCR products were diluted 3-fold into the appropriate restriction digest. All clones were tested for the presence of the Nde_I and Mlu_I sites, and for a restriction site associated with the substitution when present.

Transfer to F' plasmids. Strains TE7590 and TE7591 contain F' plasmids derived from strain TE4351, which has been described in detail (Elliott, 1992). The original plasmid were constructed by insertion of a modified Tn10 transposon into F^+. The transposon is Tn*10d-put* carrying our standard *lac* operon fusion construct and a Kan^R marker (Elliott, 1992). Strains TE7590 and TE7591 carry a *hemA-lac* protein fusion at codon 416 of the *hemA* gene and a *hemA-prfA-lac* protein fusion, respectively. The *hemA* gene of both plasmids carries a frameshift (fill-in) of the Mlu_I site at codon 19 and is unable to confer a Hem^+ phenotype. Each strain carrying a *hemA* mutation to be analyzed was subjected to two preliminary steps: (i) pCDK30 (Amp^R, $recD^+$) was introduced by electroporation and (ii) the F' plasmids described above were introduced by conjugation selecting Kan^R Tet^R Amp^R. From these strains, rare F' plasmids that had repaired the *hemA* Mlu_I site frameshift by transfer of material from the *S. typhimurium hemA* gene on the *E. coli* chromosome were detected as Hem^+ Trp^+ exconjugants with TE2640 *(E. coli hemA8*) on medium containing X-gal. Repair of the frameshift is signaled by relief from polarity and consequently higher expression of *lacZ.* Candidate clones were again screened by PCR with *S. typhimurium*-specific primers followed by digestion to reveal the presence of diagnostic restriction sites. The resulting F' plasmids were then transferred to the final strain background in *S. typhimurium* by sequential conjugation with TE2279 and then with TE518. The final strains have a chromosomal *hemA60 recA* background and

carry F' plasmids that are marked with Kan^R and express either native *hemA* or a *hemA-lacZ* protein fusion each bearing the indicated change to the coding sequence for the HemA N-terminus.

Western (immunoblot) and pulse-labeling analysis. Techniques were exactly as described in second publication in this disertation, except that the primary antibodies were mAb of the γ1 isotype, designated H17 or H23. The rates of synthesis and turnover of HemA protein were examined by pulse-labeling and immunoprecipitation as described (the second publication). Cells were grown in minimal MOPS medium contain 0.2% glycerol, 2 μM ALA, and kanamycin as necessary, to OD_{600} = 0.4. Labeling, chase, sample preparation, immunoprecipitation, and gel electrophoresis were all exactly as described (the second publication).

Results

Mutations changing the HemA N-terminus. We constructed several mutant derivatives of the *S. typhimurium hemA* gene in the hope of finding variants that are stabilized against proteolysis by Lon/ClpAP but still encode functional enzymes as judged by in vivo complementation behavior. Retention of enzymatic activity in a particular mutant would provide evidence that any defect in turnover is a specific effect. We focused on the region encoding the N-terminus of the HemA protein, because previous transplant experiments had shown that the N-terminal 18 amino acids of HemA confer instability on LacZ in the context of the HemA[1-18]-LacZ hybrid protein (Study II, 1998). The relevance of this to turnover of the native HemA protein is made more likely by the finding that both HemA[1-18]-LacZ and HemA are degraded in vivo by Lon and ClpAP but not by other proteases.

The name and sequence of each mutant *hemA* derivative constructed in this study are given in Fig 1. When scanning from the HemA N-terminus, the first charged residue that is encountered is His-10. Since hydrophobicity is suspected to play a role in Lon proteolysis (Gottesman and Maurizi, 1992), we targeted three mutations to the extreme N-terminus of HemA. In the first set of experiments, two variants were constructed with a charged doublet of amino acids placed between Thr-2 and Leu-3 of HemA. One contains two lysines (HemA-KK) at this position and the other contains two negatively charged amino acids (HemA-DE); in a third construct five residues from Leu-3 to G-7 were removed (HemA-ΔL).

Plasmid expression system in *E. coli*. These modified versions of *hemA* were placed under the control of the P_{BAD} (arabinose-inducible) promoter in the plasmid pTE570 (Brown and Elliott, 1996), which is derived from pBAD18 (Guzman et al., 1995). An *E. coli* K-12 strain mutant for both *hemA* and *ara* was transformed with each plasmid and the complementation behavior observed on plates either with or without the addition of inducer (arabinose) to a disc in the center of the plate (Table 2, next page). The HemA wild type displayed with weak complementation in the absence of inducer and strong complementation in its presence. In contrast, the HemA-DE and HemA-ΔL plasmids complemented only poorly (in the former case) or not at all, and these two constructs have not been studied further. The behavior of the HemA-KK construct was unusual in that effective complementation did not require addition of the inducer.

Western (immunoblots) with anti-HemA mAb were used to examine expression of HemA from the plasmids with complementation ability (Fig 2). The host for this experiment (TE7054) is the same as used previously: *E. coli* with the *hemA8* mutation. No signal was detected for HemA protein in this strain (Fig 2, lane c), and only a very weak signal was detected in its *hemA*$^+$ parent (lane b). When the plasmid expressing wild type HemA was introduced, the signal for HemA was substantially increased (lane e) and was approximately equal to that observed from the single copy of *hemA* in wild type *S. typhimurium* (lane a). For the *E. coli* strain with the wild type HemA plasmid, induction with arabinose greatly increased the abundance of HemA protein and also resulted in the appearance of additional bands reactive with antibody, possible degradation products that were smaller than HemA but also apparently larger polypeptides (lanes f-h). Finally, the

Table 2. Complementation test in *E. coli* of plasmids expressing modified versions of *S. typhimurium hemA*

		Hem phenotype [A]	
Plasmid	Construct	Uninduced	Induced
pTE570	P_{BAD} vector	-	-
pTE694	P_{BAD}-*hemA* (wild type)	+	++++
pTE713	P_{BAD}-*hemA* (KK)	++++	++++ [B]
pTE714	P_{BAD}-*hemA* (DE)	-	++
pTE715	P_{BAD}-*hemA* (-L)	-	-

[A] Cultures of strain TE7054 (*E. coli K-12 hemA* Δ*ara*) carrying the indicated plasmids were grown to saturation in LB + 100 μg/ml ampicillin + 150 μM ALA. After dilution in PBS, about 500 cfu were then plated on each of two NB ampicillin plates (NB medium is selective for the Hem^+ phenotype). A disc was placed in the center of one plate, and 10 μl of 20 % arabinose pipeted onto the disc. Colony size was scored after 16 hr. (+, < 0.5 mm; ++, 1.0 mm; ++++, 2.0 mm).

[B] Colonies were large at the edge of the plate, and smaller close to the disc where inducer was applied.

HemA-KK variant produced substantially more HemA protein than with the wild type plasmid (lane d) and did not show any evidence of degradation products.

Further investigation revealed some deficiencies of the plasmid-based system for the study of HemA degradation. Pulse-labeling studies revealed that the rate of synthesis of HemA-KK from pTE713 in *E. coli* was about 4-fold greater than that of HemA wild type from pTE694 (data not shown). Inspection of the sequence surrounding the *hemA* ATG start codon revealed a fortuitous stem-loop that would be predicted to sequester the ribosome binding site provided by P_{BAD} in a secondary structure with the RNA encoding the N-terminus of HemA. The increased synthesis of HemA-KK (and certain other N-terminal substitutions) compared with wild type, can probably be ascribed to disruption of this secondary structure. Given this effect, it is not simple to compare the turnover of mutant and wild type HemA proteins at the same intracellular protein level. The second and more important limitation was seen in pulse-chase experiments. Here, addition of a small amount of the inducer arabinose was necessary to observe a signal for labeled wild type HemA. At these modestly increased levels of HemA, the protein that is produced is stable, in contrast to that produced from a wild type copy of *the S. typhimurium hemA* gene carried in either *E. coli* or *S. typhimurium*. We suspect that this effect is due to titration of a limiting component required for HemA turnover.

Chromosome *hemA-kk* expression in *E. coli* and *S. typhimurium*. To circumvent these limitations, we transferred each of two constructs (HemA wild type, HemA-KK) to the chromosome of *E. coli* by linear transformation. In these *E. coli*

strains, *the S. typhimurium hemA* gene is expressed from its native promoter (Fig 3, lane a) and the *E. coli hemA* gene carries a mutation which eliminates both *hemA* function and production of cross-reactive material (lane b). Western blot analysis was used to quantitate expression of HemA protein from the HemA wild type and HemA-KK constructs (Fig 3, lane c and d) in this background. The abundance of HemA protein was substantially increased in the strain expressing HemA-KK compared to abundance of HemA wild type. However, HemA was not efficiently visualized by pulse-labeling and immunoprecipitation in these strains (not shown). In previous work, we found it necessary to employ F' plasmids to increase the expression of *hemA* to a detectable level for this kind of experiment. For this experiment, and all other experiments that follow, cultures were grown in medium supplemented with ALA to eliminate the possibility that small differences in the enzyme activity of the different HemA and HemA-LacZ might influence the stability of the proteins or any other regulatory aspect of the system.

Therefore, each of the constructs was subsequently transferred from the *E. coli* chromosome to an F' plasmid (see Methods for details), and then these plasmids were introduced to an *S. typhimurium hemA recA* mutant host by conjugation. The F' plasmids carry the indicated mutation either in the context of a native *hemA* gene or as part of *a hemA-lacZ* protein fusion that expresses the HemA[1-416]-LacZ hybrid protein. The abundance of native HemA and the HemA[1-416]-LacZ hybrid protein were examined by Western (immunoblot) analysis with an anti-HemA mAb (Fig 4). The HemA-KK variant showed an increased abundance compared to the wild type version, both in the context of native HemA (compare lanes f and d), and in the context of the HemA[1-416]-LacZ

hybrid protein (compare lanes e and c). We have not attempted to quantitate the increase in abundance observed on this blot but instead have relied on analysis of rates of synthesis and degradation.

The rates of HemA and HemA[1-416]-LacZ synthesis were measured by pulse-labeling and immunoprecipitation with an anti-HemA mAb (Fig 5). The rate of synthesis of the wild type HemA and HemA[1-416]-LacZ constructs (lanes c, d) was no different than that of the HemA-KK variants (lanes e, f). For both plasmid-encoded HemA and HemA-KK variants, expression of native HemA was not more than 3-fold higher than that observed in wild type *S. typhimurium* (lane a; data not shown). As discussed previously (the study I, 1997), the chromosomal *hemA60* allele produces a truncated immunoreactive polypeptide visible in lanes b-f. A large increase in abundance of the HemA-KK variants which is not accompanied by an increased rate of synthesis implies that the rate of turnover is greatly reduced. This was directly confirmed by a pulse-chase experiment (Fig 6). The half-life of the wild type HemA protein was about 20 min, similar to that observed previously (the study II). In contrast, the HemA-KK variant was essentially stable (half-life > 300 min).

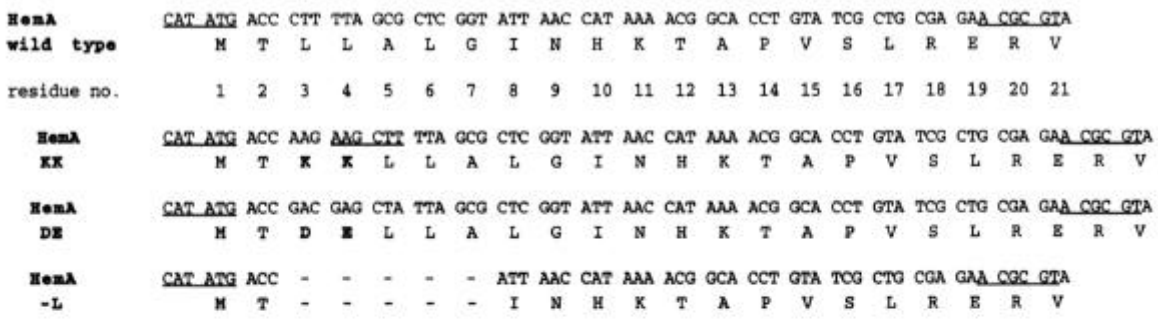

Fig. 1. Mutations constructed in this study. The DNA sequences of five mutant versions of the *hemA* gene and their predicted protein products are shown, together with that of the wild type (top line) for comparison. Each sequence begins with the Nde_1 site overlapping the ATG initiation codon and ends at an Mlu_1 site which lies at codon 19 of the wild type gene. The five mutants are referred to in the text by short designations as shown in the left-hand column. *HemA*-KK and *HemA*-DE bear insertions of two codons encoding the indicated amino acids between Thr-2 and Leu-3, and -L is a deletion of five codons encoding Leu-3 through Gly-7 in wild type *hemA*. Details of construction and plasmids bearing these constructs are given in the Methods. A mutation was inadvertently introduced to the fifth codon of HemA-DE (CTT -> CTA); this change is silent.

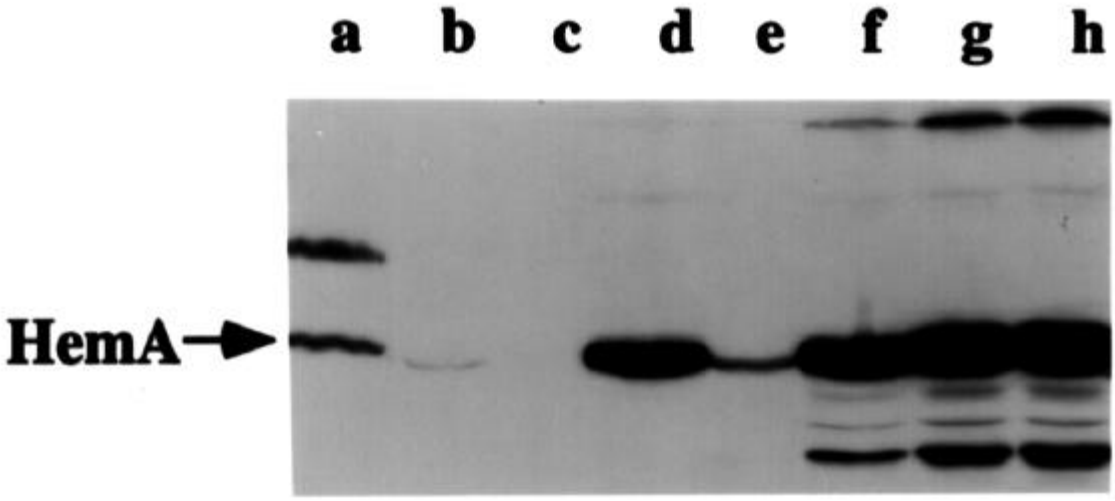

Fig. 2. Western (immunoblot) analysis of plasmid-borne *hemA* and *hemA* (KK) expressed from the PBAD promoter in *E. coli*. Lane (b) *E. coli* TE2331 (wild type), (c) *E. coli* TE7202 (*hemA8* / pTE570 [vector]), (d*) E. coli* TE7420 *(hemA8* / pTE713 *hemA* [KK]), (e) *E. coli* TE7207 (*hemA8* / pTE694 $hemA^+$ [wild type]). Lanes (f-h) were prepared as for lane (e) except that cultures were grown with various concentrations of the inducer arabinose: lane (f) 0.001%, (g) 0.005%, (h) 0.01%. Lane (a) is *S. typhimurium* LT-2 (wild type) as a positive control. Cultures were grown to OD_{600} = 0.4 and processed for immunoblotting with anti-HemA mAb H23 exactly as described (Wang 97).

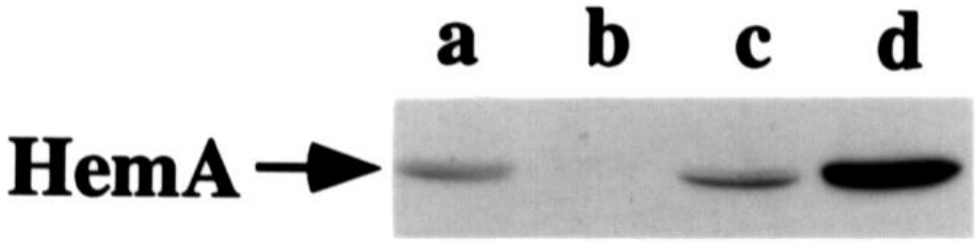

Fig. 3. Western (immunoblot) analysis of *hemA* and *hemA* (KK) expressed from a single copy in the *E. coli* chromosome. Lane (a) *E. coli* TE3057 (*hemA8 trp::put::hemA*$^{+}$), (b) *E. coli* TE7202 (*hemA8*, pTE570 [vector]), (c) TE7518 (*hemA8 trp::put::hemA* $^{+}$ (NdeI, wild type), (d) TE7519 (*hemA8 trp::put::hemA* (NdeI, KK). Cultures were grown to OD_{600} = 0.4 in minimal MOPS glycerol medium with 2 μM ALA and processed for immunoblotting with anti-HemA mAb H23 as described above.

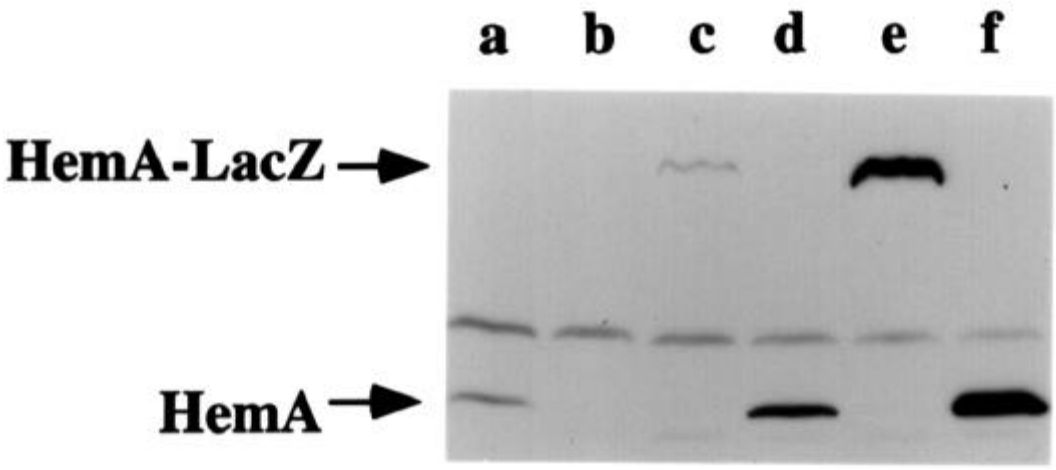

Fig. 4. Western (immunoblot) analysis of *hemA and hemA* (KK) expressed from F' plasmids in *S. typhimurium*. Lane (a) LT-2 (wild type), (b) TE 518 (*hemA60 recA1*), (c) TE7619 (*hemA60 recA* / F' Kan^R-*hemA*[1-416]-*lac* [pr], (d) TE7620 (*hemA60 recA1* / F' Kan^R-*hemA-prfA-lac* [pr]), (e) TE7621 (*hemA60 recA1* / F' Kan^R-*hemA*[1-416, KK]-*lac* [pr], (f) TE7622 (*hemA60 recA1* / F' Kan^R-*hemA (KK)-prfA-lac* [pr]). The positions of native HemA and HemA[1-416]-LacZ are indicated. Cultures were grown to OD_{600} = 0.4 in minimal MOPS glycerol medium with 2 μM ALA and 100 μg/ml kanamycin when necessary to maintain F' plasmids. Samples were processed as described above for immunoblotting with anti-HemA mAb H17. Similar results were observed with mAb H23.

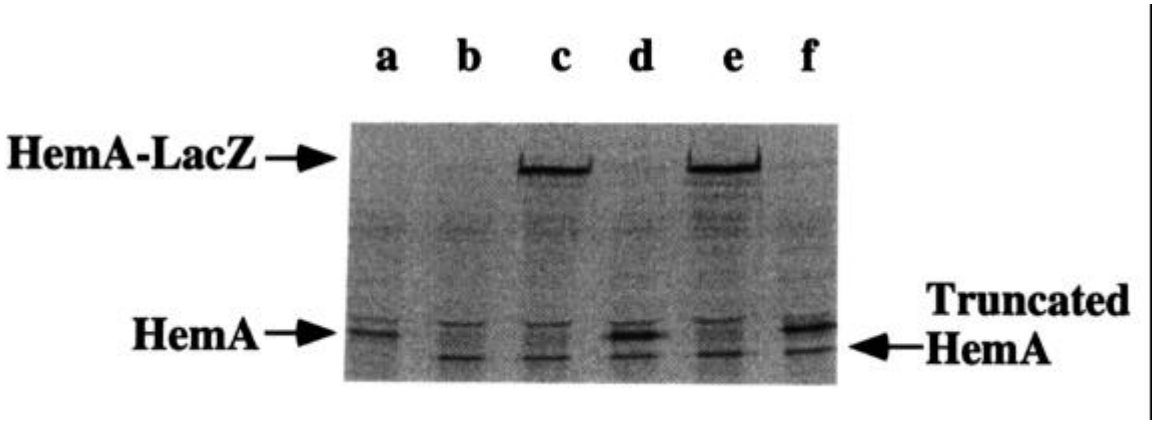

Fig. 5. Pulse-labeling and immunoprecipitation analysis of *hemA and hemA* (KK) expressed from F' plasmids in S. typhimurium. Lane (a) LT-2 (wild type, (b) TE 518 (hemA60 recA1), (c) TE7619 (hemA60 recA / F' KanR-hemA[1-416]-lac [pr], (d) TE7620 (hemA60 recA1 / F' KanR-hemA-prfA-lac [pr]), (e) TE7621 (hemA60 recA1 / F' KanR-hemA[1-416, KK]-lac [pr], (f) TE7622 (hemA60 recA1 / F' Kan^R-*hemA (KK)-prfA-lac* [pr]). The positions of native HemA and HemA[1-416]-LacZ are indicated. Cultures were the same as those analyzed by Western blot in Fig. 4. One milliliter of culture (OD_{600} = 0.4) was pulse-labeled with Tran35S-label for 5 min and then chased with unlabeled L-methionine (1.3 mM) and L-cystine (0.6 mM) for 2 min. Protein extracts were prepared, immunoprecipitated with anti-HemA mAb H17, and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

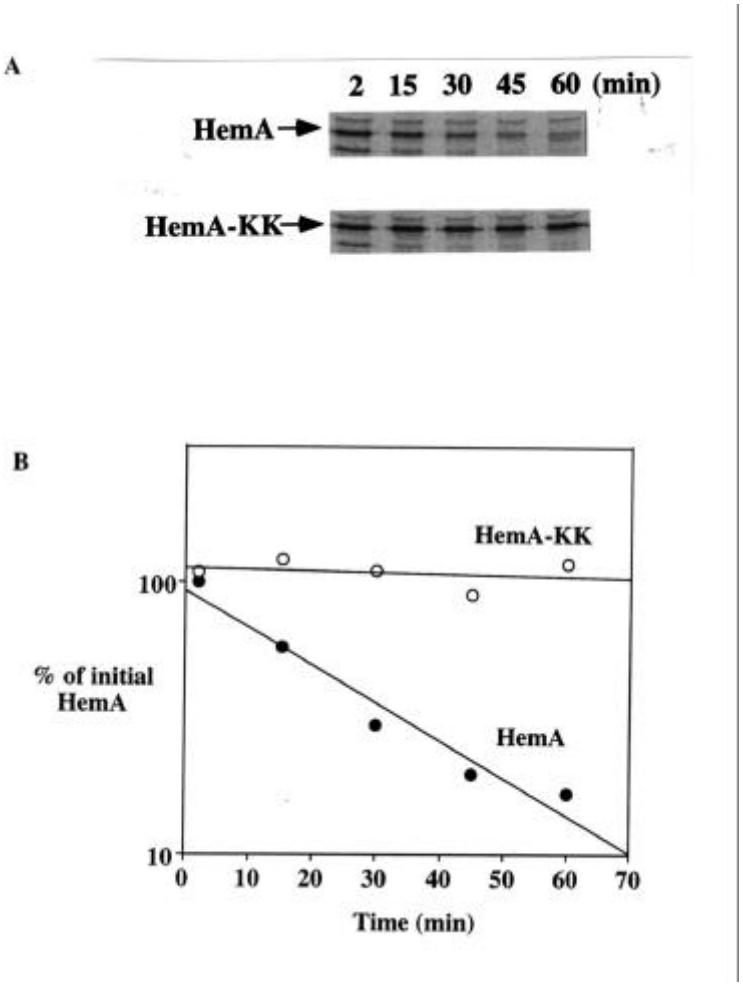

Fig. 6. Pulse-chase analysis of *hemA* and *hemA* (KK) expressed from F' plasmids *in S. typhimurium*. TE7620 (*hemA60 recA1* / F' Kan^R-*hemA-prfA-lac* [pr]) (panel A, top); TE7622 (*hemA60 recA1* / F' Kan^R-*hemA (KK)-prfA-lac* [pr]). The positions of native HemA and HemA (KK) are indicated. Cultures were grown and analyzed as described in the legend to Fig. 5, except that the chase was extended as shown above each lane. (B). An identical gel (not treated with fluor) was analyzed by using a Phosphorimager and its ImageQuant software to calculate the half-life of HemA protein. The HemA protein encoded by the wild type plasmid in TE7620 has a half-life of 20 min, the same as the chromsomally-encoded HemA protein (Wang 99). In contrast, the HemA-KK protein is stable (half-life > 300 min).

Discussion

Our previous work has demonstrated that HemA, the first enzyme of the heme biosynthesis, is an important target of heme synthetic regulation. HemA regulation is achieved by an unusual mechanism: it is conditionally unstable in a manner that is controlled by heme limitation. HemA degradation is catalyzed by the energy dependent proteases Lon and ClpAP. A hybrid HemA-LacZ protein containing just the first 18 amino acids from HemA is degraded as rapidly as native HemA protein (half-life 20 min), and is stabilized in the *lon clpP* double mutant (half-life 300 min). These results lead to a hypothesis that the N-terminal 18 amino acids of HemA constitute a degradation tag which is recognized by Lon and ClpAP.

To maintain the proper protein availability in the cell, the proteolytic system must distinguish between appropriate substrate proteins and avoid degradation of other cellular proteins. There must be certain degradation signals recognized by the proteolytic system. We would like to know if this is the case for HemA protein, which would be useful in understanding the pathway of HemA regulation.

There is some information implying that N-terminus of the substrate is more likely targeted by proteases (Tobias *et al.*, 1991; Vershavsky, 1996). Hydrophobicity is suspected to play a role in Lon proteolysis (Gottesman and Maurizi, 1992). In an attempt to confirm that the degradation tag in HemA lies in the N-terminal 18 amino acids, hydrophilic amino acid insertion or deletion mutants of the *hemA* N-terminus were constructed and tested. The HemA-KK mutant with two hydrophilic amino acids (lysine)

inserted between Thr-2 and Leu-3 of HemA retains a *hemA*$^{+}$ phenotype. Increased HemA-KK protein abundance was observed with no change of its synthetic rate. Pulse-chase labeling results confirmed that the KK-insertion prevents HemA-KK proteolysis. However, the insertion mutant with two other hydrophilic amino acids (Aspartic acid and Glutamic acid) in the same site (HemA-DE) reduces HemA function. Other than location, the biochemical feature of the "tag" seems critical. The stabilizing effect by KK insertion may directly disturb the degradation tag recognized by Lon and ClpA, or act indirectly by changing the HemA protein's structure to display degradation tag. It is also possible that another protein or effector is involved in the interaction between this tag and Lon and ClpAP. There is no doubt that the N-terminus of HemA plays an important role for HemA degradation and may be important in HemA regulation.

Greatly elevated HemA protein abundance is induced from P_{BAD}-*hemA* by arabinose (Fig. 2, page 120). This induced HemA protein is very stable (helf-life > 300 min, not shown). An unknown protein (about 120 kDa) is induced by arabinose also (Fig. 2, page 120). One cannot exclude that there may be a factor involving HemA regulation and this factor can be saturated when HemA is elevated to certain amount. The synthetic rate of HemA-KK under the promoter P_{BAD} is increased without induction of arabinose. There is a suggested secondary structure between the ribosome binding site provided by P_{BAD} and the N-terminus of HemA. KK-insertion may disrupt this structure and therefore gives the above result.

The terminal regions of protein appear to play a role in determining susceptibility to the protein degradation. The N-end rule targeting (Gonda *et al*., 1989) seems to be a general pathway for proteolysis in eukaryotic microorganisms and in animal cells. However, the significance of this pathway in bacteria is uncertain. The degradation tag can be in the N-terminus, the C-terminus, or the middle of the protein (see literature review p.18-19). Study in this paper provides another example that the N-terminus of protein is recognized by its proteases. The behavior of HemA-KK mutant here shows, once again, that a slight change in the primary sequence of a unstable protein can be sufficient to decrease protease sensitivity.

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General Discussion

The exciting findings from this project give an insight into heme biosynthetic regulation. The contributions to the heme study include: (1) Heme-limitation generated by different heme gene mutations affect the key heme enzyme, HemA, specifically; (2) The difficulties of HemA study at the protein level were surmounted by a panel of newly developed monoclonal anti-HemA antibodies and the HemA enzyme assay; (3) It provided the first direct evidence for the regulation of heme biosynthesis in enteric bacteria, and showed that HemA is the target of this regulation; (4) It revealed the unusual mechanism of HemA regulation during heme-limitation; (5) The proteases which are responsible for HemA protein degradation were discovered; and (6) The HemA degradation tag was found at the HemA N-terminal region.

Heme synthetic regulation was studied under heme-limitation in this project. Heme, as the main end product of heme biosynthetic pathway, directly changes the "heme supply" signal. The cell should promote the regulation(s) to adjust the availability of heme. Heme itself may participate in the regulation as a feedback factor. A large number of previous studies in heme research in this laboratory provide an availability of *hem*$^-$ mutations at different steps in heme pathway: like *hemL, hemB, hemE,* and *hemH.* Heme limitation can be imposed by complete starvation of heme auxotrophs (*hemB, hemE,* and *hemH*) or heme adaptation (*hemL*) based on the genetic approach. The unique *hemL* mutant leaky phenotype may be due to GST flowing through the additional non-enzymatic pathway without *hemL* encoded GST aminotransferase, which is sufficient to allow the

mutant to grow at a slower rate. This exponential but slower growth makes it possible to study HemA synthesis and degradation rate during heme-limitation. Considering that the slower growth of the *hemL* mutant might affect HemA regulation, other nutrient limitations which cause the cell slower growth were checked. Only heme-limitation specifically induced the increase of HemA level in the cell. Thus, the advantages of this heme-limitation model for heme regulation study are specific, simple, and straightforward.

Studies on the role of HemA in heme synthetic regulation at the protein level were successfully undertaken in this dissertation research. A HemA enzyme assay was used to directly measure HemA activity. Significantly elevated HemA activity was observed in *hem* mutants which are starved for heme, while the *hemA* gene transcription is not affected much. This was the first important step in this project. This finding provides the first direct evidence that heme biosynthesis is regulated by heme-limitation which targets the first heme committed enzyme HemA, and leads to the hypothesis that the *hemA* regulation in response to heme-limitation is at the post-transcriptional level.

The elevated HemA enzyme activity could be caused by either a higher level of HemA protein, or the same amount of HemA protein which is modified to give a higher activity. To distinguish these possibilities, HemA abundance in the regulatory condition needs to be measured. Measurement or purification of HemA protein was difficult at the time since HemA exists at a low level in the cell. Fortunately, a panel of anti-HemA monoclonal antibodies were generated in this laboratory with the help of Dr. Meenal Elliott. This provided a sensitive and powerful tool to detect and quantify HemA protein

even in wild type *S. typhimurium* strains by Western immunoblot. HemA protein levels seem even lower in *E. coli* which is barely detectable by Western immunoblot. The anti-HemA antibodies used in this project were carefully checked for their specificity to HemA. It is clearly shown that native HemA band (46 kDa) disappears in the *hemA60* mutant and a new band (~37 kDa), which is considered to be a truncated HemA protein encoded by *hemA60,* is recognized by these anti-HemA antibodies. HemA-LacZ hybrid proteins determined by anti-ß-galactosidase antibody are also recognized by the anti-HemA antibodies. At least four different monoclonal antibodies from the panel give the same recognition for HemA in Western Blots, and two of them worked well in pulse-labeling and immunoprecipitation.

Western immunoblot using the anti-HemA antibodies revealed that elevated HemA activity during heme limitation is due to a parallel increase (20-fold) in abundance of the protein, but this elevated HemA protein is still not detectable by SDS/PAGE stained by coomassie (data not shown). The elevated protein could be due either to higher protein synthetic rate or lower turnover rate of this protein or both. The mechanism of elevated HemA during heme-limitation was demonstrated: HemA is completely stabilized during heme-limitation with a mild change in its rate of synthesis. The native HemA protein half-life is found to be about 20 min in the unrestricted growing phase, and this is also consistent in $HemA_{1-416}$-LacZ and $HemA_{1-18}$-LacZ hybrid proteins (study II, 1998). The extreme low level and the short half-life of HemA protein account for the difficulty and the lack of research dealing with the mechanism of this enzyme in the past. This finding also points to sophisticated regulation of HemA. HemA enzyme can be increased from a

very low level by stabilization to respond quickly to the certain condition for the adaptation toward heme biosynthesis.

One of the first recognized examples of a specific proteolytic event involved in the regulation of gene expression in bacteria was reported in 1975 (Roberts and Roberts, 1975). The λ repressor undergoes proteolysis upon the induction to balance its proper availability. Since then, some other cases of gene regulation at the protein degradation level were reported, such as SulA (Dervyn *et al.*, 1990), UmuD (Gonzalez *et al.*, 1998), and RpoS (Schweder *et al*., 1996). The mechanism of proteolytic regulation were further studied in these cases. Instability of these proteins involves ATP-dependent protease (Lon or Clp) and couples with regulated transcription. The regulation by almost entirely protein conditional stability and by involving more than one protease like in case of HemA is very rare. It is possible that there are more proteins regulated similarly to HemA, but it is difficult to observe them because of their instability (Miller, 1996). It may also be true that regulation by proteolysis is only suitable for certain rare cases. The advantage of unique regulation of HemA is that it overcomes the special arrangement of *hemA-prfA-hemK* operon. The product of *prfA* gene, RF-1, is a universal peptide release factor for terminating any newly synthetic proteins at the UAG stop codon. The unusual HemA regulation gives an accurate control of HemA protein without affecting *hemA-prfA-hemK* operon expression, especially RF-1. The finding of HemA protein's nature is a good example for understanding the role of proteolytic regulation. It also provides information and experience for discovering other similar cases.

The unusual mechanism of HemA regulation leads to the proposed models to explain the molecular details of the pathway of this regulation (see discussion in study II, Fig. 12). This project reports that HemA turnover is promoted by Lon ClpAP mediated proteolysis. Both Lon and ClpAP are energy-dependent proteases. Without heme supply, the respiratory electron transport chain will be strongly diminished and result in low ATP concentrations (Rompf *et al.,* 1998). This information leads to one of the models discussed in the second publication; the activity of Lon and ClpAP might be limited if the ATP concentration is low. HemA degradation therefore slows down during heme-limitation. However, the cellular ATP level does not regulate HemA directly. There are several factors can affect HemA degradation, e.g., ATP/ADP ratio affects Lon active state (see literature review p.16-17 and discussion in study II p.97). I prefer the second model and like to discuss here more. The main hypothesis is that certain signals, such as heme itself as an end product, regulate the rate of HemA protein degradation. All the results of the study of heme regulation were observed under genetic heme-limited condition, which strongly support this model *in vivo*. Heme biosynthetic pathway produces three products (heme, siroheme and cobalamin). Siroheme or cobalamin may affect heme synthesis as the miner end products. In *hemL*, *hemB*, and *hemE* mutants, the intermediates after the blocked step may also have some effect to feedback to HemA enzyme. *hemH* mutant, which blocked the last heme pathway step, induces elevated HemA protein 15-fold comparing *hemB* and *hemE* mutants which induce 20- to 25-fold HemA increase. I do not know if this difference can count for that all three end products or the intermediates also affect heme synthetic pathway. One thing is clear that heme itself (*hemH* mutant) plays a role in HemA regulation. Other possible signals, such as heme-proteins or oxygen, were

considered indirectly affecting heme synthesis by changing free heme pool (see literature review, p.11-12). The further direct information to confirm this model is *in vitro* study of purified HemA. Heme feedback function could be determined by measuring the stability HemA without and with different concentration of heme and by detecting the association of heme and HemA.

Short half-life and low abundance of HemA seem to be advantages in the HemA regulatory strategy. In response to heme-limited signal that inhibits HemA degradation, HemA protein abundance can increase about 20-fold by its stabilization. When the elevated HemA is no longer needed or heme-limitation signal is gone, HemA protein turnover could be restored therefore lead HemA to normal concentration without the need of regulation at other levels. This hypothesis could be tested in the future by determine HemA degradation under heme-limitation comparing the same condition but adding heme or ALA at certain time.

HemA-LacZ protein fusions used in this project provide three advantages: first, HemA-LacZ fusion protein containing almost full length of HemA could be detected by either anti-HemA or anti-ß-galactosatase antibodies, and was predicted to be regulated similarly to the native HemA. If that is true, it would confirm the results observed from the native HemA. Second, these hybrid proteins give a better signal than the native HemA in the labeling experiments. This was helpful in the *E. coli* study, since native HemA signal in wild type *E. coli* strain is quite weak. Third, the LacZ fusion at different regions of HemA will allow us to screen for the segment of HemA which is sufficient for its

regulation. The first set of $HemA_{1-416}$-LacZ and $HemA_{1-18}$-LacZ fusion proteins has provided very useful information already. The N terminal 18 amino acids of HemA are not sufficient for regulation by heme-limitation, but they are adequate for recognition by Lon and/or ClpAP.

The KK insertion at the N-terminus of HemA, but not DE, completely protects HemA-KK protein from proteolysis. The biochemical feature of the degradation tag seems to be critical. Our unpublished data (Study III in this dissertation) showed that greatly induced HemA under P_{BAD} by arabinose becomes stable. What causes the HemA to be stable other than heme-limitation or mutated Lon and ClpAP? It is possible that another unidentified factor(s) is involved in the regulation and it is a limiting element. To confirm this possibility, greatly induced (> 20-fold) HemA is needed, e.g., by being expressed from a high copy number plasmid. The certain induced amount of HemA may saturate the limiting factor therefore HemA could be stabilized. The limit factor for HemA degradation could be the protease itself, like Lon which saturated by one of its substrate, SulA (Dervyn et al., 1990). Study of secondary or/and tertiary structure of HemA protein, which has not been done yet, may also provide further information to understand HemA regulation.

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