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Effects of starvation for heme on the synthesis of porphyrins in *Escherichia coli*

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Abstract A study is described of the regulation of porphyrin synthesis in *Escherichia coli* using a heme-permeable, *hemH* deletion mutant, designated VS212. This strain utilizes only exogenous hemin that is supplied in the medium and accumulates porphyrins since the final step in the synthesis of heme is genetically blocked. It is possible, therefore, to monitor the rate of synthesis of heme by examining the accumulation of porphyrins. Using this system, we found that the rate of production of porphyrins depended on the availability of heme. The lower the concentration of hemin in the medium, the higher the level of porphyrins that accumulated. We next examined the mechanism responsible for the activation of porphyrin synthesis upon starvation for heme. The main activation occurred at the step that leads to the synthesis of 5-aminolevulinic acid (ALA). Starvation for heme induced the expression of a *hemA-lacZ* fusion gene, as previously reported, but an activation pathway that is independent of the *hemA* promoter was also identified. We found that starvation for heme caused the stringent response, and such starvation promoted the synthesis of porphyrins without having any effect on the expression of the *hemA-lacZ* fusion gene. We suggest a model for the regulation of porphyrin synthesis whereby the synthesis of porphyrins is coordinated with that of proteins.

Key words Porphyrin · Heme · Stringent response · Glutamyl-tRNA reductase

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Introduction

Porphyrin-derived molecules are essential for the efficient production of energy. In chloroplasts and in photosynthetic bacteria, porphyrin is used for the production of chlorophylls and it is thus essential for photosynthesis. In mitochondria and bacteria, porphyrin is used for the production of hemes and it plays a role in respiration. Hemes are also utilized to defend cells against active species of oxygen, and as carriers of oxygen. *Escherichia coli* can grow without porphyrins, both in broth and in minimal media that contain fermentable sugars, since this bacterium has a set of glycolytic enzymes that produce ATP independently of respiration. However, mutants that lack the ability to produce porphyrins have several characteristic phenotypic features: (i) they form tiny colonies (Sasarman and Horodniceanu 1967); (ii) their growth in liquid medium stops at a certain cell density (10^7–10^8 cells/ml in LB medium), and (iii) they are no longer motile (J. Adler. personal communication). These changes in phenotype indicate that porphyrins (hemes) have an important role in maintaining the normal physiological functions of bacteria.

Porphyrins are also known as tetrapyrroles and their structural skeletons are composed of four pyrrole rings. Each pyrrole ring is synthesized by condensation of two molecules of 5-aminolevulinic acid (ALA). Thus, each porphyrin is synthesized from eight molecules of ALA (Castelfranco and Beale 1983). In many organisms, the production of ALA is regulated both at the level of gene expression and at the level of enzymatic activity. It is likely that the rate of synthesis of ALA determines the rate of synthesis of porphyrins. Two pathways are known for the formation of ALA. In the C4 pathway, which is operative in yeast and mammalian cells (Jordan 1990), ALA is formed by the condensation of succinyl coenzyme A and glycine (Shemin and Russell 1953). In the C5 pathway, ALA is synthesized from glutamate (Beale et al. 1975). This pathway operates in many

microorganisms, such as eubacteria including *E. coli* (Avissar and Beale 1989a; Li et al. 1989a,b; O'Neil et al. 1989), archaebacteria (Friedmann and Thauer 1986) and cyanobacteria (Avissar and Beale 1989b; O'Neil et al. 1988; Rieble and Beale 1988), as well as in the chloroplasts of algae and plants (Huang et al. 1984; Kannangara et al. 1988; Mayer et al. 1987; Schneegurt and Beale 1986; Weinstein and Beale 1985a,b; Weinstein et al. 1986). In the C5 pathway, glutamate is first converted to glutamyl-tRNA by glutamyl-tRNA synthetase and this is then reduced to glutamate-1-semialdehyde (GSA) by glutamyl-tRNA reductase (HemA). Then GSA is converted to ALA by GSA aminotransferase (HemL). Glutamyl-tRNA is also a substrate for protein synthesis (O'Neil and Söll 1990). In heme-deficient mutants, partial inhibition of protein synthesis accelerates the synthesis of porphyrins, a result that suggests that competition for glutamyl-tRNA may occur under heme-deficient conditions (Nakayashiki et al. 1995.).

Although there have been several reports on the regulation of porphyrin synthesis in *E. coli*, details of the regulatory mechanism remain to be clarified. In order to investigate the regulation of porphyrin synthesis, we used a heme-permeable, *hemH* deletion mutant of *E. coli*. The *hemH* gene encodes ferrochelatase, which inserts a ferrous ion into protoporphyrin IX to produce heme (protoheme). Thus, this strain cannot synthesize heme de novo and it accumulates synthesized porphyrins intracellularly, or excretes them into the medium. There are two advantages to the use of this strain for investigations of the regulation of porphyrin synthesis. First, we can estimate the amounts of porphyrins synthesized by purifying the accumulated or excreted porphyrins. Second, we can control the availability of heme by changing the concentration of hemin supplied in the medium. Taking advantages of these possibilities, we have investigated the regulation of porphyrin synthesis in this strain of *E. coli*.

Materials and methods

Bacterial strains

The *E. coli* strains used in this study are listed in Table 1.

Media and growth condition

Cells were cultured predominantly at 37° C in Luria-Bertani (LB) medium. For starvation experiments, low-phosphate tryptone medium was used. Low-phosphate tryptone medium was prepared as follows. A 0.17-ml aliquot of 1 M $MgCl_2$ was added to a 20% solution of bactotryptone (Difco, Detroit, Mich., USA). Then, a one-tenth volume of a 28% solution of NH_4OH was added and the mixture was stored at 4° C overnight. The precipitate was removed by filtration. Ammonia was removed for adjustment of the pH by heating. For experimental use, the low-phosphate 20% tryptone solution was diluted 20-fold and 0.5% NaCl was added. Hemin (Sigma, St. Louis, Mo., USA) was dissolved in 0.4 N NH_4OH at a concentration of 100 mg/ml for use as a stock solution. Cultures to be examined for accumulation of porphyrins and those that contained exogenous hemin were wrapped in aluminum foil to prevent exposure to light. Glucose was added at 0.4% as indicated. When present, ALA (Sigma) was included at 100 μg/ml. Ampicillin was added at a final concentration of 10 μg/ml as necessary.

Isolation of mutants

E. coli strains defective in *hemH* are sensitive to light since protoporphyrin IX produces active oxygen in the light (Miyamoto et al. 1991, 1992). Mutants defective in earlier steps in the synthesis of porphyrin can be isolated as light-resistant revertants of Δ*hemH* strains. Sites of mutations can be determined by complementation tests (Nishimura et al. 1993). Using this method, we isolated twenty light-resistant revertants of VS212. VR314 and VR306 were chosen as *hemA* and *hemB* mutants, respectively. Hemin-permeable mutants were isolated as follows. Aliquots of 100 μl of cultures of heme-deficient strains were spread on LB plates containing hemin at 50 μg/ml, and incubated at 37° C overnight. Several normal-sized colonies appeared on each plate. VS212 and N503 were isolated as heme-permeable mutants from cultures of VS200 and N500, respectively.

Extraction and quantitation of porphyrins

Cells from overnight cultures were pelleted by centrifugation and cells were resuspended in LB medium without hemin. Then 100-μl aliquots of culture (2×10^8 cells) were transferred to 10-ml aliquots of LB medium in 50-ml Falcon tubes (Becton-Dickinson Labware, Lincoln Park, N.J., USA). Cultures for extraction of porphyrins were wrapped in aluminum foil and incubated for 6 h at 37° C.

Porphyrins were extracted from 1-ml cultures basically as described by Cox and Charles (1973). However, when a sample contained only a small amount of porphyrins, porphyrins were extracted from 10-ml cultures and amounts per ml were estimated. During incubations for 0–6 h, little porphyrin was excreted by the various cells into the medium. Therefore, we extracted porphyrins only from pelleted cells in this experiment. Cells were resuspended in 500 μl of a mixture of ethyl acetate and acetic acid (3:1, v/v) and

Table 1 Derivatives of *Escherichia coli* K-12 used in this study

Strain	Genotype	Source
LE392	*supE44*, *supF58*, *hadR514*, *galK2*, *galT22*, *metB1*, *trpR55*, *lacY1*	Bork et al. 1976
CA274	HfrC, *lac125*, *trp49*, *relA1*, *spoT1*	Russell et al. 1970
VS200	Δ*hemH*, derived from CA274	Nakahigashi et al. 1991
VS212	Δ*hemH*, heme-permeable, derived from VS200	This study
VR306	*hemB*, Δ*hemH*, heme-permeable, derived from VS212	This study
VR314	*hemA*, Δ*hemH*, heme-permeable, derived from VS212	This study
H500	Δ*hemA*, derived from LE392	Nakayashiki et al. 1995
N500	Δ*hemA*, Δ*hemH*, derived from H500	Nakayashiki et al. 1995
N503	Δ*hemA*, Δ*hemH*, heme-permeable, derived from H500	This study

the suspension was sonicated for 90 s at room temperature. Bacterial debris was removed from the ethyl acetate-acetic acid mixture by centrifugation (15 000 rpm) at 4° C and the non-aqueous layer was washed twice with distilled water. The porphyrins in ethyl acetate were transferred to a solution of HCl by shaking with 200 µl of 3 M HCl. Absorption spectra of porphyrins were recorded between 700 nm and 375 nm (UV-160A; Shimadzu, Kyoto, Japan). Levels of porphyrins are given as OD_{408} (porphyrins)/OD_{600} (cell density).

Extraction and quantitation of ALA

One-ml cultures were centrifuged, and pelleted cells were resuspended with 100 µl of 4% TCA. Each suspension was sonicated for 90 s at 4° C. Bacterial debris was removed from TCA by centrifugation (15 000 rpm) at 4° C. Then, 48 µl of 1 M sodium acetate and 2 µl of acetylacetone were added and the mixture was boiled at 100° C for 10 min. This step converts ALA to pyrrole. After the sample had cooled, 150 µl of Ehrlich's reagent (Jordan and Seehra 1986) were added. After 30 min, OD_{555} was recorded. Levels of ALA are indicated as OD_{555} (pyrrole)/OD_{600} (cell density).

Measurement of β-galactosidase activity

The assay of β-galactosidase activity was performed as described by Miller (1972).

Quantitation of ppGpp

Cells from overnight cultures were washed with tryptone medium without hemin and 5-µl aliquots (1×10^7 cells) were transferred to 100-µl aliquots of low-phosphate tryptone medium in Eppendorf tubes. After a 1-h incubation, [^{32}P]orthophosphate (100 µCi; Amersham International, Little Chalfont, Bucks. England) was added to each culture. After 2 h, labeled nucleotides were extracted from cells and analyzed by thin-layer chromatography on polyethyleneimine-cellulose plates as described by Mitchell and Lucas-Lenard (1980).

Results

The synthesis of porphyrins is influenced by the availability of heme

In order to investigate the regulation of porphyrin synthesis, we measured the accumulation of porphyrins in VS212 cells (Δ*hemH*, heme-permeable) under various conditions (Table 2). Under anaerobic conditions, porphyrins barely accumulated, and addition of glucose under aerobic conditions had little effect. The most dramatic change was observed when hemin was omitted from the medium. Omission of hemin from the medium significantly increased the accumulation of porphyrins in VS212 cells (>20-fold). There was an inverse relationship between the concentration of hemin in the medium and the accumulation of porphyrins. Large increases were observed below concentrations of hemin of 25–50 µg/ml. These concentrations are similar to those required for normal growth.

Table 2 Accumulation of porphyrins in VS212 (Δ*hemH*, heme-permeable) cells

Medium[a]	Aeration	Porphyrins (OD_{408}/OD_{600})[b]
LB + hemin	Aerobic	0.0043
LB + hemin	Anaerobic	0.0006
LB + hemin + glucose	Aerobic	0.0065
LB	Aerobic	0.1077

[a] Hemin was added to the medium at a final concentration of 100 µg/ml. Glucose was added at 0.4%
[b] Porphyrins were quantitated by measurement of optical density at 408 nm (OD_{408}). Levels of porphyrins are given as OD_{408} (porphyrins)/OD_{600} (cell density)

Starvation for heme mainly influences the rate of production of ALA

There are several reports that starvation for heme activates transcription of the *hemA* gene (Choi et al. 1996; Darie and Gunsalus 1994). It is likely that the level of heme regulates the rate of synthesis of ALA. To examine the effect of starvation for heme on the synthesis of ALA, we used the *hemB*$^-$ strain VR306. This strain has a defective ALA dehydratase. Thus, any ALA that it synthesizes accumulates intracellularly. As shown in Table 3, the accumulation of ALA in this strain was significantly enhanced by starvation for heme.

Next we examined the effect of starvation for heme on the pathway leading from ALA to porphyrins. We used the *hemA*$^-$ Δ*hemH* strain designated VR314. This strain can not produce ALA and it synthesizes porphyrins from exogenous ALA exclusively. VR314 cells were incubated in LB medium with 100 µg/ml ALA and porphyrins were extracted after 6 h. Starvation for heme slightly enhanced the rate of accumulation of porphyrins, but the increase was much less than that in the accumulation of ALA. We concluded from these results that starvation for heme has a major stimulatory effect on the synthesis of ALA.

Effect of starvation for heme on the expression of *hemA*

There are two contradictory reports concerning the expression of *hemA*. Darie and Gunsalus (1994) reported that expression of a *lac* operon fused to the *hemA* promoter was enhanced 20-fold during starvation for heme.

Table 3 Accumulation of 5-aminolevulinic acid (ALA) in VR306 (*hemB*$^-$, heme-permeable) cells

Strain	Medium[a]	ALA (OD_{555}/OD_{600})[b]
VR306	LB	0.932
VR306	LB + hemin	0.119

[a] Hemin was added to the medium at a final concentration of 100 µg/ml
[b] Quantitation of ALA was performed as described in Materials and methods. Levels of ALA are given as OD_{555} (pyrrole)/OD_{600} (cell density)

By contrast, Choi et al. reported only two- to six-fold enhancement (1996). Using VS212 cells, we confirmed the results of Choi et al. (1996), since the extent of induction of LacZ activity upon starvation for heme was about four-fold (Table 4).

Moreover, even though LacZ activity increased only four-fold, the accumulation of porphyrins increased more than 10-fold (Table 4). We postulated, therefore, that some additional regulatory mechanism might be operative. In order to exclude any effect of the expression of *hemA*, we replaced the promoter of the *hemA* gene by the *lac* promoter and examined the effect of starvation for heme. We used the *hemA* gene fused to the *lac* promoter in p110hemA (for construction of this plasmid, see Nakayashiki et al. 1995) to transform the Δ*hemA* Δ*hemH* strain N503 and then we examined the accumulation of porphyrins. The expression of the *lac* promoter was monitored, in turn, by measuring the activity of β-galactosidase due to the chromosomally encoded *lacZ* gene. The rate of increase in the accumulation of porphyrins was reduced but a significant increase was observed upon starvation for heme (Table 5). This result suggests that a mechanism for activation of the synthesis of porphyrins exists that is independent of the *hemA* promoter. Therefore, we concluded that activation of the synthesis of porphyrins by starvation for heme occurs both as a result of the induction of *hemA* (*hemA* promoter-dependent activation) and as a result of an activation mechanism that is independent of the *hemA* promoter.

Table 4 Effects of hemin and 2-butanol on expression of a *hemA-lacZ* fusion

Medium	Porphyrins[a]	LacZ (units)[a]
LB	0.0995	472
LB + hemin	0.0087	136
LB + hemin + 2-butanol	0.0247	124

[a] Accumulation of porphyrins and activity of LacZ were measured in VS212 cells harboring plasmid pBRhemA-lacZ. Concentrations of hemin and 2-butanol in the medium were 100 μg/ml and 1.0%, respectively. Levels of porphyrins are given as OD_{408}/OD_{600}

Table 5 Effects of hemin on the synthesis of porphyrin in cells harboring a *lac-hemA* fusion gene

Strain[a]	Medium[b]	LacZ (units)	Porphyrins[c]
N503	LB	737	< 0.0001
	LB + hemin	742	< 0.0001
N503p110hemA	LB	772	0.0224
	LB + hemin	772	0.0049

[a] N503 (Δ*hemA*, Δ*hemH*) cells were used (Nakayashiki et al. 1995) N503p110hemA indicates N503 cells that carried p110hemA
[b] Hemin was added to the medium at a final concentration of 100 μg/ml. IPTG was added to the medium at a final concentration of 40 μg/ml
[c] Levels of porphyrins are given as OD_{408}/OD_{600}

Table 6 Effects of starvation for heme on the level of ppGpp

Strain	Medium[a]	ppGpp ($\times 10^3$ cpm)/OD_{600}
CA274	Tryptone broth (1.0%)	1127
	Tryptone broth (0.2%)	5164
VS212	Tryptone broth (1.0%) + hemin	1491
	Tryptone broth (1.0%) −hemin	3260

[a] Cells were incubated in low-phosphate tryptone medium and labeled with 100 μCi/ml [^{32}P]orthophosphate. Radioactivity (cpm) in ppGpp was quantitated as described in Materials and methods

The stringent response promotes the synthesis of porphyrins

By adding a low concentration of antibiotics to the medium, we demonstrated previously that partial inhibition of protein synthesis accelerates the synthesis of porphyrins under heme-deficient conditions (Nakayashiki et al. 1995). If starvation for heme were to repress protein synthesis by provoking the stringent response, such a response might promote the synthesis of porphyrins independently of the *hemA* promoter. The idea that the starvation for heme might repress protein synthesis is supported by the observation that heme starvation reduced the growth rate of VS212 cells. We therefore examined this possibility further.

As shown in Table 6, starvation for heme caused the accumulation of ppGpp in cells, a result that suggests that the stringent response might operate in response to heme deficiency. Since VS212 is a *relA*$^-$ strain, starvation for heme must provoke the stringent response by a *relA*-independent pathway. We investigated the effect of the stringent response on the synthesis of porphyrins. Addition of 2-butanol to the medium has been reported to provoke the stringent response by a *relA*-independent pathway (Mitchell and Lucas-Lenard 1980), and 2-butanol had little effect on the expression of a *lac* operon fused to the *hemA* promoter (Table 4). Therefore, we used 2-butanol to investigate the effect of the *relA*-independent stringent response on the synthesis of porphyrins. As shown in Fig. 1, addition of 2-butanol increased the accumulation of porphyrins up to eight-fold, a result that suggests that the stringent response might promote the synthesis of porphyrins. The rate of increase was almost the same as that observed in the case of the activation that was independent of the *hemA* promoter (Table 5).

Discussion

We have investigated the regulation of the synthesis of porphyrins in *E. coli* using a Δ*hemH* heme-permeable strain, and we confirmed that the synthesis of porphyrins is activated in response to heme deficiency. As reported previously, induction of transcription of *hemA*

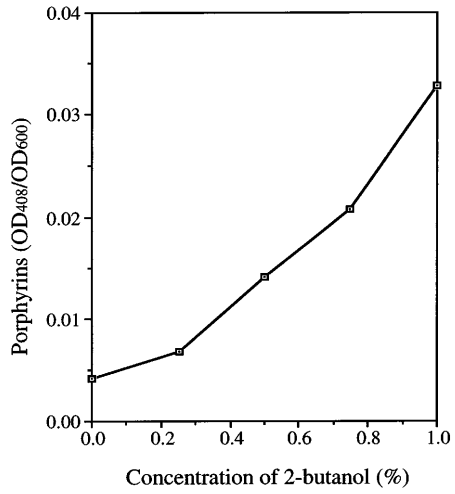

Fig. 1 Effects of 2-butanol on the accumulation of porphyrins in VS212 cells. Cells were incubated in LB medium that contained 100 μg/ml hemin, and 2-butanol at the indicated concentrations. Porphyrins were extracted after a 6-h incubation. Levels of porphyrins are given as OD_{408} (porphyrins)/OD_{600} (cell density)

was observed but activation by a mechanism that was independent of the *hemA* promoter was also observed. The activation that was independent of the *hemA* promoter appeared to be due mainly to the stringent response caused by starvation for heme. However, post-transcriptional activation of *hemA* or activation of other steps in the synthesis of porphyrins might also be involved.

Although Δ*hemH* heme-permeable strains are useful for examining the regulation of heme synthesis, several problems are associated with their use. First, such mutations may change the physiological condition of cells. In particular, when cells are blocked in early steps in the synthesis of porphyrin, they cannot form siroheme and cobalamin, the cofactors of sulfite and nitrite reductases. Second, we can monitor only the accumulation of porphyrins, and not the rate of heme synthesis.

Figure 2 shows a hypothetical model for the regulation of porphyrin synthesis upon starvation for heme. Starvation for heme provokes the stringent response, which represses protein synthesis. Repression of protein synthesis increases the rate of porphyrin synthesis relative to the rate of protein synthesis via the allocation of more glutamyl-tRNA to porphyrin synthesis (competition for glutamyl-tRNA between HemA and EF-Tu is assumed to occur under heme-deficient conditions). By contrast, starvation for heme induces expression of the *hemA-prfA* operon. The *prfA* gene encodes polypeptide-releasing factor 1 (RF1). RF1 might cooperate with the stringent response to repress protein synthesis (Nakayashiki et al. 1995).

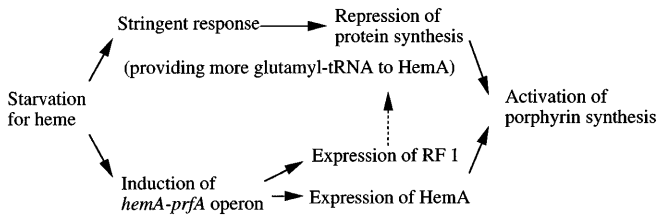

Fig. 2 A hypothetical model for the regulation of porphyrin synthesis upon starvation for heme. Starvation for heme accelerates the synthesis of porphyrins by two different pathways. The upper pathway operates via the stringent response. The lower pathway results in induction of *hemA*

The requirement for heme depends on growth conditions. Cells probably need high levels of heme for rapid growth in rich medium or under aerobic conditions. Thus, cells need to have the ability to regulate the level of heme to reflect the conditions of their environment. It seems reasonable that a shortage of heme should promote the synthesis of heme. *E. coli* cells that carried multicopy plasmids that encoded heme proteins (e.g., cytochromes) were found to overproduce heme (Woodard and Dailey 1995). This result suggests that the level of heme in cells is regulated not by the total amount of heme in cells but by the amount of unused heme or by the intracellular demand for heme. A certain level of free heme might be required to repress induction of the synthesis of porphyrins.

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