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Aspects of the regulation of heme biosynthesis in *Escherichia coli* K-12

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City University of New York, 1990

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ASPECTS OF THE REGULATION OF HEME BIOSYNTHESIS IN *ESCHERICHIA COLI* K-12

by
HEYWOOD UMANOFF

A dissertation submitted to the Graduate Faculty in Biochemistry in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York.

1990

APPROVAL PAGE

This manuscript has been read and accepted by the Graduate Faculty in Biochemistry in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

Date

Charlotte S. Russell
Chair of Examining Committee

August 24, 1990
Date

Executive Officer

Supervisory Committee

ABSTRACT

ASPECTS OF THE REGULATION OF HEME BIOSYNTHESIS IN *ESCHERICHIA COLI* K-12

by
Heywood Umanoff

Advisors: Dr. Charlotte Russell and Dr. Sharon Cosloy

Hemin-permeable mutants were isolated from *hemA* (glutamyl-tRNA dehydrogenase) and *hemB* (5-aminolevulinic acid dehydratase) mutants by nitrosoguanidine mutagenesis and selection on hemin-containing and unsupplemented media. When grown in hemin-containing media neither mutant exhibited porphobilinogen deaminase (PBG D) (*hem C* gene product) activity in crude extracts. Hemin-permeable prototrophs were obtained by complementation of the *hemA* and *hemB* mutant alleles with a phasmid transducing system. Complementation of each mutant allele with its corresponding functional gene resulted in restoration of PBG D activity in the extract. It was determined that the presence of porphobilinogen (PBG) was required for PBG D activity.

PBG D was partially purified from an overproducing *E. coli* strain. Incubation of this enzyme sample with either 10% formic acid or 1 M HCl resulted in the formation of porphyrins from enzyme-bound pyrroles, confirming earlier reports (Jordan and Warren, 1987). Incubation of the enzyme with p-hydroxymercuribenzoate, a known inhibitor of PBG D, did not release a novel dipyrromethane cofactor from the enzyme.

Hemin did not affect ALA synthesis when added to growing cultures of either a hemin-permeable *hemB* mutant or a hemin-permeable prototroph. Nor did it affect ALA D or PBG D activities in the extract, when hemin was added to

growing cultures of various hemin-permeable mutants. Hemin did not affect the activity of partially-purified PBG D.

Anaerobic growth of C600 in rich media resulted in a marginal increase in PBG D activity but had no affect on ALA D activity. Glucose decreased PBG D activity by approximately two-fold when added to aerobically growing cultures of C600, but had no affect on ALA D activity.

The effects of the heme pathway-related metabolites, L-glutamatic acid, 5-aminolevulinic acid (ALA), porphobilinogen (PBG), protoporphyrin IX, and hemin, on the *in vitro* transcription-translation of the *E. coli hemA*, *hemB*, and *hemC* genes was studied. None of these compounds affected gene expression.

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ABBREVIATIONS

amp	ampicillin
ALA	δ-aminolevulinic acid or 5-aminolevulinic acid
ALA D	δ-aminolevulinic acid dehydratase
ALA S	δ-aminolevulinic acid synthase
ATP	adenosine triphosphate
bp	base pair
BuOH	butanol
cys	cysteine
dH_2O	deionized water
DEAE	diethylaminoethyl
DEPC	diethylpyrocarbonate
DTT	dithiothreitol
EDTA	ethylenediametetraacetic acid
EtBr	ethidium bromide
EtOH	ethanol
g	grams or gravitational force (when multiplied by a factor)
glu	glutamate
GSA	glutamate-1-semialdehyde
hr(s).	hour(s)
KAc	potassium acetate
leu	leucine
ME	β-mercaptoethanol
MeOH	methanol
met	methionine
mg	milligram
min.	minute(s)
NADPH	nicotinamide adenine dinucleotide phosphate, reduced
PHMB	p-hydroxymercuribenzoate
PBG	porphobilinogen
PBG D	porphobilinogen deaminase or uroporphyrinogen I synthase
proto	protoporphyrin IX
rpm	revolutions per minute
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
sec(s).	second(s)
TCA	trichloroacetic acid
thia	thiamine (vitamin B_1)
Tris	tris(hydroxymethyl)aminomethane
$tRNA^{glu}$	glutamyl-transfer RNA
uv	ultraviolet

1 INTRODUCTION

1a Purpose of This Study

The major goal of the experiments carried out in this thesis was to understand how the heme pathway is regulated in *E. coli*. To that end several mutants for hemin permeability and heme biosynthesis were isolated for the purpose of gene complementation and characterization. The lack of PBG D activity in some of these mutants which were *hemC*$^+$ led to experiments to determine the effects of intermediates of heme biosynthesis on PBG D activity.

Based on the central role of heme as a regulator of heme biosynthesis in the C4 pathway, its role as a regulator in *E. coli* of the synthesis of early heme-pathway intermediates and porphyrins was investigated. Current research in other laboratories elucidated the role of PBG in a dipyrromethane cofactor of PBG D. The chemical nature of the linkage of the dipyrromethane cofactor to the apoenzyme was investigated. The effects of possible regulators on transcription of three early *hem* genes in *E. coli* and on the translation of their corresponding mRNA's was investigated using an *in vitro* transcription-translation system.

Since oxygen levels have such profound effects on the metabolism of *E. coli* and had been shown to affect the levels of heme synthesis of growing cultures (Ishida and Hino, 1972), the effect of aerobic vs. anaerobic growth on the levels of activity of ALA D and PBG D was investigated. The effect of glucose, a major regulator of gene expression in *E. coli*, on these same activities was also investigated.

1b Tetrapyrroles; Structure and Biological Significance

Tetrapyrroles are important molecular species found in widely diverse organisms in which they serve as enzyme cofactors or energy transferring molecules. The tetrapyrroles include hemes, chlorophylls, corrins, and chlorins. The linear tetrapyrrole hydroxymethylbilane (HMB) is the precursor of the porphyrinogens; it is formed enzymatically from the condensation of the pyrrole units of porphobilinogen (PBG) by PBG deaminase (PBG D) (see Fig. 1). Porphyrinogens are precursors to the porphyrins. Uroporphyrinogen III is the common precursor to all tetrapyrroles, including protoporphyrin IX and heme (Fig. 1).

Heme (see Fig. 2) serves as the prosthetic group for hemoglobin and myoglobin, cytochromes including P_{450}, catalase, and peroxidase. Chlorophyll (Fig. 2) serves as the light energy-converting molecule in photosynthesis in the thylakoid membrane of green plants. In animals and plants vitamin B_{12} (Fig. 2) is the cofactor for enzymes (notably methylmalonyl-CoA mutase) catalyzing exchange reactions in the synthesis of succinyl-CoA from propionyl-CoA and catalyzing the degradation of methionine, valine, and isoleucine. In *Salmonella typhimurium* and *Escherichia coli*, two of the few organisms that can synthethize it, vitamin B_{12} is a cofactor for several enzymes including homocysteine methyltransferase, an auxillary enzyme for methionine biosynthesis (Cauthen et. al., 1966; Elliott and Roth, 1989). The chlorin siroheme (Fig. 2) is a cofactor for sulfite reductase and nitrite reductase in *E. coli* and *S. typhimurium*, enzymes involved in the biosynthesis of cysteine from inorganic sulfate and of ammonia from NO_3^-, respectively (Elliott and Roth, 1989; Ingledew and Poole, 1984).

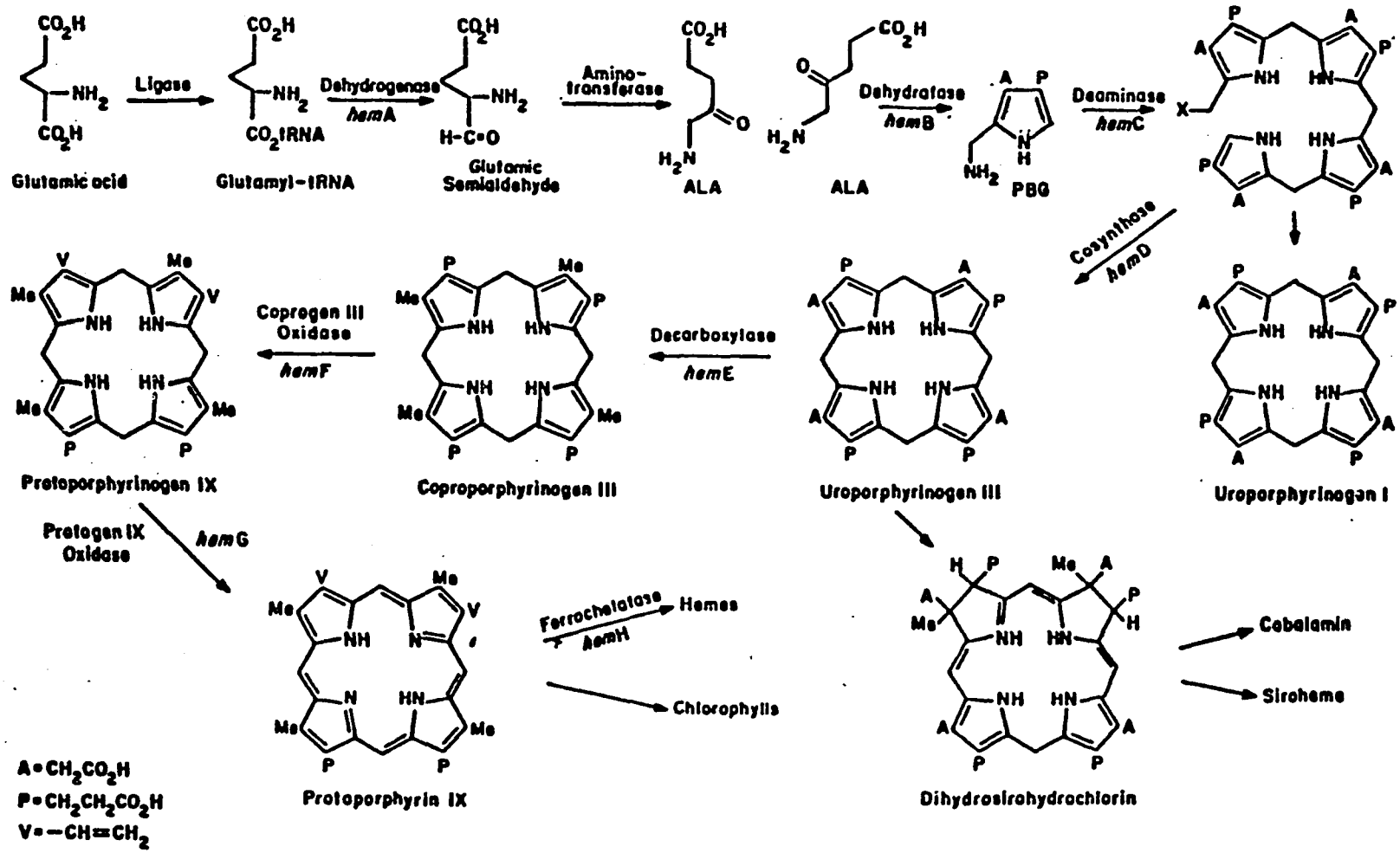
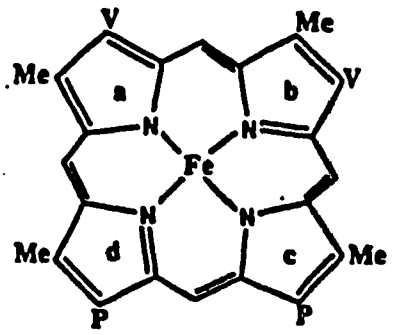
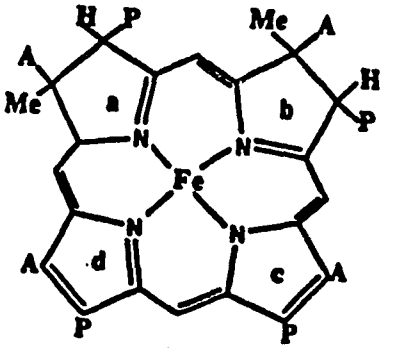


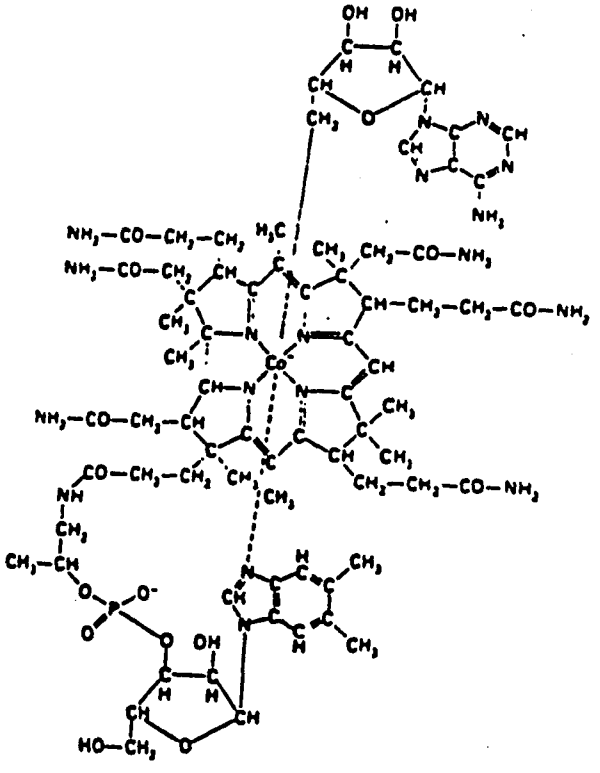
Fig. 1 The heme biosynthestic pathway depicting the C5 pathway to ALA synthesis and branches to the major tetrapyrroles.



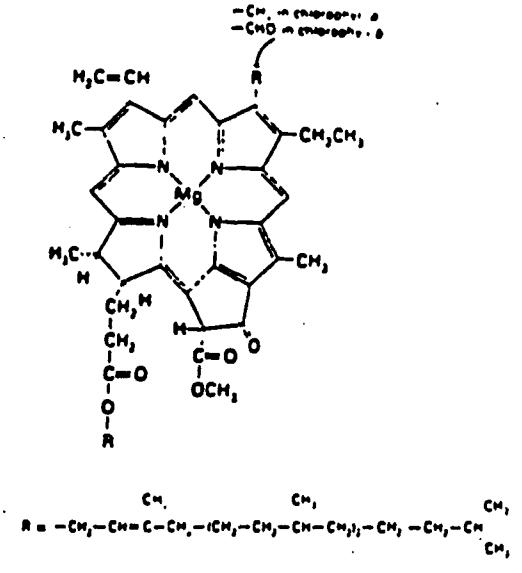
heme



siroheme



cobalamin



chlorophyll

Fig. 2 Structures of the major tetrapyrrole endproducts originating from the heme pathway (cobalamin and chloroyphyll structures are from Stryer, 1981).

1c Heme; Structure and Characteristics

In heme four bonds between Fe^{+2} and the pyrrole nitrogens are in one plane while the fifth and sixth coordinates are perpendicular to that plane. In hemoglobin and myoglobin the fifth and sixth coordinates of iron represent bonds to a histidine residue on the apoprotein and to O_2. In the tissues, however, following the release of O_2 in response to lowered oxygen partial pressure, the Fe^{+2} is restored to a pentacovalent species.

Hemin is the form taken by heme when Fe is in the +3 oxidation state; in hemin chloride the fifth coordinate position is occupied by chlorine; in hematin a hydroxy group occupies that same position. The behavior of hemin in solution is complex. At alkaline pH heme exists mainly as monomers and dimers (Inamura et. al., 1989), but polymers can exist under certain conditions. Dimers and polymers are formed by an oxygen bridge between two or more Fe atoms on two or more heme molecules. Complexes between hemin and other molecules in solution, such as Tween 80, have been identified (Inamura et. al., 1989).

1d Heme; Biological Significance and Regulatory Functions

In hemoglobin and myoglobin the Fe^{+2} atom of heme binds O_2 for transport to the tissues; CO binds to Fe^{+2} with even greater affinity than O_2. In cytochromes, the iron of heme functions as an electron carrier and and goes through successive oxidation (to the ferric state)-reduction cycles in response to an electropotential gradient which is coupled to ATP synthesis. Cytochromes aa_3 and P_{450} contain heme A as a cofactor bound to a hydrophobic cleft in the protein through the interaction of a long hydrophobic tail. Cytochrome P_{450}, a

monooxygenase, along with auxillary enzymes, carries out hydroxylation reactions necessary for the detoxification of various substances in the liver.

Heme is an important regulator of cellular activity at all levels (see Fig. 3). It negatively regulates ALA S activity and positively regulates heme oxygenase in the liver cell at the transcriptional level (Padmanaban et. al., 1989). In yeast, a heme regulon exists comprised of genes coordinately activated and repressed. Under aerobic growth conditions, in the presence of heme, genes whose products are involved in oxidative metabolism are activated, including ROX1, encoding a heme-induced repressor of genes whose products are involved in anaerobic metabolism (Lowry et. al., 1984).

Translational activity in reticulocytes is regulated by hemin. A heme-regulated kinase phosporylates eukaryotic initiation factor 2-α to inhibit protein synthesis (Fagard and London, 1981). When levels of heme are low the phosphorylation of initiation factor 2-α is inhibited resulting in the stimulation of protein synthesis.

At the enzyme level heme inhibits ALA S in the liver as well as in *R. spheroides*. Hemin was shown to relieve repression of ferritin synthesis in a vertebrate *in vitro* translation system. When partially-purified ferritin-repressor protein (FRP) was incubated with hemin and added to the translation reaction mixture, amounts of newly synthesized ferritin increased proportionally to the time of hemin-FRP incubation (Lin et. al., 1990).

Heme prevents ubiquitin-tagged proteins from degradation in a cell-free, ATP-dependent proteolytic system from a reticulocyte lysate (Padmanaban et. al., 1989). The transport of ALA S from the cytosol into the liver mitochondrion appears to be inhibited by heme while the transport of cytochrome c of yeast from the cytosol to the outer surface of the inner mitochondrial membrane is

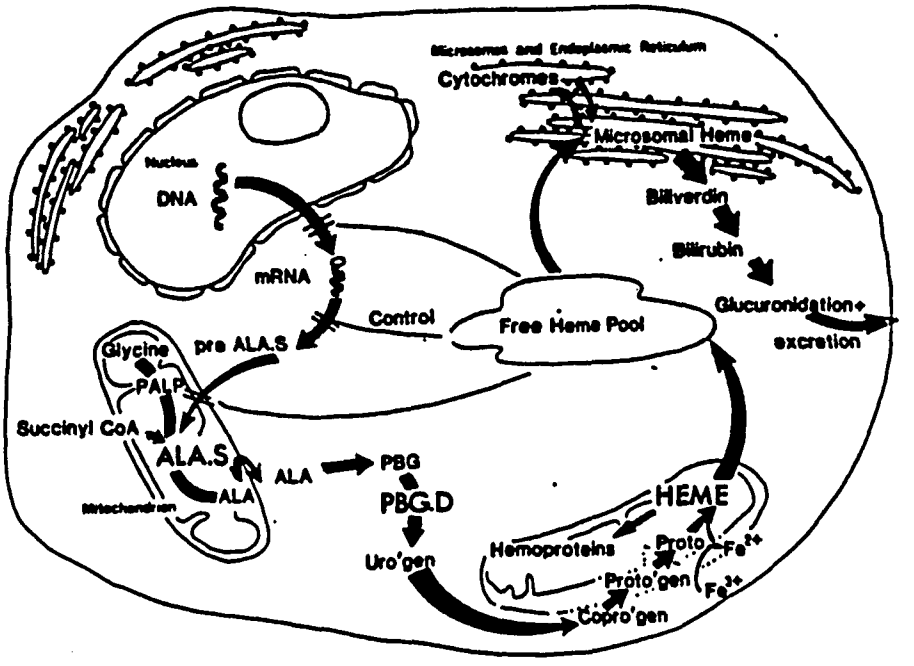


Fig. 3 The role of the "free heme pool" in the control of heme biosynthesis. PALP, pyridoxal phosphate (from Moore et. al., 1987).

stimulated by heme (Padmanaban et. al., 1989). Hemin also enhanced the differentiation of a mouse 3T3 adipose cell line (Padmanaban et. al., 1989).

1e The Heme Biosynthetic Pathway

Several of the enzymatic reactions in the biosynthesis of heme are conserved among animals, plants, and microorganisms, while others are unique to particular organisms. The heme pathway in *E. coli* and *S. typhimurium*, as it is currently known, has a major branch at uroporphyrinogen III leading to cobalamin and siroheme synthesis (Fig. 1). This branch is lacking in the heme pathway of animals and plants. The enzymatic steps which synthesize δ-aminolevulinic acid (ALA), the first intermediate of the pathway, also differ between animals, plants, and microgorganisms. The steps which convert ALA to uroporphyrinogen III are thought to be well conserved. On the other hand, the coproporphyrinogen III oxidase and protoporphyrinogen IX oxidase reactions show variation in the enzyme involved and in co-substrate requirements among different organisms.

1e.1 ALA synthesis via the C4 pathway

One important difference between animals, plants, and microorganisms in the synthesis of heme involves the steps for ALA synthesis. The C4 pathway (see Fig. 4) designates the single step enzymatic conversion of succinyl-CoA and glycine to ALA via an ALA synthase activity which is dependent on pyridoxal phosphate (Laver et. al., 1958) . The role of ALA in heme biosynthesis was based on the production of labelled porphyrins from labelled glycine and acetate (Bloch and Rittenberg, 1945) and on the demonstration that labelled ALA is incorporated into protoporphyrin (Shemin and Russell, 1953).

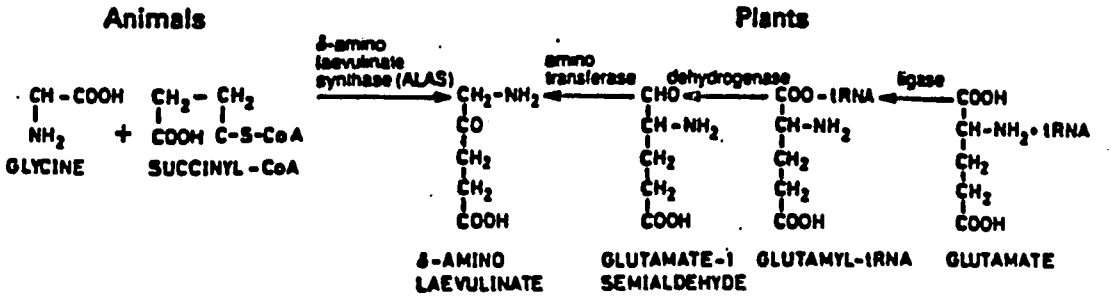


Fig. 4 Scheme of the C4 (animals) and C5 (plants) pathways to ALA synthesis (from Padmanaban et. al., 1989).

This ALA synthase activity is present in animal tissues (Bottomly and Smithee, 1968; Gibson et. al., 1958), some fungi (Pora et. al., 1972) including *Saccharomyces cerevisiae*, the photosynthetic flagellate (algae) *Euglena gracilis*, and many eubacteria (true bacteria) including among others, *Rhizobium melilotti* (Leong et. al., 1982) (anaerobic), *Rhodopseudomonas spheroides* (Lascelles, 1964) (facultative), *Pseudomonas denitrificans* (anaerobic), and *Propionibacterium shermanii* (anaerobic) (Kannangara et. al., 1988).

1e.2 **ALA synthesis via the C5 pathway**

The C5 pathway (see Fig. 4) of green plants designates the three-step conversion of glutamate to ALA: a glutamyl-tRNA synthase ligates glutamate to its appropriate tRNA and requires ATP (Kannangara et. al., 1984); a glutamyl-tRNA dehydrogenase converts the glutamyl residue of the tRNA to glutamate semialdehyde (GSA) in an NADPH dependent step; and an aminotransferase then exchanges the amino group of GSA with the carbonyl group, resulting in the formation of ALA.

The C5 pathway is thought to be the more ancient of the two pathways for the synthesis of ALA (Avissar et. al., 1989). This pathway is utilized by higher plants (Beale and Castelfranco, 1974; Beal et. al., 1975), and several algae (Huang and Wang, 1986; Oh-hama, 1986) including *Euglena gracilis* , which has been reported to use both pathways (Weinstein and Beale, 1983).

The distribution of the C4 and C5 pathways among microorganisms and eukaryotes is shown in Fig. 5, in which *E. coli* has been classified in the γ-subgroup of the purple bacteria (Avissar et. al., 1989). Among the eubacteria the C5 pathway is utilized by several cyanobacteria (facultative, photosynthestic eubacteria) (Friedmann and Thauer, 1986), the green sulfur and green non-

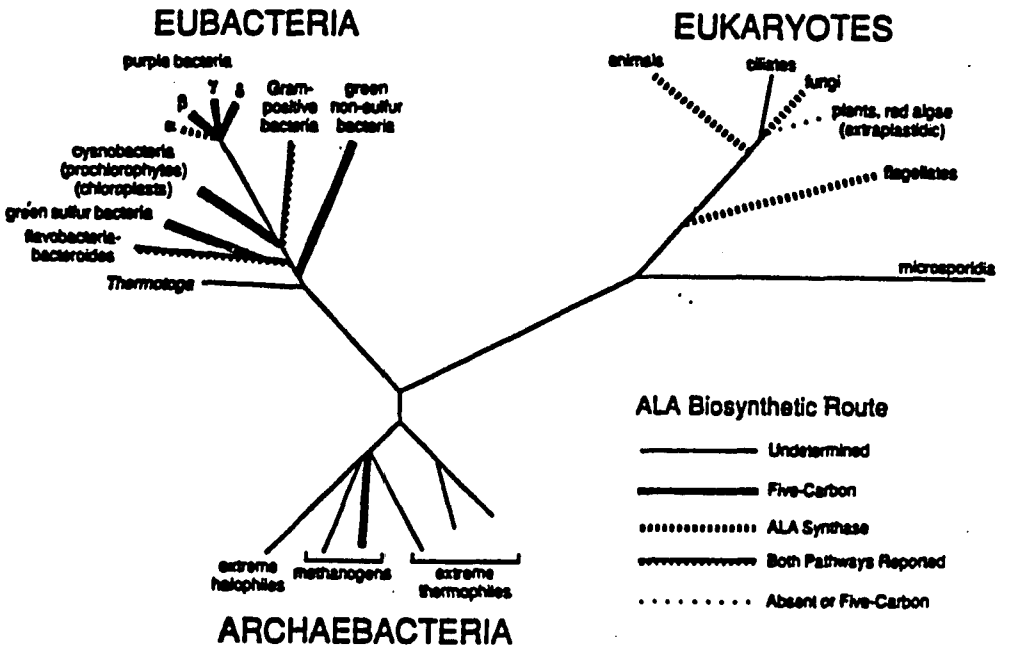


Fig. 5 A phylogenetic tree depicting the distribution of the C4 and C5 pathways (from Avissar and Beale, 1989).

sulfur bacteria, and three of the four subgroups of purple bacteria (Woese, 1987). Among archaebacteria, *Methanobacterium* (methane producer, anaerobic) *thermoautotrophicum* is reported to use the C5 pathway (Gillis et. al., 1983).

It had been thought for some time that *E. coli* utilized the C4 pathway for ALA synthesis (Ishida and Hino, 1972). However, research into the synthesis of ALA in *E. coli* has indicated very little or no ALA synthase activity from succinyl-CoA and glycine (Avissar and Beale, 1989; unpublished results). On the other hand, there is mounting evidence that *E. coli* utilizes the C5 pathway for the synthesis of ALA. Li et. al. (1989) analyzed the labelling pattern of ^{14}C-glutamate and ^{14}C-glycine incorporation into ALA. The results showed that glutamate was incorporated into ALA while glycine was not . O'Neill et. al. (1989) demonstrated the synthesis of ALA from glutamate in crude extracts of *E. coli* and *B. subtilus* . And Avissar and Beale (1989) identified glutamyl tRNA synthetase, glutamyl-tRNA dehydrogenase, and aminotransferase activities in crude *E. coli* extracts. *S. typhimurium* is reported to utilize two pathways for ALA synthesis (Elliott and Roth, 1989): a major pathway utilizing at least two gene products and a minor pathway distinct from the former, which can operate during anaerobic growth for the synthesis of siroheme and cobalamin (vitamin B_{12}).

1e.3 **Remaining steps of the heme biosynthetic pathway**

Two molecules of ALA are condensed to form porphobilinogen (PBG) by ALA dehydratase (ALA D), via a Schiff base intermediate. PBG is the basic unit of the porphyrin nucleus. Four PBG molecules are condensed head-to-tail by PBG deaminase (PBG D) in deamination reactions which release NH_3 and

H_2O, resulting in the formation of the linear tetrapyrrole, hydroxymethylbilane (HMB).

HMB is cyclized by uroporphyrinogen III cosynthase to uroporphyrinogen III. HMB can spontaneously condense to form uroporphyrinogen I which differs from uroporphyrinogen III by the symmetrical arrangement of the acetate and propionate side chains of the D pyrrole ring with respect to the rest of these side chains in the porphyrin nucleus. Uroporphyrinogen III cosynthase rotates ring D of HMB during cyclization, resulting in the asymmetric side-chain arrangement found in uroporphyrinogen III (Stark et. al., 1986).

Uroporphyrinogen III decarboxylase converts uroporphyrinogen III to coproporphyrinogen III. This enzyme catalyzes the conversion of the acetate substituents in each pyrrole ring of the porphyrin nucleus to a methyl group via successive deacarboxylations (Straka et. al., 1982). The conversion of uroporphyrinogen I to coproporphyrinogen I by the same uroporphyrinogen decarboxylase is thought to result in a metabolic dead end.

Coproporphyrinogen III is converted to protoporphyrinogen IX by the action of coproporphyrinogen III oxidase (Grandchamp and Nordman et. al., 1982). This enzyme converts two of the four propionyl substituents of the porphyrin ring to vinyl groups by oxidative decarboxylation. Protoporphyrinogen IX oxidase converts protoporphyrinogen IX to the fully conjugated protoporphyrin IX by the removal of six hydrogen atoms in the porphyrinogen ring system, a reaction which can also occur spontaneously, although less efficiently (Jacobs and Jacobs et. al., 1982). Finally, ferrochelatase inserts Fe^{+2} into protoporphyrin IX, resulting in heme.

1f Location of Heme Pathway Enzymes

In the eukaryotic cell, ALA S is found in the mitochondrial matrix following its transport from the cytosol (see Fig. 3). The transport is dependent on an ALA S amino-terminal presequence, receptors on the mitochondrial surface, an electrochemical potential gradient across the membrane, and ATP (Haldi and Guarente, 1989). ALA is transported across the mitochondrial membrane into the cytosol where all the reactions responsible for converting ALA to coproporphyrinogen III occur. Coporporphyrinogen III is transported into the mitochondrion where the final three reactions of heme biosynthesis occur. Therefore protoporphyrinogen, protoporphyrin IX, and heme are found within the mitochondrion, as are ALA S, coproporphyrinogen oxidase, protoporphyrinogen IX oxidase, and ferrochelatase.

In *E. coli* less is known about the localization of the enzymes for heme biosynthesis. There is evidence to suggest that one or more of the enzymes involved in ALA synthesis are membrane associated (Li et. al., 1989b), but if so then probably only loosely so since after cell disruption and centrifugation, ALA is synthesized in the supernatant fraction (Avissar and Beale, 1989; O'Neill et. al., 1989).

Protoporphyrinogen oxidase activity in *E. coli* has been found in the membranous fraction (sedimented at 139,000g for 150 min) of a crude extract and appears to be coupled to electron transport, since cytochrome-deficient strains and quinone-deficient strains could not carry out the aerobic oxidation of protoporphyrinogen (Jacobs and Jacobs, 1979).

Activities for ALA D, PBG D, and uroporphyrinogen III cosynthase, coproporphyrinogen III oxidase, ferrochelatase, and uroporhyrinogen decarboxylase have all been assayed in the supernatant fraction of crude

sonicates (Chartrand et. al., 1979; Sasarman et. al., 1979). Therefore if any of these enzymes are membrane associated they are not strongly so.

1g Genetics of Heme Biosynthesis in *E. coli*

1g.1 Organization of *hem* genes

The heme biosynthetic pathway in *E. coli* consists of ten currently identified enzymatic activities for which only nine genes have been mapped to positions on the chromosome. The heme genes are distributed around the chromosome (see Fig. 6) with only one operon having been identified, the so-called uroporphyrin operon, which involves cotranscription of *hemC*, *hemD*, and possibly *hemG*. Genes for ALA synthesis have been mapped to two locations: one at 27 minutes in the purB-trp region (Sasarman et. al.,1968) and one at 6-9 minutes in the pro-thr-leu region (Wulff, 1967).

1g.2 *Hem* mutants

The enzymatic steps for heme biosynthesis in *E. coli* were identified largely through the selection and characterization of mutants unable to synthesize heme. Such mutants grow very poorly aerobically, either in liquid media or on agar media (as microcolonies) if no heme is supplied. They exhibit little or no catalase activity (Sasarman et. al., 1968). Certain heme-deficient mutants exhibit better anaerobic growth compared to aerobic growth, presumably because of the effects of toxic oxygen radicals formed during aerobic growth which would accumulate in catalase-deficient strains. Accumulation of particular intermediates have been found depending on the mutation involved.

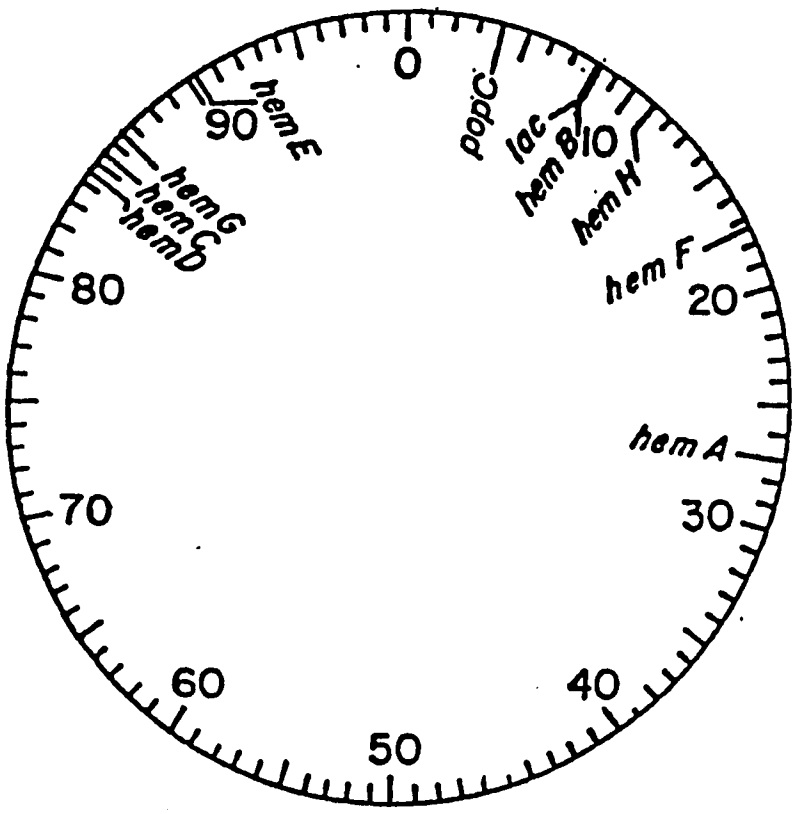


Fig. 6 Location of the *hem* genes on the map of the *E. coli* chromosome.

The identification of an ALA-requiring mutant of *E. coli* by Wulff (1967) confirmed a role for ALA as an early intermediate in the *E. coli* heme biosynthetic pathway. Cox and Charles (1973) used a *popA* (now *hemG*) mutant to isolate secondary mutants which accumulated porphyrin intermediates and precursors. McConville and Charles, using nitrosoguanidine (NTG) mutagenesis, identified and characterized several mutants for hemin-permeability (wild-type *E. coli* is not permeable to hemin, Sasarman et. al., 1968) and heme biosynthesis including *hemB*, *hemC*, and *hemH* mutants (McConville and Charles 1979a, 1979b, 1979c). Sasarman, using a neomycin selection for respiratory mutants, identified and characterized several other heme mutants in the pathway including a *hemA* mutant which mapped to a different location on the *E. coli* chromosome than did Wulff's *hemA* mutant (Sasarman et. al., 1968), a *hemD* mutant which accumulated uroporphyrin I and coproporphyrin I and mapped to 83 min on the chromosome in P1-mediated transduction experiments (Chartrand et. al.,1979), and a *hemG* mutant which accumulated uroporphyrin III and coproporphyrin III and mapped to 85 min on the chromosome (Sasarman et. al., 1979). Jordan identified a gene, hemX, which is cotranscribed along with *hemC* and *hemD* from the *hemC* promoter. The precise role of the hemX gene product remains to be determined, but it is thought to be *hemG*, which had been mapped to the same chromosomal position (Jordan et. al., 1988a).

Heme mutants have been isolated in numerous other organisms including, among others, *Rhodobacter capsulatus* (a purple nonsulfur bacterium) (Wright et. al. 1987), *R. spheroides* (Hatch and Lascelles, 1972), *Staphylococcus aureus* (Fuente et. al., 1986) and *Saccharomyces cerevisiae* (Bard and Ingolia, 1984; Kurlandzka and Rytka, 1985).

1h Aspects of *E. coli* (and *S. typhimurium*) Metabolism Pertinent to the Regulation of Heme Biosynthesis in This Study

1h.1 Facultative metabolism

E. coli exhibits a remarkable adaptibility to environmental conditions by virtue of its facultative metabolism. But growth of cultures under differing oxygen concentrations or on different carbon sources results in significant metabolic changes. The enzymes of the Krebs cycle are much more active during aerobic growth than anaerobic growth (Gray et. al. 1966a). Cytochrome levels are, interestingly, high under anaerobic as well as aerobic conditions since *E. coli* and *S. typhimurium* utilize uroporphyrinogen III, anaerobically, for vitamin B_{12} and siroheme synthesis and heme for cytochromes used in anaerobic electron transport (Ingledew and Poole, 1984; Elliott and Roth, 1989). However the pattern of cytochromes synthesized changes; cytochrome c is synthesized under anaerobic growth but is absent under aerobic growth and levels of cytochrome b_1 are elevated under aerobic growth compared to anaerobic growth (Gray, 1966a; Ishida and Hino, 1972).

1h.2 Respiratory chains in *E. coli*

The *E. coli* respiratory system includes various dehydrogenases which supply reducing equivalents to ubiquinone or menaquinone. The quinones are thought to carry the reducing equivalents supplied by the dehydrogenases to the cytochromes and ultimately to oxygen or, under anaerobic conditions, to one of two terminal electron acceptors, fumarate or nitrate, in conjunction with either fumarate reductase or nitrate reductase, respectively. Ubiquinone is in

greatest amounts during aerobic growth while menaquinone is the predominant species under anaerobic growth (Ingledew and Poole, 1984).

1h.3 **Nitrate and fumarate reductases**

Nitrate reductase converts nitrate (NO_3^-) to nitrite (NO_2^-). The nitrite formed is readily reduced to NH_3 for amino acid biosynthesis under anaerobic conditions by nitrite reductase, which contains a siroheme cofactor. The genes for nitrate and nitrite reductases are repressed by oxygen, while NO_2^- induces the nitrite reductase gene (Ingledew and Poole, 1984). Fumarate reductase reduces fumarate to succinate.

Therefore either fumarate or nitrate can serve as a terminal electron acceptor in the *E. coli* anaerobic respiratory chain. More ATP is generated from nitrate as terminal acceptor than from fumarate (Ingledew and Poole, 1984). Both fumarate and nitrate reductases are bound to the cell membrane although the site of nitrate reduction is cytoplasmic and the need for a nitrate-nitrite porter system has been proposed (Ingledew and Poole, 1984) since *E. coli* cannot synthesize NO_3^-.

1h.4 **Transcriptional regulation during anaerobic growth**

Many metabolic changes are brought about by a shift from an aerobic to an anaerobic environment in *E. coli*. Aerobically, pyruvate is converted to lactate by lactate dehydrogenase, whereas anaerobically, transcriptional activation of the pyruvate formate-lyase gene causes almost all available pyruvate to be converted to acetyl-CoA and formate (Knappe, 1987). Formate is converted to CO_2 and H_2 by the action of formate hydrogen-lyase which forms a complex with pyruvate formate-lyase (the formate hydrogen-lyase complex) (Knappe, 1987).

The *fnr* gene product is a positive regulator of transcription for several anaerobic respiratory enzymes including nitrate reductase, nitrite reductase, fumarate reductase, formate hydrogen-lyase, and cytochrome c_{552} (Newman and Cole, 1978) and was mapped at 29.5 minutes (Strauch et. al., 1985).

In *S. typhimurium* three oxygen regulatory locii (*oxr*) have been identified: *oxrA*, *oxrB*, and *oxrC*. *OxrA* maps in the same region of the *S. typhimurium* chromosome as *fnr* does in *E. coli* and it is thought to be the corresponding locus to *fnr* (Strauch et. al., 1985). *OxrA* and *oxrB* regulate genes serving respiratory functions, while *oxrC* regulates genes serving fermentative or biosynthestic functions (Jamieson and Higgins, 1986). This finding suggests the possibility of identifying another *E. coli* regulatory locus analogous to *oxrC* of *S. typhimurium* as well as other hitherto unidentified oxygen-regulated locii.

1h.5 **Effect of carbon source on *E. coli* metabolism**

The effect of carbon source in the growth medium on enzyme activity in crude extracts is well documented. Glucose was shown to repress levels of all Krebs cycle enzymes in cultures of aerobically grown *E. coli* in rich media (Gray et. al., 1966b) before the molecular mechanism of catabolite repression involving catabolite activator protein and cyclic AMP was understood. Metabolites of glucose, in addition to glucose itself, were known to repress the formation of enzymes involved in the catabolism of those metabolites (Gray et. al., 1966b).

1i Regulation of Heme Biosynthesis in *E. coli* and Other Organisms

1i.1 Regulation of the C4 pathway to ALA synthesis

ALA S is considered to be the rate limiting and major regulatory enzyme in the mammalian liver. ALA S is feedback inhibited by heme and negatively regulated at the transcriptional level. The transport of the precursor form of ALA S from the cytosol to the liver mitochondrion is inhibted by heme. ALA S in human HepG2 hepatoma cells was inhibited by heme in a dose-dependent manner when heme was added to cells in growing culture (Iwasa et. al., 1989). Erythroid ALA S, however, is not feedback inhibited by heme (Kappas et. al., 1983). This ALA S synthase has a different molecular weight from and is encoded by a different gene on a different chromosome than liver ALA S (Yamamoto et. al., 1988).

It has been suggested that iron, in entering the heme pathway after release from transferrin, rather than heme may be the main regulator of heme biosynthesis in erythrocytes. Laskey et. al. (1986) showed that a synthetic iron carrier stimulated ^{14}C glycine incorporation into heme for DMSO-induced Friend cells, but not for uninduced cells. Beru and Goldwasser (1985) found that upon erythropoietin treatment for the differentiation of erythroid cells Fe uptake and heme biosynthesis increased. PBG D levels, not ALA S levels, increased 3.5-fold over a four day period and experiments using actinomycin D and cycloheximide in this system suggested that the increase in PBG D levels was due to transcriptional activation.

Early studies on ALA S in the photosynthetic bacteria *R. spheroides* (Lascelles,1964) showed repression by heme at the enzyme level as well as the transcriptional level. Similar effects were seen with the anaerobic *Micrococcus denitrificans* (Granick and Beale, 1978). The effects of heme on the regulation of ALA S and on heme biosynthesis in general in other bacteria have not been extensively studied.

1i.2 Regulation of the C5 pathway for ALA synthesis

Little is known about the regulation of ALA synthesis via the C5 pathway. In green plants the synthesis of ALA from glutamate is inhibited in the dark in the plastid of the plant leaf. There is a dependence on ATP, NADPH, and $tRNA^{glu}$ for the synthesis of ALA. GTP stimulated the conversion of glutamate to ALA in reconstitution assays with barley chloroplast tRNA and C5 pathway enzymes (Kannangara et. al., 1988).

Three glutamyl-tRNAs have been identified in greening barely, one of which is involved in ALA and protein synthesis. The other two actually code for glutamine. After ligation of glutamate to these glutamine-coding tRNAs, the glutamate is converted to glutamine by an amidotransferase reaction using glutamine as the nitrogen donor (Kannangara, et. al., 1988). It has yet to be determined in *E. coli* whether the same $tRNA^{glu}$ used for ALA synthesis is also used for protein synthesis.

1i.3 Regulation of ALA D

Two molecules of ALA are converted to PBG via a Schiff base intermediate covalently attached to the ε-amino group of an essential lysine residue at the active site of ALA D. The enzyme has been isolated from several organisms including mammals (Anderson and Desnick, 1979; Wu et. al., 1974),

plants (Liedgens et. al., 1983), and a variety of microorganisms including *R. spheroides* (Nandi and Shemin,1968).

ALA D occurs as an octamer. The subunit molecular weight ranges from 32,000 to 42,000 daltons. The enzyme requires Zn^{+2} for activation and a reducing environment usually supplied by DTT or mercaptoethanol in enzyme assays. Levulinic acid is a competitive inhibitor of ALA D and iodoacetamide inactivates it.

ALA D genes from various organisms have been cloned, including human (Wetmur et. al., 1986), rat (Bishop et. al., 1986), mouse (Bishop et. al., 1989), yeast (Myers et al., 1987), and *E. coli* (Li et. al., 1988). The *E. coli* gene shares good homology with the human liver, rat liver, and yeast genes with very high homology for the Zn-binding region (16 amino acids) and the active site lysine region (4 amino acids) (Li et. al., 1989a).

The *E. coli* gene is reported to be moderately expressed and contains two possible promoter sites within a 367 base pair region, one of which contains a 7 base pair direct repeat (Li et. al., 1989a).

1i.4 **Regulation of PBG D**

1i.4a *Enzyme level regulation*

PBG D, the third enzyme in the heme biosynthetic pathway, carries out the head-to-tail condensation of 4 molecules of PBG to the product HMB. The enzyme has been isolated and purified from many sources including, among others, human (Frydman and Feinstein, 1974), wheat germ (Higuchi and Bogorad, 1975), *Euglena gracilis* (Battersby et. al., 1983a), and *E. coli* (Jordan et. al., 1988b.). The enzyme occurs as a monomer with molecular weights ranging from 36.000 in *E. coli* (Jordan et. al., 1988b; see Fig. 15) to 44,000 in

human liver (Grandchamp et. al., 1987) based on SDS-PAGE . The amino acid and gene sequences are well conserved across these diverse organisms. Following the first incoming PBG molecule (which is designated as ring A in the resulting porphyrin nucleus, see Fig. 7), the next three molecules are covalently attached to the one entering before it at the free α-H position (Battersby et. al. 1983a). The next PBG to bind to the enzyme causes the release of the linear tetrapyrrole from the protein (Fig. 7) (Battersby et. al., 1983b). The product HMB is a competitive inhibitor of the enzyme (Battersby et al 1983b).

More recent research has shed light on the nature of the attachment of the linear tetrapyrrole to PBG D. PBG in the growth medium was required for PBG D activity in the extract of an *E. coli hemB* mutant, incapable of endogenous PBG synthesis (Umanoff et. al., 1988). Work by several groups (Jordan and Warren 1987; Miller et. al., 1988; Scott et. al., 1988b) have demonstrated the presence of a unique dipyrromethane cofactor covalently linked to cysteine-242 of the apoenzyme. This cofactor incorporates two PBG molecules bridged by a methylene group and serves as the anchoring group for the first PBG molecule to be incorporated into the product HMB (see Fig. 8).

Two enzyme binding sites are proposed: a catalytic site site which holds the first two PBGs which get incorporated into the dipyrromethane cofactor and which also holds the growing pyrrole chain, and a substrate site which holds each of the four incoming PBGs. After the deamination reaction the enzyme-substrate (ES) complex is translocated to the catalytic site occupied by the newly incorporated PBG (Warren and Jordan, 1988).

When the purified enzyme is treated with formic acid or HCl, porphyrins are formed (Jordan and Warren, 1987; see Results) from cofactor and/or the emerging enzyme-bound linear pyrrole species. Scheme I below illustrates a likely mechanism for porphyrin formation in the presence of acid

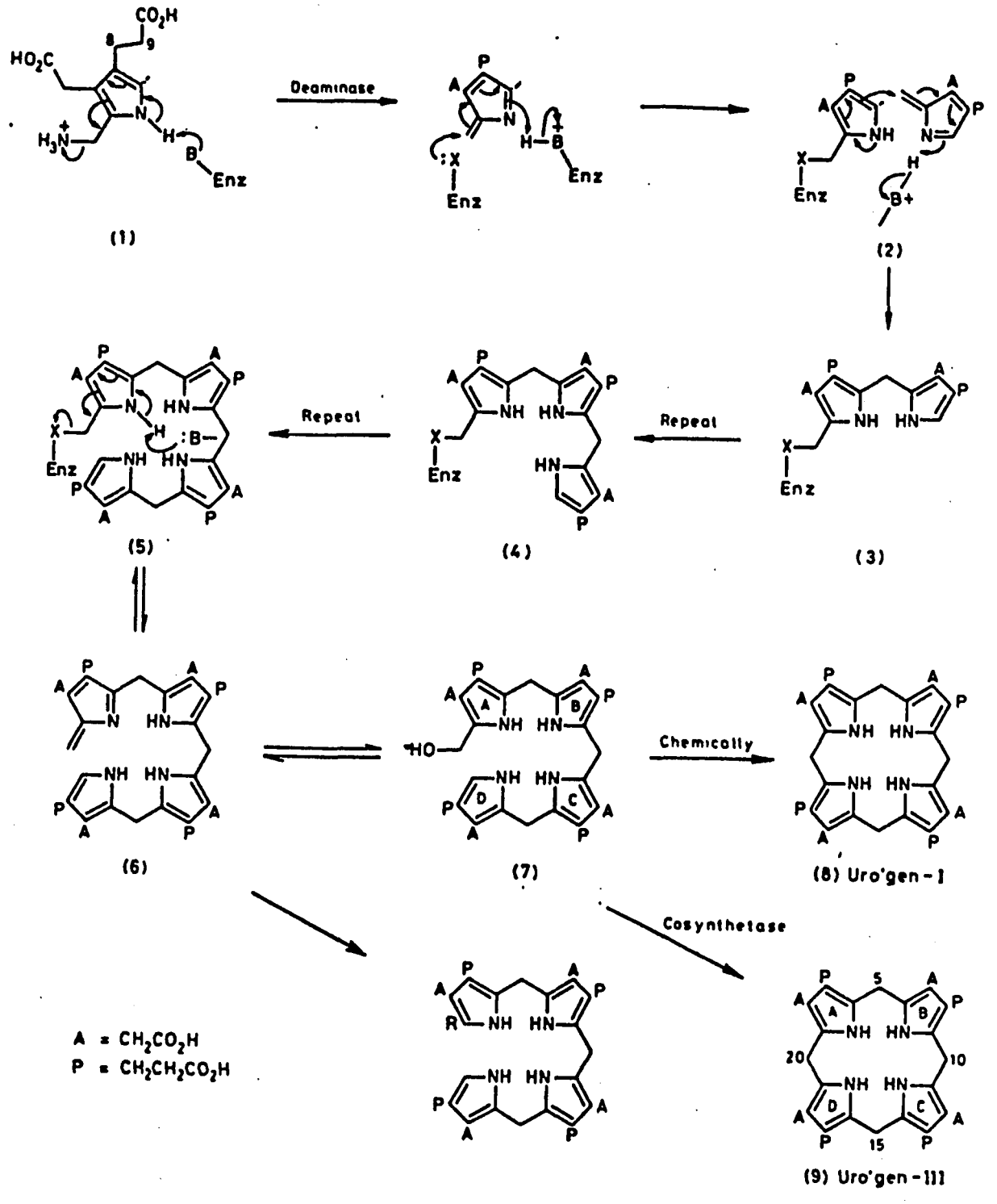


Fig. 7 Enzymatic mechanism of PBG D in the formation of the linear tetrapyrrole, hydroxymethylbilane (7), and the uroporphyrinogens (from Battersby et. al., 1983a).

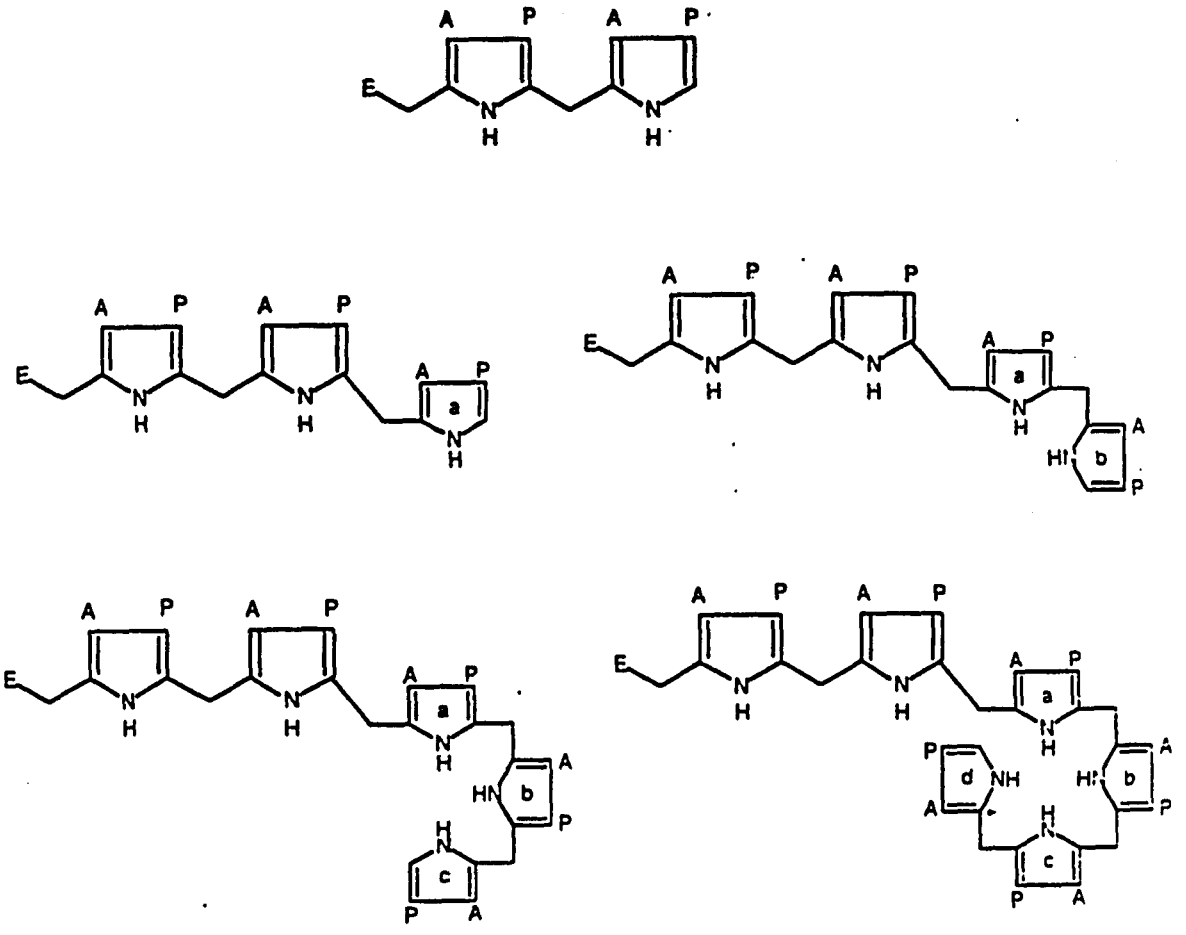
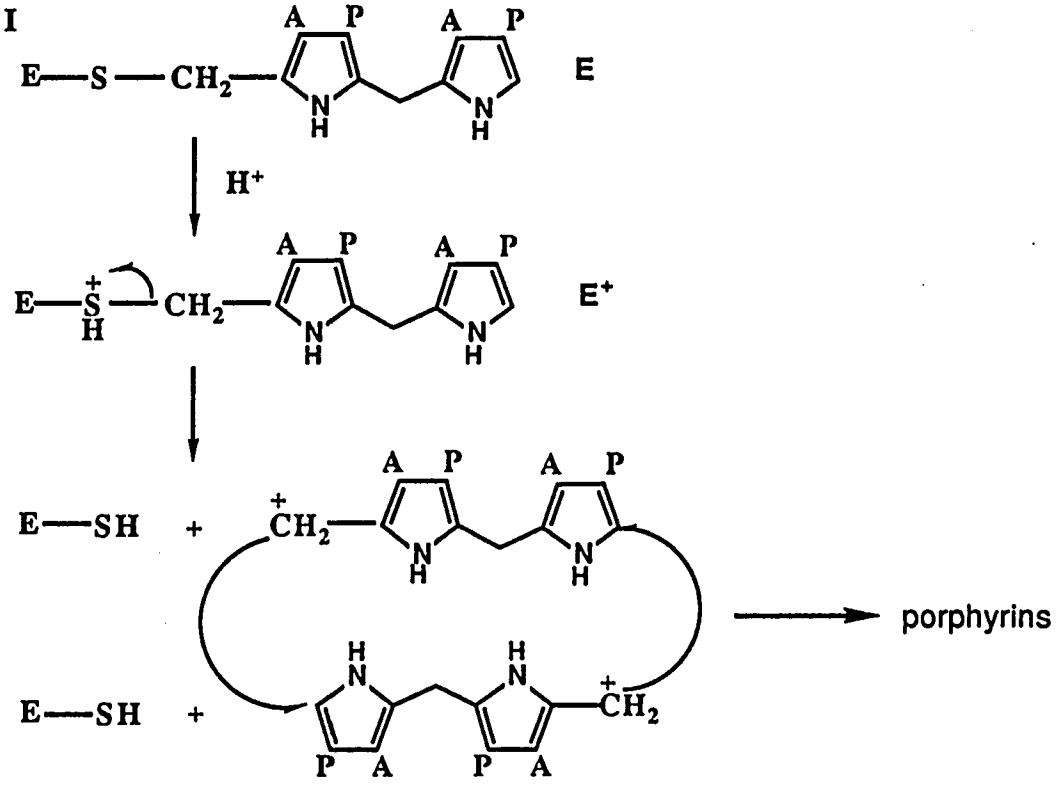
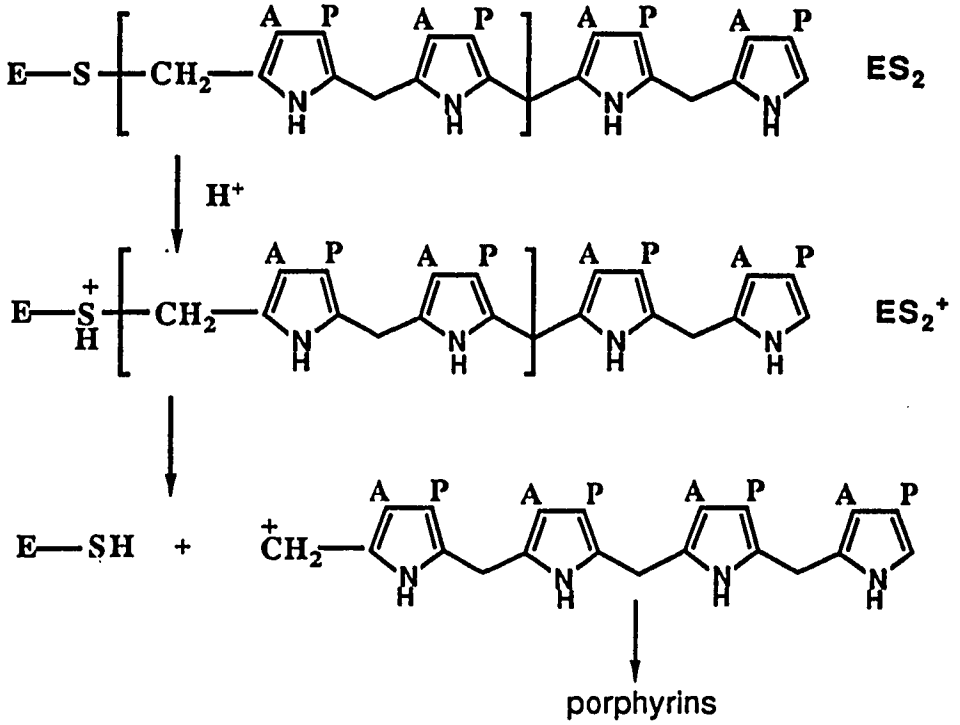


Fig. 8 Schematic representation of the dipyrromethane cofactor covalently bound to PBG D and its role as an anchoring group for the condensation of four PBG molecules in the synthesis of HMB (from Jordan and Warren, 1987).

from the bound cofactor of the enzyme and scheme II illustrates porphyrin formation from the ES_2 complex.



II



^{13}C-labelled PBG (Miller et. al., 1988) and ^{13}C-ALA (Scott et. al., 1988a and 1988b) were shown to be incorporated into a dipyrromethane cofactor on the enzyme. Site-directed mutagenesis of the *hemC* gene of *E. coli* has demonstrated that the dipyrromethane cofactor is covalently bound to cysteine-242 of the apoenzyme, since mutation of this residue to serine resulted in the loss of enzymatic activity (Scott et. al., 1988b). It has been proposed that the cofactor is attached by PBG D itself since the same deamination reaction occurs in the synthesis of the cofactor as in the synthesis of HMB bound to it (Jordan and Warren, 1988).

Purified PBG D from various sources is inhibited by metal (II) cations including Zn^{+2}, Hg^{+2}, Ca^{+2}, and Mg^{+2} as well as p-chloromercuribenzoate and p-hydroxymercuribenzoate(Llambias and Batlle, 1971; Frydman and Feinstein, 1974; Russell and Rockwell, 1980). Avian PBG D was also inhibited

by ATP and ADP (Llambias and Batlle, 1971) and PBG D from *R. spheroides* was inhibited completely for porphyrin formation from PBG by I_2 at 10 μM (Jordan and Shemin, 1973).

1i.4b *Transcriptional Regulation*

In animals PBG D exists in a housekeeping or ubiquitous form and an erythroid form. Most cells require heme for cytochromes in the mitochondria and for liver cytochrome P_{450}. The red blood cell requires large amounts of heme for hemglobin. Upon erythropoietin-induced differentiation of rat bone marrow cells rates of iron uptake and heme biosynthesis increased, and the increase in PBG D levels paralleled the increase in heme biosynthesis, suggesting a regulatory role for erythropoietic PBG D (Beru and Goldwasser, 1985).

The human, mouse, and rat PBG D genes show good sequence homology. The gene is comprised of 15 exons, the last 13 of which are common to the ubiquitous and erythroid forms of the enzyme. Exons 1 and 2 are transcribed from separate promoters and exhibit tissue-specific expression (Beaumont et. al., 1989) resulting in two isoforms of PBG D mRNA. When transcription is initiated from the housekeeping promoter just 5' to exon 1in non-erythroid tissue, the resulting pre-messenger RNA is spliced adjacent to exon 3 in a manner that removes exon 2. Translation of the ubiquitous PBG D mRNA at a translational start signal in exon 1, produces a protein with an additional 17 amino acids at the amino terminal end compared to the erythropoietic isoform. Transcription from the promoter just 5' to exon 2 is activated by several nuclear factors, one of which, NF-E2, binds specifically to this promoter sequence and not to the upstream promoter of exon 1. This binding site may be the one for

Ery-F1, a protein that binds to regulatory regions of alpha and beta globin genes in chicken (Evans and Felsenfeld, 1989).

1i.5 Regulation of the remaining steps of heme biosynthesis

1i.5a *Effect of oxygen on porphyrin and heme accumulation in whole cultures*

Studies on the synthesis of porphyrins during aerobic and anaerobic growth of microorganisms, and on the synthesis of porphyrins and heme from resting cultures and extracts prepared from those cultures (using ALA as a substrate) have highlighted the differences between organisms for specific reactions in later steps of the pathway. *E. coli* is able to grow well in anaerobic cultures containing nitrate or fumarate as a terminal electron acceptor for the anaerobic respiratory chain. *E. coli* synthesizes approximately 6 times the amount of heme in aerobically grown cultures than in anaerobically grown cultures in a rich medium (Ishida and Hino, 1972). *E. coli*, *Pseudomonas denitrificans*, and *Micrococcus denitrificans* can synthesize heme and cytochromes under aerobic or anaerobic growth conditions, but the facultative anaerobes of *Staphylocccus* and *Bacillus* produce very small amounts of heme and accumulate coproporphyrin when grown anaerobically. *Staphylococcus epidermidis* does not synthesize cytochromes under anaerobic growth (Jacobs and Jacobs, 1976). Therefore these organisms may utilize alternate anaerobic respiratory chains.

The inhibition of heme synthesis in *E. coli* cultures grown anaerobically could be overcome by aerobic incubation of the resting culture in the presence of ALA: after incubation of cells for 4 hrs. heme levels were 4-fold higher than for cells without incubation (Ishida and Hino, 1972). This finding suggests the

greater efficiency of oxygen as a substrate for the reactions involving conversion of ALA to heme. In mammals oxygen is thought to be the required co-substrate for the coproporphyrinogen oxidase and protoporphyrinogen oxidase reactions.

1i.5b *Conversion of coproporphyrinogen to protoporphyrinogen*

Coproporphyrinogen oxidase activity has been found in several microorganisms including *E. coli*, and oxygen was thought to be the required electron acceptor for this enzyme since the few oxidizers tested in anaerobic enzyme assays failed to restore activity (Jacobs et. al., 1971). However nitrate and fumarate were not tested as possible acceptors in enzyme assays. This suggested at least the greater efficiency of oxygen (if not a requirement for it) as a substrate in the coproporphyrinogen oxidase reaction. It is not yet clear whether *E. coli* utilizes coproporphyrinogen oxidase or another enzyme for the conversion of coproporphyrinogen to protoporphyrinogen during anaerobic growth. Nitrate and fumarate may serve as effective co-substrates for the coporporphyrinogen oxidase reaction as they have been shown to do for the protoporphyrinogen oxidase reaction in anaerobic enzyme assays (Jacobs and Jacobs, 1976).

In *Staphylococcus epidermidis* and *Bacillus subtilus* no coproporphyrinogen oxidase activity was found, aerobically or anaerobically, using coproporphyrinogen III as a substrate despite the fact that both organisms can synthesize heme. This result, again, suggests that alternate enzymatic reactions (or pathways) for heme synthesis exist. *R. spheroides* is known to utilize two enzymes systems to convert coproporphyrinogen to protoporphyrinogen: one operates aerobically and the other anaerobically (Davies et. al., 1973).

1i.5c *Conversion of protoporphyrinogen to protoporphyrin*

Nitrate and fumarate were shown to be effective co-substrates for the conversion of protoporphyrinogen to protoporphyrin under anaerobic assay conditions in crude *E. coli* extracts obtained from anaerobically grown cultures in rich media, containing glucose, and with either fumarate or nitrate added to the growth medium as terminal electron acceptor (Jacobs and Jacobs, 1976). On the other hand, *S. epidermidis* extracts showed no capacity for the conversion of protoporphyrinogen to protoporphyrin (or heme) under similar conditions using nitrate or fumarate as a co-substrate (Jacobs et. al., 1971; Jacobs and Jacobs, 1976).

In the obligate anaerobe and sulfur reducing *Desulfovibrio gigas*, the conversion of protoporphyrinogen to protoporphyrin in crude extracts was shown to occur in the presence of several electron acceptors, the best of which were sulfite, NAD^+, and $NADP^+$. This protoporphyrinogen oxidizing activity was coupled to a membrane fraction and system comprising fumarate and nitrite reductases (Klem and Barton, 1985). These studies suggest different specificities for cofactors in different microorganisms for the conversion of protoporphyrinogen to protoporphyrin.

2 EXPERIMENTAL

2a MATERIALS

2a.1 Culture media and supplements used in the growth of strains

Ingredients for the media for the growth of *E. coli* were purchased from Difco (Detroit, MI). LB media was 10.0 g tryptone, 5.0 g yeast extract, 5.0 g sodium chloride per liter of dH_2O. TB media was 10.0 g tryptone, 2.5 g NaCl, 1 ml of 1 M $MgSO_4$, per liter of dH_2O. Minimal media was 7.87 g dibasic potassium phosphate, 3.37 g monobasic potassium phosphate, 0.75 g ammonium sulfate, 0.35 g sodium citrate, and 0.0375 g $MgSO_4$ per 800 ml of water.

Casamino Acids, heme (Type III, equine), ampicillin, cycloserine, kanamycin, actinomycin, and p-dimethylaminobenzaldehyde were purchased from Sigma (St. Louis, MO). 5-Aminolevulinic acid, porphobilinogen, and protoporphyrin IX were from Porphyrin Products (Logan, UT).

2a.2 Bacterial Strains, plasmids, and phage

Bacterial strains used in this study are shown in Table 1. SASX41B(*hemA* [glutamyl-tRNA dehydrogenase], met), isolated by Sasarman (1968), was obtained from Barbara Bachman at the *E. coli* Genetic Stock Center, as was C600(F-, *thi-1, thr-1, leu B6, lac Y1, ton A21, sup E44,* λ^-). SASX41B was used as the parent strain for NTG mutagenesis to the resulting hemin-permeable mutant, HU227 (Umanoff et. al., 1988). RP523 (C600, *hemB*, heme-permeable) (Li et. al., 1988) was isolated by Rui Proenca, an undergraduate in the laboratory of Dr. Sharon Cosloy.

Table 1. Strains used in the study of the regulation of *E. coli* heme biosynthesis.

***E. coli* strains**	**Relevant genotype or phenotype[a]**	**Reference[b]**
C600	Hem^+	CGSC
RP522	C600, but *hemB*	Li et. al. 1988
RP523	RP522, but hemin permeable	Li et. al. 1988
HU108	RP523, but PBG permeable	This laboratory
HU1000	RP523 (pSE103)	Li et. al. 1988
HU1029	HU1000 (phHU29), $hemB^+$	Umanoff et. al. 1988
Hfr Cavalli	Hem^+	L. Mindich
SASX41B	*hemA*	CGSC
HU227	SASX41B, but hemin permeable	Umanoff et. al. 1988
HU2000	HU227 (pSE103),	Umanoff et. al. 1988
HU2001	HU2000 (phHU201), $hemA^+$	Umanoff et. al. 1988
TB1	TB1 (pBG101, *hemC*)	Scott et. al., Biochemistry, 1988b
Plasmid or phage		
lambda SE6	E. coli genomic library	ATCC
pSE103	lambda CI857, Kan^r	ATCC
phHU16	$HemB^+$	Umanoff et. al. 1988
phHU201	$HemA^+$	Umanoff et. al. 1988
pJL68	$HemA^+$ (from pTZ19U)	Li et. al. 1989
pJL2	$HemB^+$ (from pTZ18U)	Li et. al. 1988
pBG101	$HemC^+$ (from pUC8)	C.A. Roessner

[a] Other genetic markers are: C600 and RP523, *thr-1* , *leu*B6, *thi*-1, *ton*A21, *supE*44, λ^- , F^-; Hfr Cavalli, *metB ,gly*; SASX41B, Hfr Cavalli, PO2A.

[b] CSGC, E. coli Genetic Stock Center, Yale University, New Haven, CT; ATCC, American Type Culture Collection, Rockville, MD; L. Mindich, Public Health Research Institute, New York, NY; C.A. Roessner, Texas A & M University, College Station, TX.

Plasmid pSE103 (λ CI857, *kan*^{r}) (Elledge and Walker,1985), carrying the λ temperature-sensitive repressor protein, was purchased from the American Type Culture Collection and was used to transform HU227 and RP523, resulting in HU2000 and HU1000, respectively (Umanoff et. al., 1988). An *E. coli* genomic library in λSE_6 (Elledge and Walker,1985) was purchased from the American Type Culture Collection and used to transduce HU1000 and HU2000, resulting in the heme prototrophs HU1029 and HU2001, respectively--strains complemented for their heme-related enzyme mutations (Umanoff et. al., 1988) (see Table 1). Hybrid plasmids pJL68 (Li et. al., 1989c) and pJL2 (Li et. al., 1988), obtained from C.A. Roessner were added as templates for the *in vitro* reactions.

2a.3 Enzymes, biochemicals, and special reagents

In vitro transcription and translation of cloned heme genes was carried out using a kit purchased from Amersham (Arlington Heights, IL) containing an *E. coli* S-30 extract, nucleotides, tRNA, amino acids, buffer, and an energy-generating system. Newly synthesized proteins were labelled with ^{35}S- met, purchased from NEN (Boston, MA). Econofluor scintillation fluid and Protosol base for gel digestion were also purchased from NEN (Boston, MA).

Molecular weight standard proteins, Trizma base, p-hydroxymercuribenzoate, and p-dimethylaminobenzaldehyde were from Sigma. DEAE (Cellex-D) and ammonium persulfate were from Bio-Rad (Richmond, CA). Sephadex G-100 was from Pharmacia (Piscataway, NJ). Mercaptoethanol was from J. T. Baker (Phillipsburg, NJ). Acrylamide and bisacrylamide were used from stock solutions purchased from National Diagnostics (Manville, NJ) under the trade names of Acrylagel and Bisacrylagel.

Acetylacetone and N-methyl-N-nitro-N'-nitrosoguanidine (NTG) were from Aldrich (Milwaukee, WI). All other chemicals were from standard suppliers and were reagent grade or better. X-omat XAR5 film for autoradiography was from Kodak.

2a.4 Instrumentation

2a.4a *Klett meter, shaker baths, water baths, block heaters*

Cell growth in incubating cultures was monitored on a Klett-Summerson colorimeter. Incubation of cultures in Erlenmeyer or side-arm flasks was carried out at 37 °C, unless otherwise indicated, in a New Brunswick Scientific Gyrotory Water Bath Shaker (Model G-16) for smaller volumes or in Eberbach floor model reciprocal or rotary shakers for larger volumes.

Incubation of enzyme assays in Eppendorf tubes was in either the New Brunswick Gyrotory shaker bath, but without shaking, a Lab-Line Imperial III water bath (Lab-line Instruments, Inc., Melrose Park, IL), or in a Blue M Magni Whirl constant temperature bath. DNA restriction digestions were in either a Thermolyne Dri-bath (Sybron Corp.) or a Multi-Blok Heater (Lab-line Instruments, Inc.).

2a.4b *Centrifuges and centrifuge tubes*

Whole cultures were centrifuged either in 500 ml centrifuge bottles in the Sorvall RC-5C Superspeed refrigerated centrifuge, at 4 °C unless otherwise indicated, or in 250 ml centrifuge bottles in the Sorvall RC-5B Superspeed refrigerated centrifuge, at 4 °C, unless otherwise indicated. Centrifuge bottles were carried in Sorvall GS3 (500 ml bottles) and GSA (250 ml bottles) rotors.

Smaller volumes cultures were centrifuged in 50 ml and 15 ml centrifuge tubes in the RC-5B or RC-5C in a Sorvall SS-34 rotor, at indicated temperatures.

Five hundred ml polypropylene bottles were from Sarstedt (W. Germany). Two hundred fifty ml bottles were from Nalgene or Sorvall and were either polypropylene or polyethylene. Fifty ml and 15 ml tubes were from Sorvall (Du Pont, Newtown, CT) or Nalgene (Nalge Co., Rochester, NY) and were either polyethylene or polypropylene. Sterile 50 ml and 15 ml polypropylene Corning centrifuge tubes were also used, when required, in the RC-5B or RC-5C in the SS-34 rotor. Overnight cultures and some small volume samples (approximately 10 ml in volume) in glass test tubes or plastic tubes were sometimes centrifuged in a Sorvall General Laboratory Centrifuge (GLC-2) at room temperature, or in a Sorvall RT6000 refrigerated table-top centrifuge.

Ultracentrifugation of samples used for the preparation of template DNA for *in vitro* transcription-translation reactions were carried out in a Sorvall OTD 75B ultracentrifuge.

Small volume DNA samples and enzyme assay samples on the order of 1 ml in volume were centrifuged in polypropylene Eppendorf tubes in either a Beckman Microfuge 11, Beckman Microfuge B, or Brinkmann Eppendorf Centrifuge 5415.

2a.4c *Sonicators and probes*

Cell samples for the preparation of extracts or for ALA quantitation were sonicated on either a Heat Systems W-375 or Heat Systems W-385 sonicator equipped with a 0.5-inch disrupter and microtip (for small volume samples), 0.5-inch disrupter with 0.5-inch tip (for large volume samples), or 2.5-inch cup horn (for variable sample volumes) as indicated.

2a.4d *Spectrophotometers*

Spectra resulting from enzyme activity, ALA accumulation, or determination of protein concentration were recorded on either a Cary 15 (dual beam) or a Perkin-Elmer Lambda 3B (dual beam) spectrophotometer. Sample DNA concentration was determined on an LKB Ultrospec II uv/visible spectrophotometer. Protein concentration was determined on the Perkin-Elmer, or on a Spektralphotometer PM6 spectrophotometer at a fixed wavelength.

2a.4e *Column chromatography for partial purification of PBGD*

DEAE-cellulose chromatography utilized a Buchler peristaltic pump for the elution of protein adsorbed to the column. Fractions from the DEAE-cellulose column as well as from the Sephadex G-100 column were collected and monitored using LKB instruments: Ultrorac Fraction Collector (Instrument Group 7,000), 8300 Uvicord II detector, Biocal Recorder, and Uvicord II Ultraviolet Absorbtiometer Control Unit (Type 8301A).

2a.4f *DNA preparation, gel electrophoresis, gel drying, scintillation counting*

DNA samples were dried either under N_2, or in a SpeedVac Concentrator connected to a SpeedVac Condensation Trap and Savant VP 100 two-stage high vacuum pump (Savant Instruments, Farmingdale, NY). Electrophoresis was carried out using a Buchler 3-1500 Constant Power Supply (Buchler Instruments, Saddle Brook, NJ) and Bio-Rad Protean II electrophoresis unit. Polyacrylamide gels were dried at 60 °C on a Hoefer Drygel Sr. slab gel dryer (Model SE1160) (Hoefer Scientific Instruments, San

Francisco, CA). Scintillation counting was done on an LKB Wallach (Finland) 1219 Rackbeta Spectral liquid scintillation counter.

2b METHODS

2b.1 Preparation of Reagents

Hemin stock solution (4 mg/ml) was prepared by mixing 0.1 g heme, 2.5 ml Tween 80, 22.5 ml dH_2O, and 4 drops 10 N NaOH until a clear, dark green solution was obtained. This was stored at 4 °C for several months.

Modified Ehrlich's stock solution was prepared by dissolving 0.32 g $HgCl_2$ in 81.8 ml glacial acetic acid and 20 ml 70% perchloric acid while stirring at 40 °C. This solution was stored at 4 °C for one month. Modified Ehrlich's reagent was prepared fresh by mixing 50 ml of the stock solution with 1.0 g p-dimethylaminobenzaldehyde.

2b.2 Mutagenesis

2b.2a *Isolation and selection of hemin-permeable mutants*

NTG mutagenesis (Davis, 1948) was carried out to select for hemin-permeability mutants, since wild type *E. coli* strains are not permeable to hemin. The resulting mutation is thought to affect cell membrane proteins, permitting passage of large molecules through the membrane.

Ten ml LB media containing ALA (26 µg/ml final conc.) in a 125 ml sidearm flask was inoculated with 0.12 ml of an SASX41B overnight culture, and grown to a cell density of 4.1×10^8 cells per ml (70 Klett units) at 37° C in a rotary shaker bath. The culture was centrifuged in the GLC-2 at maximum

speed and room temp. for 15 min. The cell pellet was washed twice with and resuspended in an equal volume of 0.1M citrate buffer (pH 5.5) and centrifuged in the GLC at maximum speed and room temp. for 15 min. The final pellet was resuspended in 9.0 ml citrate buffer to which 1 ml NTG solution (1 mg/ml) was added. The culture was incubated for 20 min. at 37° C in a rotary shaker bath, then centrifuged in the GLC at maximum speed, at room temperature, for 15 min. The pellet was washed with an equal volume of 0.1M potassium phosphate buffer (pH 7.0) and resuspended in 5 ml of the same buffer.

Aliquots of 0.1 ml were spread onto nine minimal plates supplemented with hemin (15 µg/ml final conc.) and met (50 µg/ml final conc.). The plates were incubated for two days at room temp. (over weekend) and one day in the incubator at 37° C, after which time several good-sized colonies were selected. The hemin-permeability mutation was verified by streaking these colonies in corresponding sectors on a set of minimal plates, where one was supplemented with hemin and met while the other contained no hemin, only met. The parent *hemA* strain was used as a control. Plates were incubated at 37° C overnight, and colonies showing good growth on the hemin-containing plate and no growth without hemin were then checked for requirements on appropriately supplemented minimal plates. A similar procedure was used to obtain a hemin-permeable, *hemB* mutant (Li et. al., 1988).

2b.2b *Isolation and selection of a PBG-permeable mutant*

Since it could not grow in a culture containing PBG at 10 µg/ml, RP523 was mutagenized to obtain a PBG-permeable mutant, using the basic procedure above. RP523 was grown in a 20 ml Penn Assay containing 2 µg/ml hemin to a Klett of 70 at 37 °C in a rotary shaker bath. After taking the final Klett

reading, 15 ml of culture remained. The washed pellet from this culture was NTG-mutagenized as described above. From the final cell resuspension in 0.1 M potassium phosphate buffer (pH 7.0), 200 µl aliquots were spread onto LB plates containing PBG at 10 µg/ml, and incubated at 37 °C for approximately 20 hrs. after which several good-sized colonies appeared. These were streaked for single-colonies, checked for requirements, and one was chosen for further study.

2b.2c *Isolation and selection of secondary heme pathway-related mutations*

A second round of NTG mutagenesis was used to isolate additional mutations in genes related to heme biosynthesis. These mutants were isolated by their ability to grown on hemin-containing media with a concomitant inability to grow on ALA-containing media. This phenotype was presumbably due to the mutants' inablitiy to convert ALA to heme as a consequence of the mutagenesis.

A 10 ml LB culture containing 0.37% (w/v) glucose, 75 µg/ml met, and 8.7 µg/ml ALA was inoculated with 0.4 ml of an overnight of HU227 and was grown to a density of 6.6 x 10^8 cells/ml (113 Klett units, about 4 hrs.) at 37° C in a rotary shaker bath. The culture was centrifuged in the GLC at full speed, at room temperature, for 15 min. The pellet was washed twice with equal volumes of 0.1M citrate buffer (pH 5.5), and the final pellet was resuspended in 9.0 ml of the same buffer. One ml NTG solution (I mg/ml) was added and the culture was incubated for 20 min. at 37° C in a rotary shaker bath. The mutagenized cells were centrifuged in the GLC at full speed, at room temperature, for 15 min., then washed with an equal volume of 0.1M potassium phosphate buffer (pH 7.0), and resuspended in 5 ml of the same buffer. Aliquots of 0.5 ml were used to

inoculate ten test tubes, each containing 4.5 ml SSA, 0.2% (w/v) glucose, 8.0 µg/ml thia, 100.0 µg/ml met, 22.0 µg/ml hemin, and all tubes were incubated at 37° C overnight in a rotary shaker bath.

An ampicillin enrichment technique was utilized to increase the probability of seleting heme-deficient mutants. The overnights were centrifuged in the GLC, as before; pellets were washed twice in SSA and each resuspended in 5 ml SSA. A 1 ml aliquot from each tube was used to inoculate individual tubes containing 10 ml SSA, 0.075% (w/v) glucose, 0.6 mg/ml sodium succinate, 8.0 µg/ml thia, 8.7 µg/ml ALA, and 100.0 µg/ml met. All tubes were incubated at 37° C in a rotary shaker bath until a reading of 20 Klett units was obtained (about 7-8 hrs.). 1 ml ampicillin-cycloserine solution was then added to yield final concentrations of 36.0 µg/ml ampicillin and 90.0 µg/ml cycloserine per tube. When the cell turbidity had dropped 10 Klett units (about 1 hr.), all cultures were transferred to 30 ml polyethylene centrifuge tubes and centrifuged in the SS-34 rotor at 10,000 rpm, 4° C, for 10 min. All pellets were washed twice in 5 ml SSA and resuspended in 5 ml SSA. Aliquots of 0.3 ml from each culture were used to inoculate corresponding test tubes containing 4.0 ml SSA, 0.14% glucose, 5.7 µg/ml thia, 71.4 µg/ml met and 16.1 µg/ml hemin. The tubes were incubated at 37° C overnight in a rotary shaker bath.

The following day another round of ampicillin-cycloserine selection was completed under the same condition as the first, except that the final pellet resuspension was in 10 ml SSA. It was necessary to make 10^{-2} and 10^{-3} dilutions in SSA for each tube before plating. Five 0.1 ml aliquots from each culture tube (two at 10^{-2} and three at 10^{-3}) were plated onto each of five minimal plates supplemented with hemin (15 µg/ml) and met (50 µg/ml) [50 plates for 10 tubes] to be used as masters for replicaplating. The plates were

incubated at room temp. for 2.5 days. Each master plate was replica-plated onto a set of two plates, one containing hemin and met, and the other ALA and met. Replicaplates were incubated at 37° C overnight. Colonies which showed growth on the hemin-containing plate and no growth on the ALA-containing plate were selected as potential heme-deficient mutants.

2b.3 Transformation and selection of transformants

The transformation procedure used was that of R.W. Davis et. al. (1980). Transformants for pSE103 were selected on kanamycin (50 µg/ml)-containing media and checked for resistance to λ infection. Transformants for pJL68 (*hemA* $^{+}$) and pJL2 (*hemB* $^{+}$) were selected for ampicillin resistance and growth on media lacking hemin. Transformants for pBG101(*hemC* $^{+}$) were selected for their increased PBG D activity compared to C600.

2b.4 Transduction with λSE$_6$

The transduction procedure used was a modification on that of Shaw and Berg (1979). An overnight culture of the pSE103-transformed mutant, to be complemented for its potential heme deficiency, was prepared in TB medium containing 0.2% maltose and hemin at 4 µg/ml. The next day the cells were centrifuged and washed in an equal volume of 0.1 x MC buffer (0.1 M $MgSO_4$, 0.005 M $CaCl_2$) to remove residual hemin. Cells were resuspended in the same volume of buffer, and shaken at 30 °C for 1 hr. Two hundred µl of λ SE_6 dilutions in 0.1 x MC at 10^{-1}, 10^{-2}, 10^{-4}, and 10^{-6} were mixed by hand in sterile Eppendorf tubes with 200 µl of cells and incubated for 20 min. at 30 °C without shaking. Two hundred µl from each transduction tube were spread onto LB plates and incubated at 30 °C along with phage control and cell control plates.

2b.5 Growth of cells and preparation of crude extracts

2b.5a *For the effects of supplements in the growth medium on PBGD activity*

For inoculation, overnight cultures were grown in minimal medium supplemented with 0.2% glucose, 0.2% Casamino acids, 50 µg/ml each of met and cys, 1.0 µg/ml thia, and 2µg/ml hemin. These cells were washed, suspended in the same medium without hemin, diluted 1:40 in 25 ml of the same medium with the supplements shown in results (see effect of supplements on PBG D activity and effect of hemin on PBG D activity*in vivo*), and grown to a density of 6.0×10^8 to 7.0×10^8 cells per ml, or for a maximum of 6.0 hrs. for those cultures that could not attain that density. The cells were centrifuged in an SS-34 rotor in 50 ml tubes at 6,000 rpm, at 4 °C, for 6 min. The pellet was washed twice in 50 ml of 0.1 M Tris, pH 7.6 and suspended in 2.0 ml of the same buffer.

Sonication was carried out on ice on a Heat Systems Ultrasonics W-385 sonicator using a microtip and ten 10-second pulses (with a 10-sec. pause between pulses) at an output setting of 4. Sonicates were transferred to 15 ml tubes and centrifuged in an SS-34 rotor at 15,000 rpm, at 4 °C, for 15 min. The supernatants were transferred to 5 ml polycarbonate Falcon tubes and mixed by inversion, then aliquotted to 1.5 ml Eppendorfs and frozen at either -70.0 °C or -20.0 °C.

2b.5b *For the effect of PBG concentration on the growth of HU108*

For inoculation, a 20 ml LB culture containing 4 µg/ml hemin was grown overnight and the cells centrifuged in 50 ml centrifuge tubes at 6,000 rpm, at room temperature, for 6 min. The cells were washed in LB, and resuspended in

20 ml of LB. These cells were used to inoculate 20 ml LB cultures, in 125 ml side-arm flasks, containing either 10, 25, 50, 75, or 100 µg/ml PBG. The cultures were grown at 37 °C in a rotary shaker bath.

2b.5c *For quantitation of ALA in extracts and/or medium*

HU1029 or HU108 were grown in 50 ml LB cultures at the appropriate hemin concentration, inoculated 1:40 from corresponding overnights, until early stationary phase. 50 ml complete minimal media cultures contained Casamino acids at 0.2 %, thia at 1 µg/ml, and glucose at 0.2%, final concentrations. The HU1029 cultures were centrufuged at 6,000 rpm, at 4 °C , for 6 min. in 250 ml bottles. HU108 cell suspensions were centrifuged at 7,000 rpm, at 4 °C, for 7 min. on the RC-5C. The medium (supernatant) was decanted and stored at -20 °C or -70°C. The pellet was transferred to 50 ml centrifuge tubes and washed in 0.1 M Tris, pH 7.6. Pellets were frozen at -70 °C.

Pellets were resuspended in 2.0 ml 0.1 M Tris, pH 7.6 and transferred to Falcon tubes. HU1029 cell suspensions were sonicated for approximately 4 min. on a Heat Systems model W-375 ultrasonicator with cup horn at an output setting of 6, transferred to 15 ml tubes, and centrifuged at10,000 rpm, at 4 °C , for 20 min. in an SS-34 rotor on the RC-5B. HU108 sonicates were centrifuged on a Sorvall RT6000 refrigerated table-top centrifuge at highest speed, at 4 °C, for 20 min. The supernatant was the crude extract and was aliquotted and frozen at -70 °C.

2b.5d *For partial purification of PBG D*

Five 4 L Erlenmeyer flasks, each containing 2 L of LB and Penicillin G at 50 µg/ml, were inoculated 1:200 with an overnight of pBG101 in the same

medium. Cells were grown for 49 hrs. at 37 °C with continuous rotary shaking. The cultures were centrifuged at 4 °C at either 6,000 rpm for 6 min. (GSA rotor in the RC-5B) or 8,000 rpm for 8 min. (GS3 rotor in the RC-5C). Cell were washed in 0.1 M Tris, pH 8.2 and the pellets were frozen at -40 °C.

2b.5e *For the effect of oxygen on PBG D and ALA D activities*

A 200 ml LB aerobic culture and a 550 ml LB anaerobic culture were inoculated 1:100 from a C600 overnight in LB. The anaerobic culture was placed in a Becton-Dickinson anaerobic chamber with an H_2 + CO_2 generator and an anaerobic indicator strip. After 30 min., both cultures were shaken on an Eberbach reciprocal platform shaker at 37 °C for 18 hrs. post-inoculation.

The flasks were removed from the shaker and plunged into an ice-water bath to discourage any aerobic growth of the anaerobic culture once it was removed from its anaerobic environment, before processing the cells. Cultures were spun down in a GSA rotor at 7,000 rpm, at 4 °C, for 8 min. Half of each culture was washed twice in either 50 ml of ALA D buffer (0.05 M Tris containing 3.3 mM $MgCl_2$, 10 mM $ZnCl_2$, and 0.01 M ß-mercaptoethanol) for the ALA D assay or 50 ml of PBG D buffer (0.1 M Tris, pH 8.2) for the PBGD assay. Each aerobic pellet, corresponding to half the original culture, was resuspended in 4.0 ml of either ALA D or PBG D buffer before sonication. Each anaerobic pellet was also resuspended in 4.0 ml of the appropriate buffer. Sonication was carried out as described above for PBG D activity.

2b.5f *For the effect of nalidixic acid on expression of PBG D in C600 and HU1029*

C600 and HU1029 were inoculated 1:100 into 50 ml LB medium from corresponding LB overnights and grown to mid-log phase (Klett 60) in a rotary

shaker bath at 37 °C. At that time 10 ml aliquots of the large culture were transferred to 125 ml Erlenmeyer flasks and naladixic acid was added from a stock solution to yield final concentrations of 0, 20, 40, 60, and 80 µg/ml. The cultures were grown to stationary phase, at which time the cells were transferred to 15 ml tubes and centrifuged at 5,000 rpm, at 4 °C, for 5 min. Cells were washed in 10 ml 0.1 M Tris, pH 8.2 and the pellets were frozen at -70 °C.

The pellets were resuspended in 1.0 ml 0.1 M Tris, pH 8.2 and transferred to 5.0 ml polycarbonate Falcon tubes. Sonication was carried out on a Heat Systems Sonicator Model W-375 fitted with a 2.5-inch cup horn at 0 °C for 4 min. at an output setting of 6.5. Sonicates were transferred to 15 ml tubes and centrifuged in an SS-34 rotor at 15,000 rpm, at 2 °C, for 20 min. The supernatants were the crude extracts which were aliquotted and frozen at -70 °C.

2b.5g *For isolation of hemA, hemB, and hemC -containing-plasmids pJL68, pJL2, and pBG101, respectively*

A 250 ml LB culture containing ampicillin at 50 µg/ml was inoculated 1:100 from a corresponding 5 ml LB overnight culture. The cultures were grown for 16 hrs. at 37 °C with either rotary or reciprocal shaking. Cells were centrifuged either in 500 ml bottles in a GS3 rotor at 8,000 rpm, at 4 °C, for 8 min. or in 250 ml bottles in a GSA rotor at 8,000 rpm, at 4 °C, for 6 min.

2b.6 Partial purification of PBGD

2b.6a *Extract preparation*

TB1(pBG101) pellets, frozen at -40 °C for 7 days, were thawed and each resuspended in 50 ml 0.1 M Tris, pH 8.2. Each 50 ml aliquot was sonicated on a Heat Systems W- 385 sonicator fitted with a 0.5-inch disruptor horn and 0.5-inch tip for sixteen 10-sec pulses (with a 10-sec pause after each pulse) at an output setting of 4.2. The four sonicates were pooled into a 250 ml Erlenmeyer flask, on ice. The sonicate was then aliquotted into nine 50 ml tubes and clarified by centrifugation in an SS-34 rotor at 15,000 rpm, at 4 °C, for 20 min.

The supernatants were evenly aliquotted among 5 125 ml Erlenmeyer flasks (for more efficient heat transfer) and heat-treated at 62 °C, with shaking, for 12 min. in a rotary shaker bath. The clear supernatants turned milky white and a heavy protein precipitate appeared during the heating period. The heat-treated supernatants (HTSU) were aliquotted among 8 50 ml tubes and centrifuged at 12,000 rpm, 4 °C, for 20 min. The resulting clear, yellowish supernatant (approximately 200 ml) was transferred into two 250 ml centrifuge bottles and frozen at -40 °C.

2b.6b *Column chromatography*

All column chromatography was carried out at 4 °C, unless otherwise indicated. A DEAE glass column (Kontes), 2.0 cm indiameter x 32.0 cm in bed height, with a calculated bed volume of 402 ml, was prepared. It was connected to a Buchler peristaltic pump and equilibrated overnight with over two bed volumes of 0.05 M Tris, pH 8.2 at a flow rate of 14.8 ml/cm^2 hr. One hundred sixty ml of HTSU (15.2 mg/ml) was loaded onto the DEAE column and the flow rate was then set at 16.8 ml/cm^2 hr. The column was washed with 0.05 M Tris, pH 8.2 until the recorder indicated that eluted protein had returned to baseline levels (over 6 hrs.), and then with 25 mM KCl in 0.05 M Tris, pH 8.2 until

baseline protein levels returned (over 4 hrs.). PBG D was eluted from the column using a KCl gradient from 25 mM to 919 mM in 0.05 M Tris, pH 8.2. One hundred twenty (twelve racks) 8.6 ml fractions were collected in all and these fractions were assayed for enzyme activity.

Fractions 56-95 were pooled and concentrated by ultrafiltration, using an Amicon ultrafiltration unit and PM10 membrane, to 40 ml under 40 psi N_2 and frozen at -20 °C. Fractions 96-115 were pooled and concentrated, separately, to 23 ml before freezing at -20 °C.

For further purification of PBGD a Sephadex G-100 glass column (Pharmacia), 2.60 cm in diameter x 86.0 cm (final bed height), with a final bed volume of 457 ml, was prepared. The column had been equilibrated with approximately three column volumes of 0.05 M Tris, pH 8.2 and the flow rate adjusted to 7.5 ml/cm^2 hr. Ten ml (180 mg protein) of the concentrated fractions 56 to 95 from the DEAE column were applied to the G-100 column and 5.5 ml fractions were collected overnight. Active fractions were determined and fractions 29 to 44, totalling approximately 100 ml, were concentrated down to 6 ml, aliquotted, and frozen at -20 °C. A higher molecular weight form of PBG D was found in fractions 20-28. These were pooled, totalling approximately 50 ml, and concentrated down to 8 ml before aliquotting and freezing.

2b.7 Protein Determination

Protein concentration was determined by the Bio-Rad assay. A standard set of protein samples was obtained by mixing increasing amounts of gamma-globulin standard protein, in a total of 0.1 ml of protein solution plus dH_2O, with 5.0 ml of a 1:5 dilution of the Bio-Rad reagent. The $O.D._{595}$ nm was recorded for the reagent dye-protein complex, and a standard curve for O.D. vs. protein

concentration was generated by linear regression. Sample extracts containing known volumes in 0.1 ml of extract plus dH_2O were treated in the same manner and protein concentration for these extracts was determined by interpolation to the standard curve.

2b.8 ALA quantitation in medium and extracts of HU1029 and HU108

HU1029 and HU108: Eight hundred µl of medium was mixed with 200 µl dH20 and 100 µl 50% TCA in a 15 ml glass screw-top test tube. Five hundred µl of freshly made 10% acetylacetone (in 1 M NaAc, pH 4.7) was added followed by heating at 80 °C for 15 min., during which time ALA-pyrrole is formed from the Knorr condensation of ALA with acetylacetone. Reference tubes were prepared identically, except 500 µl dH_2O was added instead of 10% acetylacetone. Tubes were cooled to room temperature and 300 µl Na_2HPO_4 was added to bring the pH to 7.1. Then 2 ml CH_2Cl_2 was added and extraction of contaminating pyrroles was carried out by vortexing each tube vigorously for 10 sec. All tubes were centrifuged on a clinical table-top centrifuge at two-thirds speed for 1 min. Eight hundred µl of the aqueous (top) phase was then transferred to new glass screw-top tubes and reacted with 800 µl modified Ehrlich's reagent.

Ehrlich's reagent forms a complex with ALA pyrrole having a characteristic pink color and an absorbance maximum at 552 nm. The spectra for each tube was recorded between 650 nm and 450 nm on the Perkin-Elmer spectrophotometer approximately 10 min. after adding Ehrlich's reagent. ALA in the medium or supernatant was converted to nanomoles per ml by using a molar extinction coefficient of 6.5×10^4 L $M^{-1}cm^{-1}$ at 552 nm. (Lien and Beattie, 1982).

For ALA determination in extracts of HU1029, 600 µl extract was mixed with 400 µl dH_2O and 100ml 50% TCA in a 1.5 ml Eppendorf tube. After mixing by hand, all tubes were centrifuged on the Microfuge B at the highest speed for 5 min. The supernatant was transferred to a glass screw-top test tube and reacted with 500 µl 10% acetylacetone or 500 µl dH_2O (for reference tubes) as above. All tubes were processed as above.

For ALA determination in extracts of HU108, 400 µl extract was mixed with 600 µl dH_2O and 100 µl 50% TCA. All samples were processed as for HU1029 extracts except that 600 µl of the final aqueous phase was reacted with an equal volume of modified Ehrlich's reagent.

Total ALA in the medium or supernatant was obtained by multiplying the total volume by the concentration of ALA in nanomoles per ml. Total ALA in the extract alone for HU108, or in the extract plus medium for HU1029, was normalized to protein concentration (see Tables 7a and 7b).

2b.9 Enzyme Assays

2b.9a *PBG D assay for effects of supplements*

One hundred fifty µl of crude extract was incubated with 44 µl PBG solution (l mg/ml in 0.1 M Tris, pH 8.2) at 37 °C for 2.0 hrs., unless otherwise indicated. The assay was terminated with the addition of 1.0 ml 1.0 M HCl containing I_2 at 0.1 mg/ml. All assay tubes were spun at high speed on a Beckman Microfuge B for 5.0 minutes to precipitate denatured protein. The supernatant was transferred to a semi-micro cuvette and mixed with 50 µl 2.0% $Na_2S_2O_3$ to reduce excess I_2. The spectrum was recorded between 450 nm

and 350 nm on a Perkin-Elmer Lambda 3B spectrophotemeter. Activity is reported as $O.D._{405}$ per mg protein.

2b.9b *PBG D assay for the effects of oxygen and glucose*

One mg protein (approximately 50 µl of crude extract), PBG at 1.48×10^{-3} M (50 µl), and PBG D buffer to bring the total volume up to 150 µl were added to assay tubes and incubated at 37 °C for 2.5 hrs. The reaction was terminated by addition of 1.0 ml of 1.0 M HCl containing 0.1 mg/ml I_2. The reaction tubes were centrifuged in the Beckman Microfuge at high speed for 5 min. The supernatant was processed, spectra recorded, and activity determined as described above for the PBG D assay for effects of supplements in growth medium.

2b.9c *PBG D assay for the effects of nalidixic acid*

The total assay volume was 100 µl, 10 µl of which was 1 mg/ml PBG added to initiate the reaction, and 90 µl of which was extract plus buffer. The amount of each extract added to the reaction was inversely proportional to the $O.D._{595}$ obtained from the Bio-Rad assay, with 90 µl of extract used for the least concentrated extract. Volumes of extract corresponded to between 0.164 mg and 0.193 mg protein. The incubation period was for 2 hrs. 15 min. at 37 °C. Reactions were terminated with 1.0 ml 1.0 M HCl containing I_2 at 0.1 mg/ml and spectra were recorded and activity determined in the usual manner as described for effect of supplements.

2b.9d *PBG D assay for detection of activity in potential heme-deficient mutants*

The presence of PBG D in cell-free crude extracts of potential heme-deficient mutants was assayed using a modified procedure of Anderson and

Desnick (1982). Ten ml LB in a 125 ml sidearm flask, supplemented with heme (23.2 µg/ml), ALA (0.25 µg/ml) and met (50 µg/ml), was inoculated with a 1:50 dilution of an overnight of a potential heme-deficient mutant. The culture was incubated in a rotary shaker bath at 37° C until a density of 7.1 x 10^8 cells/ml was reached (120 Klett units). The cells were centrifuged in an SS-34 rotor at 6,000 rpm, 4° C, for 10 min. and washed twice in 0.1M Tris-HCl, pH 8.2. The pellets were frozen overnight.

The next day the pellets were thawed and resuspended in 0.5 ml of 0.1M Tris-HCl (pH 8.2). The suspensions were sonicated in Falcon tubes using a Heat Systems Sonicator W-375 with 2.5-inch cup horn, at a power output of 35% for approximately 12 min., until the suspension cleared.

Protein concentrations in each extract were determined by recording the absorbance at 280 nm of a 3.0 µl sample in 1.0 ml 0.1M Tris-HCl, using a Spektralphotometer PM-6 spectrophotometer. Aliquots of equivalent protein concentration were then determined and added to 1.5 ml Eppendorf tubes followed by the appropriate volume of 0.1M Tris-HCl (pH 8.2) needed to bring the reaction volume to 50 µl.

The reaction was initiated by addition of PBG to a final concentration of 100 µg/ml, and incubated at 37° C for 2 hrs. The reaction was terminated by the addition of 0.8 ml 1.0 M HCl. Tubes were centrifuged at high speed at room temp. for 5 min., and the spectrum of the supernatants from 600 nm to 350 nm were recorded on the Perkin-Elmer spectrophotometer .

2b.9e *ALA D assay in crude extracts of selected strains*

One hundred µl of crude extract was incubated with 50 µl ALA (8.8 mM in 0.05 M Tris, pH 7.6 containing 3.3 mM $MgCl_2$, 10 mM $ZnCl_2$, and 0.01 M ß-

mercaptoethanol), at a final substrate concentration of 2.93 x 10^{-3} M, at 37 °C for 1.5 hrs., unless otherwise indicated. The assay was terminated with 0.5 ml 1.0 M HCl. Assay tubes were centrifuged at high speed in a Beckman Microfuge B for 5 minutes to precipitate protein. The supernatant (650 µl) was transferred to a new Eppendorf tube and 650 µl of modified Ehrlich's reagent was added. Spectra were recorded between 650 nm and 450 nm on the Perkin-Elmer. Activity is reported as O.D. $_{553}$ per miligram protein.

2b.9f *ALA D assay for the effect of oxygen*

One hundred µl of crude extract (containing 2.0 mg protein), 72 µl ALA (1.28 x 10^{-3} M) and ALA D buffer to bring the volume up to 220 µl were added to assay tubes and incubated at 37 °C for 2.25 hrs. The reaction was terminated by addition of 1.0 ml of 1.0 M HCl. The reaction tubes were then centrifuged in the Microfuge B at high speed for 5 min. The entire supernatant was transferred to corresponding glass screw-top test tubes before addition of 1.2 ml modified Ehrlich's reagent. Spectra were recorded and activity determined as described above for the ALA D assay.

2b.9g *ALA D assay for detection of activity in potential heme-deficient mutants*

The ALA D assay (a coupled assay depending on the presence of functional PBG D) was carried out under the same conditions as those described for the PBG D assay for detection of activity in potential heme-deficient mutants, except that the reaction was initiated with the addition of ALA to a final concentration of 230 µg/ml.

2b.9h *PBG D assay for in vitro hemin effect*

Twelve and one-half ml of a 4.0 mg/ml hemin stock solution was prepared by adding 0.52 mg heme, 1.25 ml Tween 80, 11.25 ml sterile H_20, and 3 drops of 10.0 N NaOH from a Pasteur pipette, with enough stirring to yield a clear, dark green solution. A 1:64.5 dilution of this hemin stock solution in sterile H_20 yielded a 9.50×10^{-5} M hemin stock solution used for the experiment. A control Tween-80-NaOH stock solution was prepared in an identical manner, except without heme; and a 1:64.5 dilution of the Tween-80 stock solution was used as the control Tween-80 solution for the experiment. It was added to each reaction tube in the proportion required to keep the Tween-80 and NaOH concentrations equal in each tube, regardless of the hemin concentration in the tube.

To each reaction tube in order was added: 72.8 µl of reaction buffer (0.1 M Tris, pH 8.2), 3 µl of a 1:30 dilution of partially purified PBG D in the reaction buffer (0.028 mg protein), and either 0, 1.21, 2.42, 12.1, or 24.2 µl of 9.50×10^{-5} M hemin stock solution to yield final reaction concentrations of hemin of 0, 0.766, 1.53, 7.66, and 15.3 mM, respectively. Then the following volumes of the Tween-80 control solution were added separately to each reaction tube, in order of increasing reaction tube hemin concentration: 24.2 µl (to the tube containing no hemin), 23.0, 21.8, 12.1, and 0 µl, respectively. All tubes were mixed by hand and incubated on ice for 5 min. to allow for any interaction between hemin and PBG D. Then 50 µl PBG (I mg/ml in reaction buffer) was added to all assay tubes and 50 µl reaction buffer added to each corresponding extract control tube, bringing the total reaction volume to 150 µl.

All tubes were incubated for 1 hr. 26 min. in a 37 °C water bath without shaking. Reactions were terminated by addition of 1.0 ml 1.0 M HCl containing 0.1 mg/ml I_2. Tubes were centrifuged in the Microfuge B at high speed for 5

min. to remove denatured protein. The supernatant was processed, spectra recorded, and activity determined as described above for the PBGD assay for effects of supplements in growth medium.

2b.10 Incubation of partially-purified PBG D with HCOOH, HCl, and Ehrlich's reagent

To detect porphyrin formation from enzyme-bound pyrroles in the presence of acid, 50 µl aliquots of partially purified PBG D were incubated with 675 µl of 1.0 M HCl containing I_2 at 1 mg/ml for up to 1 hr. 45 min. The same experiment was carried out using 10% HCOOH and overnight incubation. A 0.5 ml aliquot of the enzyme preparation was mixed with an equal volume of Ehrlich's reagent. After centrifuging in a 15 ml tube at 15,000 rpm, 4 °C, for 15 min. to precipitate protein, the supernatant spectrum was scanned from 650 nm to 450 nm on the Perkin-Elmer over a 133 min. period.

2b.11 p-Hydroxymercuribenzoate (PHMB) experiments with partially-purified PBG D

To detect whether the thioether linkage of the active-site cysteine residue to the dipyrromethane cofactor of PBG D could be cleaved by a mercury-containing reagent, the enzyme preparation was incubated in the presence and absence of PHMB. Seventy µl (.268 mg protein) in the volume of 0.1 M Tris, pH 8.2 needed to bring the total reaction volume to 500 µl, was incubated with no addition, with PHMB alone (190 µl for a final concentration of 1 mM), with ME (β-mercaptoethanol) alone (20 µl for a final concentration of 50 mM), or with both PHMB and ME. PHMB was added first followed by a 33 min. incubation period at room temperature with the enzyme. ME was then added followed by a 7 min. incubation. Then a 36 µl aliquot of the reaction mixture was removed for a 2 hr. assay of PBG D activity with 14 µl 0.1 M Tris, pH 8.2,

and 50 µl 1 mg/ml PBG. This reaction was terminated by addition of 1.0 ml of 1.0 M HCl containing 0.1 mg/ml I_2. The reactions were then centrifuged for 5 min. on the Microfuge B and the supernatants scanned from 450 mn to 350 nm on the Perkin-Elmer, following addition of 50 µl of 2.0% $Na_2S_2O_3$. A second aliquot of 50 µl was removed from the reaction mixture for a 2 hr. incubation with 50 µl 1.0 M HCl for porphyrin formation from cofactor and/or enzyme-bound pyrroles released from the enzyme by acid. ME was added to the aliquots not already containing it to 50 mM, before porphyrin formation following HCl addition, since it was found to enhance porphyrin formation. Following the incubation period, 0.5 ml of 1.0 M HCl containing I_2 was added.

The remaining 414 µl of reaction mixture was loaded by Pasteur pipett onto a 5 ml G-25 spun column fitted with a polyethylene disc and pre-equilibrated in 50 mM Tris, pH 8.2 according to the method of Penefsky (1977). The syringe-columns were fitted into 15 ml polypropylene Corning tubes before loading of the reaction mixture, and then the tubes, carrying loaded columns, were placed in a clinical table-top centrifuge (with a swing-bucket rotor) and spun at half maximal speed (approximately 1,700 rpm) for 2 min. Approximately 400 µl of eluate were collected, and, again, a 36 µl aliquot of the eluate was removed for assay of PBG D activity as for the samples before G-25. Then a 50 µl aliquot was removed for porphyrin formation, ME added back to all tubes to 50 mM, and 50 µl HCl was added before incubation and recording of spectra as for the samples before G-25.

2b.12 Plasmid Isolation

For complementation of heme mutants with *hemA*, *hemB*, or *hemC* plasmids, or for transformation with pSE103 containing the CI857 λ repressor

gene, a rapid boiling method based on that of Holmes and Quigley (1981) was used. Five ml (or 10 ml for the low-copy pSE103) LB overnights containing either ampicillin or kanamycin at 50 µg/ml were centrifuged and resuspended in 100 µl of 25% sucrose. Three hundred µl M-STET (5% Triton X-100, 50 mM EDTA, 50 mM Tris, pH 8.0, 5% sucrose) was added to the cell suspension and mixed by vortexing. The mixture was transferred to an Eppendorf tube and 30 µl 10 mg/ml lysozyme (in dH_2O) added. The tube was placed in a boiling water bath for 1 min., placed on ice, then centrifuged at 14,000 rpm for 15 min. in either a Beckman or Brinkman Microfuge. The supernatant was transferred to a new Eppendorf tube and the DNA precipitated by addition of two volumes of isopropanol and freezing at -70 °C for 30 min. The DNA pellet was dried either under N_2 or vacuum (Savant SpeedVac Concentrator), and resuspended in either dH_2O or TE buffer (10mM Tris, 1mM EDTA, pH 7.4).

For isolation of plasmid DNA to be used as a template for the synthesis of new protein in the *in vitro* transcription-translation reaction, a procedure published by Promega Biotec (Madison, WI) was used, with modifications where indicated. The cell pellet from a 250 ml LB overnight culture was transferred into a 30 ml centrifuge tube and resuspended in 6 ml of freshly prepared lysing solution containing: 25 mM Tris-HCl, pH 7.5, 10 mM EDTA, 15% sucrose, 2 mg/ml lysozyme, and incubated for 20 min. on ice. 12 ml of 0.2 M NaOH, 1% SDS was added and mixed well by inversion followed by a 10 min. incubation on ice. Then 7.5 ml of 3 M sodium acetate, pH 4.6 was added and mixed well by inversion, followed by a 20 min. incubation on ice. The preparation was centrifuged at 15,000 rpm, 4 °C , for 15 min. and the supernatant (approximately 20 ml) transferred to a 250 ml centrifuge bottle. Fifty µl RNase A (1 mg/ml) was added to the supernatant followed by a 20 min.

incubation at 37 °C. Two phenol/$CHCl_3$ extractions were carried out and two volumes EtOH were added to the supernatant of the final extraction in a new 250 ml centrifuge bottle. The preparation was mixed by inversion and frozen at -70 °C.

The ethanol (EtOH) precipitate was centrifuged at 9,000 rpm (12,000 x g) for 10 min. at 0 °C in the GSA rotor to pellet DNA. After draining the EtOH, 7.0 ml TE, pH 7.4 and 7.1 g CsCl was added followed by incubation at 37 °C with gentle shaking for 30 min. The plasmid preparation was transferred to a glass beaker and 800 µl of 10 mg/ml ethidium bromide (EtBr) was added. The CsCl-DNA solution was transferred to 5 ml polyallomer ultracentrifuge tubes; all tubes were weighed in their buckets to 0.1 g, topping off with 1.0 g/ml CsCl in TE when necessary. The samples were centrifuged in a Sorvall AH650 rotor on a Sorvall OTD 75B ultracentrifuge at 40,000 rpm, 20 °C, for 36 hrs. The resulting plasmid band was removed from the tube using a syringe with a 21-gauge needle and extracted six times with H_2O-saturated butanol (BuOH) to remove excess EtBr.

To remove CsCl, 400 µl of the BuOH-extracted DNA preparation was applied to a G-50 (autoclaved and diethylpyrocarbonate [DEPC]-treated) column packed in a sterile Pasteur pipett, which had been previously equilibrated with three column volumes of DEPC-treated elution buffer containing 10 mM Tris, pH 8.0, 1 mM EDTA, and 0.1 M NaCl. The sample was chased through the column by addition of elution buffer. Two ml of eluant was collected for each DNA preparation and isopropanol-precipitated at -70 °C. DNA pellets were washed in 70% EtOH, dried under N_2, and resuspended in DEPC-treated H_2O containing 20 µM potassium acetate (KAc), pH 7.5. The pJL2 (*hemB* $^+$), pBG101 (*hemC* $^+$), and pJL68 (*hemA* $^+$) plasmid preparations

had an OD_{260}/OD_{280} of 1.63,1.67 and 1.28, respectively, for 1:80 dilutions of each sample read on an LKB Ultrospec II, resulting in final concentrations of 300, 330, and 148 µg/ml, respectively.

Based on early results of autoradiographs from the*in vitro* transcription-translation reactions, the pJL68 plasmid preparation seemed to be a poor template, possibly due to its lower DNA concentration. A new preparation was prepared according to a spun column procedure (Maniatis et. al., 1982).

A sterile, polyethylene 5 cc. syringe was packed with Sephadex G-50 (autoclaved and DEPC-treated) and equilibrated with DEPC-treated DNA elution buffer (10 mM Tris, pH 8.0, 1 mM EDTA, 0.1 M NaCl). One ml of the previously prepared *hemA* plasmid preparation, frozen at -70 °C, was applied to the column. The syringe was inserted into a sterile 15 ml Corning polyethylene tube and centrifuged in the Sorvall RT6000 centrifuge in a H1000B rotor at 1,600 x g for 4 min. at 2 °C. The eluate was EtOH-precipitated at -70 °C for 45 min. The DNA pellet was washed in 70% EtOH, dried under N_2, and resuspended in 150 µl of DEPC-treated H_20, pH 7.5. This preparation had an OD_{260}/OD_{280} of 1.74, corresponding to a final concentration of 244 µg/ml, and performed well in the ensuing *in vitro* transcription-translation reactions.

2b.13 Plasmid digestions

The *hemA*, *hemB*, and *hemC* plasmid preparations were analyzed on 0.7% agarose gels electrophoresed for 16 hrs. at 40 V, using a Buchler power supply. Bam H1 digestions were carried out to linearize each plasmid. Digestion of 4 µl DNA, 1 µl (10 units) Bam H1, 2 µl digestion buffer, and dH_20 to bring the total volume to 20 µl was carried out for 3 hrs. at 37 °C. Gels were

stained for 30 min. in 0.5 mg/ml EtBr and photographed under ultraviolet (uv) light.

2b.14 *In vitro* transcription-translation reactions

2b.14a *Reagent preparation and concentrations*

All reagents for the *in vitro* transcription-translation reactions were stored at -70 °C. The *E. coli* S-30 extract and ^{35}S-methionine had been previously aliquotted. Regulator solutions were previously prepared in DEPC-treated H_2O. The hemin solution was prepared by adding 1.11 mg heme, 27.8 µl of Tween 80, 6 µl of 5 N NaOH, and half the desired final stock solution volume of DEPC-treated H_2O before mixing and adjusting the pH of the solution to 8.4. The protoporphyrin IX solution was prepared in a similar manner and adjusted to pH 7.4. The final pH for the glutamate, ALA, and PBG stock solutions were 7.6, 7.4, and 7.4, respectively. One µl of these stock solutions were added to buffered reaction mixtures.

The reaction concentrations for L-glutamic acid, ALA, PBG, protoporphyrin IX, and hemin were 1.32×10^{-4} M, 1.19×10^{-4} M, 8.88×10^{-5} M, 7.11×10^{-6} M, and 6.32×10^{-6} M, respectively. The reactions were carried out according to the Amersham procedure (17), modified as indicated, in sterile Eppendorf tubes, and incubated at 37 °C for 1 hr.

2b.14b *Preparation and incubation of reactions*

A previously determined amount of dilution buffer (1 mM Tris, 0.1 mM EDTA, pH 8.0) was the first reagent added to each reaction tube. Then 2.50 µl

of the S-30 extract followed by 1.00 µl of the appropriate potential regulator were added. Either 0.50 µl of *hemA* (122 ng) DNA or 1.00 µl of *hemB* (300 ng) or *hemC* (330 ng) DNA were then added followed by a pulse-spin in the Eppendorf Microfuge and a 5 min. incubation on ice to allow for any possible interactions between regulatory proteins, regulator, and DNA.

A mix of supplement (containing nucleotides for transcription, tRNA for translation, and energy-generating system, and inorganic salts), amino acids minus met, and ^{35}S-met was prepared for 16 reactions (for loading and running two gels simultaneously) by adding 71.25 µl, 28.50 µl, and 19.00 µl of each solution, respectively to one Eppendorf tube and mixing by inversion, followed by a pulse-spin. Then 6.22 µl of this mixture was added to each tube corresponding to 3.75 µl of supplement, 1.50 µl amino acids minus met, and 1.00 µl ^{35}S-met per reaction tube, and bringing the total reaction volume to 15.00 µl. All tubes were then incubated at 37 °C for 1 hr.; addition of 2.50 µl of met chase solution, a pulse-spin, and a further 5 min. incubation at 37 °C followed. Then 17.5 µl of 2 x electrophoresis loading buffer (0.0125 M Tris, 20% glycerol, 0.139 M SDS, 10% ß-mercaptoethanol, 2mg/100ml bromophenol blue) was added to each tube followed by a pulse-spin, heating at 100 °C for 5 min., and freezing the reactions at -20 °C overnight.

2b.14c *SDS-PAGE electrophoresis and autoradiography*

The reactions were run in 20 cm long 10% polyacrylamide resolving gels with a 5% stacking gel, both 2.7% in bisacrylamide, according to the method of Laemmli (1970). Twenty µl of each reaction was loaded per lane. Electrophoresis was carried out at 27.5 mA per gel, constant current, using a Buchler constant power supply for approximately 6 hrs. Gels were stained in

Coomassie Blue R-250 staining solution (0.125% Coomassie Blue R-250, 50% MeOH, 10% acetic acid) for 30 min. on a Red Rocker reciprocal rocker. Destaining was for two 30 min. periods in 250 ml of 50% MeOH, 10% acetic acid, with reciprocal rocking and a change of solution after the first 30 min. Then gels were destained overnight without rocking in 5% MeOH, 7% acetic acid.

Gels were dried for 1 hr. at 60 °C followed by a 30 min. cooling period. The dried gel was autoradiographed at -70 °C for up to 40 hrs. Molecular weights for the heme enzymes were determined from the migration of a standard protein mixture run on these gels.

2b.13d *Scintillation counting*

To quantitate the amount of newly-synthesized *hem* gene product from each *in vitro* reaction, the band corresponding to each was excised from the dried gel and counted on an LKB Wallach 1219 scintillation counter.

First, the band for a particular heme enzyme as well as the ß-lactamase band from the same lane (which was also encoded on the vector template DNA and served as an internal control) were cut from all lanes on an autoradiograph. An X-acto blade was used for this purpose. Next, the cut autoradiograph was aligned with the dried gel from which it originated and was used as a template to excise the corresponding regions from the dried gel. The dried gel strips were inserted into glass minivials for scintillation counting. One hundred µl dH_2O was added and the strip hydrated for 30 min. Then 6.0 ml of Econofluor scintillant made 6.0% in Protosol was dispensed into each vial. All vials were shaken by hand for 5 sec. and then placed on a reciprocal shaker for overnight shaking at 37 °C. The next day the vials were mixed by inversion, allowed to

cool to room temperature, and counted in the LKB Wallach 1219 scintillation counter. The scintillation counting data were analyzed by a SAS General Linear Models program for block analysis.

3 RESULTS

3a Mutant Construction and Characterization

3a.1 Construction of hemin- and PBG-permeable mutants

E. coli cannot utilize hemin from the growth medium, presumably because it lacks a transport system for it. Strains with mutations in the heme pathway could not grow well aerobically unless they were hemin-permeable.

An *E. coli hemA* mutant, SASX41B, was mutagenized with NTG and hemin-permeable mutants were selected on hemin-containing media. One, HU227, was selected for further study. Similarly, an *E. coli hemB* mutant, RP522, was mutagenized with NTG to hemin-permeability. A resulting mutant, RP523, was chosen for further study (Table 1). It was possible for these strains to grow aerobically despite their respective heme pathway-related mutations since exogenous hemin could enter the cell and, presumably, be incorporated into cytochromes and catalase (Table 2).

Attempts to grow RP523 or HU227 in media supplemented with PBG at a final concentration of 10 μg/ml failed, probably due to a low level of permeability to PBG. Therefore RP523 (*hemB*, hemin-permeable) was mutagenized with NTG for permeability to PBG. Mutants were selected on PBG-containing media and one, HU108, was selected for further study. HU108 grew well in LB supplemented with PBG to a final concentration of 25 μg/ml (see Fig. 9).

3a.2 Characterization of hemin-permeability mutations.

The mutations affecting cell permeability in HU227 and RP523 were selected for by growth on hemin-containing media. These mutations were independently selected, therefore they were thought to be different from each

Table 2. The effect of ALA, PBG, or hemin on the growth[a] of RP523 (hemB) and related wild-type strains.

Strain	Hemin permeability	Growth supplement[b]	Generation time[c] (min)
C600	-	none	41
		ALA	41
		PBG	41
		hemin	41
RP523	+	none	109
		ALA	82
		PBG	76
		hemin	36
HU1029	+	none	56
		ALA	56
		PBG	53
		hemin	39

[a]Growth conditions were the same as described in Table 4.

[b]ALA at 20 µg/ml; PBG at 40 µg/ml.

[c]Generation times were calculated from the slope of the linear portion of the growth curve.

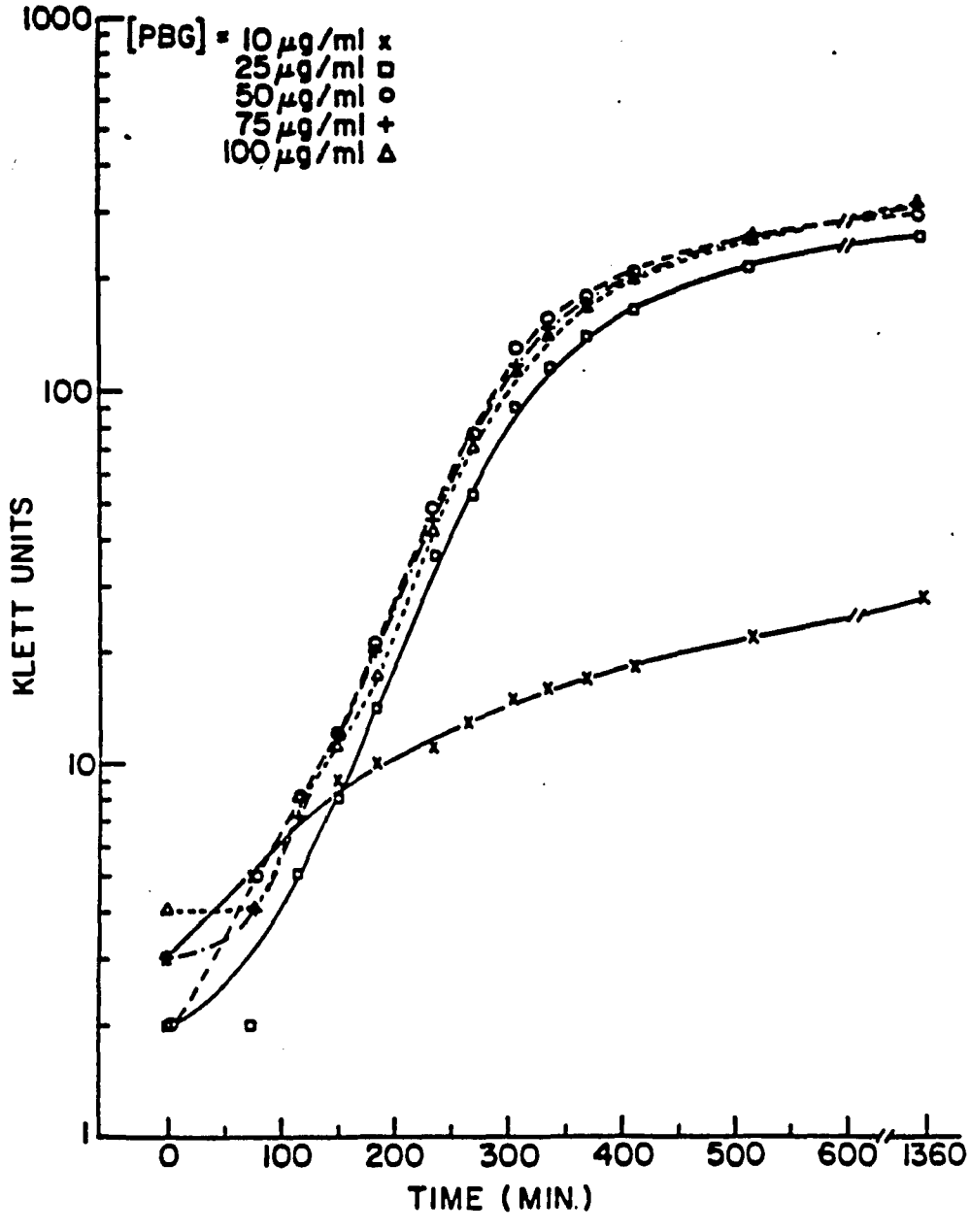


Fig. 9 Growth curves of HU108 grown in LB medium containing increasing concentrations of PBG.

other. It is thought that the mutations create a hole in the membrane which allows the passage of molecules which would normally not enter the cell.

A preliminary characterization of the hemin-permeable mutations in strains HU227 and RP523 and their Hem^+ derivatives, HU2001 and HU1029 was carried out. C600, RP523, and HU227 were streaked onto minimal agar media containing hemin and citrate as a sole carbon source: RP523 grew on citrate while C600 (a hemin-impermeable control) and HU227 did not. In a separate experiment C600, HU1029, and HU2001 were streaked onto LB plates containing either hemin alone as a positive control or hemin and actinomycin D at 10 μg/ml, or onto McConkey (a medium containing crystal violet) plus hemin plates. C600 grew in the presence of actinomycin whereas neither HU1029 nor HU2001 grew. C600 grew on McConkey media whereas HU2001 grew marginally and HU1029 failed to grow. Also RP523 grew well in complete minimal media supplemented with PBG at 40 μg/ml whereas HU227 grew poorly in the same media (Table 4, and see discussion of the availability of PBG as a requirement for PBG D activity).

The permeability mutations in RP523 and HU227 were mapped by conjugation with F' strains. Each mapped to a different location on the chromosome and to regions containing known markers for cell envelope proteins (Cosloy, unpublished results), the possible target of the mutation.

3a.3 **Isolation of secondary heme mutants**

Mutagenesis of HU227 to obtain heme pathway-related mutations in addition to the original *hemA* mutation, resulted in the isolation of at least four independent mutants (depending on how many were siblings) unable to grow on ALA-containing media, but which could grow on hemin-supplemented media. Two of these mutants accumulated porphyrins when grown on ALA-containing

media, while the others did not. Those mutants which were assayed exhibited ALA D and PBG D activities (Table 3), therefore their mutations were not in *hemB* or *hemC.*

A more complete characterization of these mutants was carried out by Hua Zhu, a graduate student in the laboratory of Dr. Sharon Cosloy. He found that several mutants accumulated uroporphyrin when grown on ALA-containing media, including K7-5, KC-10, K6-4, KA-9, 2Ba3-1, 10L2-1, and 10L3-1. All of these mutants, with the exception of 2Ba3-1, were complemented by an F' strain carrying the *hemE* gene. One mutant, 10L2-1, was assayed for uroporphyrinogen decarboxylase activity and did not form coproporphyrinogen from uroporphyrinogen III although the positive control strain, C600, did. On the basis of these results Zhu concluded that the additional heme mutation in the mutants listed above (excepting 2Ba3-1) was in the *hemE* gene.

3a.4 Construction of Hem$^+$ transductants from hemin-permeable heme mutants

A heme mutant was complemented for its respective heme pathway enzymatic deficiency first by transformation with pSE103, carrying the temperature sensitive lambda (λ) repressor protein, followed by transduction with an *E. coli* genomic library in λSE$_6$. Lambda SE$_6$ is a hybrid vector containing a λ genome lacking the λ attachment site, and the CI gene encoding λ repressor protein and containing plasmid sequences derived from pSC101, including a low-copy number origin of replication and a kanamycin resistance marker (Elledge and Walker, 1985). In the presence of λ repressor protein (introduced on pSE103) λSE$_6$ is thought to exist as a circular, single or low-copy lysogen. Because the vector contains phage and plasmid sequences it has been referred to as a phasmid.

Table 3. Strains derived from HU227 which have at least one additional heme pathway-related mutation resulting in an inability to grow on ALA-containing media.

Strain	Growth on ALA	Growth on Hemin	ALA D	PBG D[a]	Porphyrin Accumulation
SASX41B	+	+	+	+	-
27	+	+	+	+	-
K7-5	-	+	+	+	-
K7-6	-	+	ND	+	ND
K7-7	-	+	ND	+	ND
K7-8	-	+	ND	+	ND
KC-10	-	+	ND	+	ND
K6-4	-	+	ND	+	ND
KA-9	-	+	ND	+	ND
1Ba2-1	-	+	+	+	-
2Ba2-1	-	+	+	+	+
2Ba3-1	-	+	+	+	ND
10L2-1	-	+	+	+	+
10L2-2	-	+	ND	+	-
10L3-1	-	+	+	+	-

HU227 was mutagenized with NTG for inability to grow on ALA-containing media as described in Materials and Methods. Following ampicillin enrichment, mutants were selected by replicaplating from an LB hemein plate onto a pair of LB plates, one hemin-containing, the other ALA-containing. Those colonies which grew on the hemin plate but not on the ALA plate were used for further characterization. ALA D and PBG D assays were carried out as described in Materials and Methods. Porphyrin accumulation was determined by examining liquid cultures containing ALA or colonies on agar media containing ALA for fluoresence under uv illumination. Mutants within a group originated from the same batch of mutagenized cells and, therefore, may be siblings. ND means the experiment was not done.

[a]Growth of secondary heme mutants was in media containing ALA as well as hemin, since under these growth conditions ALA is required to observe PBG D activity.

Heme prototrophic derivatives of strains HU227 and RP523 were constructed. Transductants for each mutant were isolated on non-hemin containing media and one from each mutant was chosen for further study: HU2001 (harboring phHU201) resulted from transduction of the *hemA*, hemin-permeable mutant and HU1029 (harboring phHU16) resulted from transduction of the *hemB*, hemin-permeable mutant (Table 1). Each transductant was able to grow well aerobically without hemin supplementation

3a.5 **Mutant growth characteristics**

Growth in supplemented or unsupplemented complete minimal media of HU227, RP523, and their wild-type derivatives was studied. In general the wild-type (with respect to heme biosynthesis), hemin-impermeable strain, C600, showed no variation of generation time when grown in minimal media either unsupplemented or supplemented with either ALA, PBG, or hemin (Table 2). RP523, on the other hand, was unable to grow well in unsupplemented minimal media or in media supplemented with ALA; it reached only low cell densities when grown under these conditions. RP523 grew better in PBG-supplemented media. It grew best in hemin-supplemented minimal media, to approximately 6×10^8 cells per ml, and with a generation time less than that obtained for C600 (Table 2). HU1029, the phasmid-complemented, *hemB*$^+$ transductant of the *hemB* mutant, RP523, grew well in unsupplemented minimal media or in media supplemented with ALA or PBG, but somewhat more slowly than C600 did with the same supplements. HU1029 exhibited the shortest generation time in hemin-supplemented media in which it grew as well as RP523 in hemin-supplemented media (Table 2).

3b Availibility of PBG is Required for PBG D Activity

3b.1 Effect of supplements in the growth medium on PBG D activity

When grown in complete minimal medium supplemented with hemin, HU227 (*hemA*) exhibited no PBG D activity, however, it did exhibit ALA D activity. When grown in the same medium supplemented with hemin, RP523 (*hemB*) also exhibited no PBG D activity (Table 4).

The phasmid-complemented transductants of these strains, HU2001(*hemA*$^+$) and HU1029 (*hemB*$^+$) were able to grow aerobically without hemin supplementation and each had PBG D activity in addition to being restored for their original enzymatic defects--glutamyl-tRNA dehydrogenase and ALA D, respectively. When grown aerobically in complete minimal media, however, the PBG D specific activities for these transductants was approximately half that found for C600 (Table 4).

Therefore, in a *hemA* mutant and a *hemB* mutant, complementing the mutant alleles via transduction resulted in restoration of PBG D activity. This observation suggested a requirement for one of the early intermediates of heme synthesis for PBG D activity, specifically ALA or PBG.

The lack of PBG D activity in HU227 and RP523 when grown on hemin-containing media was unexpected. When HU227 was grown in medium supplemented with ALA, PBG D activity was restored (Table 4). This observation suggested that the availability of ALA or PBG was necessary for PBG D activity in *E. coli*.

RP523 could not be grown with ALA supplementation (Table 2) since the *hemB* mutation prevented the conversion of ALA to PBG. RP523 grew well, however, in minimal medium supplemented with PBG to a final concentration of

Table 4. Effect of supplements in the growth medium[a] on PBG D activity in heme-deficient and wild-type strains.

Strain	Genotype or phenotype	Hemin permeability	Growth[b] supplement	Specific[c] activity
C600	Hem^+	-	None	0.88
			ALA	0.92
			PBG	0.95
RP523	*hemB*	+	None	0.01
			ALA	0.01
			PBG	0.58
HU1029	Hem^+	+	None	0.53
			ALA	0.39
			PBG	0.58
Hfr Cavalli	Hem^+	-	None	1.00
			ALA	0.59
			PBG	1.03
HU227	*hemA*	+	None	0.02
			ALA	0.42
			PBG	0.12
HU2001	Hem^+	+	None	0.45
			ALA	0.54
			PBG	0.50

[a]Cells were grown in 25 ml minimal medium supplemented with 0.2% Casamino Acids, 50 µg/ml each of methionine and cysteine, and 1 µg/ml thiamine. Cells were grown to a density of 6×10^8 to 7×10^8 cells per ml or for a maximum of 6 hrs. for cells that could not achieve that density.

[b]ALA at 20 µg/ml; PBG at 40 µg/ml.

[c]Specific activities are in units of A_{405} per mg protein per 2.5 hr. incubation, and are the averages of at least two individual determinations.

40 µg/ml and under these conditions PBG D activity was comparable to the level obtained for HU1029 in unsupplemented medium. Similarly, when HU108, the more PBG-permeable derivative of RP523, was grown in LB supplemented with hemin, it exhibited no PBG D activity, but when grown in LB supplemented with PBG at 10 µg/ml, PBG D activity was restored (Table 5). These experiments clearly established the requirement for PBG availability in order to obtain PBG D activity from the cell extract.

3b.2 Effect of *p*-hydroxymercuribenzoate (PHMB) on the cofactor of partially-purified PBG D

When a sample of partially purified *E. coli* PBG D was incubated overnight with 10% formic acid, porphyrins formed which were detected both by fluorescence of the sample under uv illumination and spectrophotometrically, by absorption at 405 nm. This confirmed results reported by Jordan and Warren (1987). It was found that incubation of the enzyme preparation with 1 M HCl for 2 hrs. at room temperature yielded similar results.

Reaction of the same enzyme preparation with Ehrlich's reagent resulted in a spectrum which changed over time. Shortly after incubation two absorption maxima were present at 562 and 492 nm. Over a 1 hr. period the peak at 562 nm disappeared while that at 492 nm became more prominent, behavior typical of a diprrylmethane. Similar results were reported by Jordan (Jordan and Warren, 1987).

Four experiments were carried out under the conditions described in Materials and Methods to determine the effect of PHMB treatment on the cofactor of PBG D. A sample of partially purified PBG D was incubated with or without PHMB followed by a shorter incubation with or without β–mercaptoethanol (ME). Aliquots of the incubation mixtures were removed for

Table 5. Effect of supplementation in the growth medium on ALA D and PBG D activities in selected PBG-permeable and impermeable mutants.

Strain	Genotype or Phenotype	Permeability to PBG	Growth Supplement[a]	Specific Activities[b]	
				ALA D	PBG D
RP523	*hemB*	-[c]	none	0	0
			ALA	0	0
			PBG	0	0
			PBG[d]	ND	0.58
HU108	*hemB*	+	none	0	0
			ALA	0	0
			PBG	0	1.6
HU1029	*hemB*$^+$	-	none	86	1.6
			ALA	91	1.7

[a]Cells were grown overnight in 40 ml LB medium with supplements added as indicated. The final concentration of ALA and PBG in the growth medium was 10 µg/ml.

[b]Specific activities for ALA D are defined as total nanomoles of PBG produced per mg protein per 2 hr. incubation. ALA D activity was determined by the method of Sassa (ref p. 3) except 150 µl extract was used with 44 µl ALA (1.5 mg/ml) and the buffer was 0.1 M Tris, pH 7.6. Specific activities for PBG D are defined as A_{405} per mg protein per 2.25 hr. incubation. PBG D activity was determined in the usual manner except 150 µl extract was used with 44 µl PBG (1mg/ml) and the buffer was 0.1 M Tris, pH 7.6.

[c]RP523 has limited permeability to PBG.

[d]PBG at 40 µg/ml in minimal medium with the supplements listed in the growth medium for Table 4.

PBG D assay and for porphyrin formation from the dipyrromethane cofactor and/or polypyrroles bound to cofactor after addition of HCl. The remaining sample was centrifuged through a G-25 column and aliquots were removed for the PBG D and porphyrin assays.

The results from these experiments are shown in Table 6. Two specific activities reported are thought to be erroneous, specifically the value for Tube 1 before G-25 in experiment 3, and the value for Tube 4 after G-25 in experiment 3.

Generally, the results of the four experiments show that the activity of the untreated enzyme did not change as a consequence of the spun column treatment (see before and after values for Tube 1). It was not surprising to find that PHMB inhibited PBG D activity since it was expected to react with available sulfhydryl groups on the enzyme, as shown for the before values for Tube 3. After G-25 treatment the PHMB-treated enzyme showed some recovery of activity. However it was only a fraction of the activity of the untreated enzyme after G-25 treatment. It was also not surprising to find that ME was able to reverse the inhibitory effect of PHMB treatment both before and after G-25 as seen for Tube 4 values, since its concentration in the reaction was fifty-times that of PHMB. PHMB would react with the excess free sulfhydryl groups of ME, thereby freeing the enzyme cysteine residues.

Levels of porphyrin produced during incubation of the enzyme with HCl are shown in Table 6. This non-enzymatic porphyrin formation was found to be enhanced in the presence of ME (data not shown), therefore ME was added back to each sample before incubation with HCl was initiated. For the untreated enzyme (Tube 1 values) as well as for ME, PHMB, and PHMB plus ME treatments (Tubes 2, 3, and 4, respectively), porphyrin levels were always lower

Table 6. Effect of p-hydroxymercuribenzoate on the activity of partially-purified PBG D and on porphyrin production (PP) from PBG D before and after G-25 treatment.

Tube		1		2		3		4	
enzyme		+		+		+		+	
buffer		+		+		+		+	
PHMB		-		-		+		+	
ME		-		+		-		+	
Expt.		Before	After	Before	After	Before	After	Before	After
1	PBG D[a]	147	118	93.9	212	0.00	48.0	96.4	303
	PP[b]	1.5	0.32	1.2	0.13	0.62	0.15	0.90	0.18
	PBG D/PP[c]	100	370	77	1700	0	320	110	1700
2	PBG D[a]	317	344	106	330	0.00	55.5	330	270
	PP[b]	1.7	0.48	1.5	0.12	0.92	0.20	1.04	0.10
	PBG D/PP[c]	190	720	71	2750	0	280	320	2600
3	PBG D[a]	15[a]	165	333	223	0.830	53.3	333	63.6
	PP[b]	0.92[b]	0.072	0.72	0.096	0.60	0.056	0.54	0.072
	PBG D/PP[c]	16[c]	2300	460	2300	1.4	950	620	880
4	PBG D[a]	333[a]	330	339	322	1.08	46.7	336	303
	PP[b]	0.28[b]	0.12	0.82	0.12	0.19	0	0.84	0.14
	PBG D/PP[c]	1200[c]	2750	410	2700	5.6	x	400	2200
	PBG D[d]	203±75	240±57	218±68	270±31	0.477±.28	50.9±2.1	274±59	235±58
	PP[e]	1.1±0.32	0.25±0.094	1.0±0.16	0.12±0.0072	0.58±0.15	0.10±0.045	0.83±0.11	0.123±0.024
	PBG D/PP[f]	380±280	1500±580	250±100	2400±240	1.75±1.3	x	360±110	1800±370

PBG D was partially purified by heat treatment, DEAE chromatography, and Sephadex G-100 gel filtration. Samples were incubated alone, with PHMB, with ME, or with both PHMB and ME. Aliquots from each reaction tube were removed for PBG D assays. The remaining sample was processed on a G-25 column, and aliquots were again removed for PBG D assay as described in Materials and Methods. Each reaction volume before G-25 was 500 µl. 414 µl of each reaction mixture was applied to a spun column and approximately 400 µl was recovered from each after centrifugation.The values reported in this table are in units of activity per ml

Table 6 continued

(or ratios of activity per ml). A unit of PBG D activity is defined to be A_{405} per 1.5 hr. incubation period. A unit of porphyrin production (PP) is defined to be A_{405} per 2 hr. incubation of the PBG D preparation with 1M HCl. x, requires division by zero.

[a]Values in this row are in units of PBG D activity per ml of reaction mixture.

[b]Values in this row are in units of porphyrin activity per ml of reaction mixture.

[c]Values in this row are ratios of units of PBG D activity per unit of porphyrin activity and were determined by dividing the former value by the latter. This ratio is proportional to molecules of enzyme per molecule of porphyrin formed from enzyme, assuming no differences in porphyrin-forming ability between different enzyme-substrate species of PBG D.

[d]Values in this row are the means for units of PBG D per ml ± standard error for all four experiments.

[e]Values in this row are the means for units of porphyrin activity per ml ± standard error for all four experiments.

[f]Values in this row are the means for ratios of units of PBG D activity per unit of porphyrin activity ± standard error for all four experiments.

(from 4 to 13 times lower depending on the experiment and values being compared) after spun column treatment than before.

The expectation that levels of porphyrin formation from treated samples should be low when PBG D activity from that sample was low was not supported by the data. Even for the untreated sample (Tube 1), the mean for porphyrin formation after G-25 treatment was reduced about fourfold from the value before treatment. Yet mean PBG D activity values for the untreated sample before and after G-25 are similar. The mean values for porphyrin formation for the other samples (Tubes 2, 3, and 4) after G-25 treatment were four to six times lower than their respective means before G-25, yet the corresponding means for PBG D activity before and after G-25 are similar, except for Tube 3 (PHMB-treated sample) where some activity is restored after G-25. It is also difficult to interpret the fact that the mean values for porphyrin formation after G-25 for tubes 2, 3, and 4 were two to three times lower than the after G-25 value for the untreated sample.

A considerable amount of variability of results between replicate experiments can be seen in Table 6. Some of this variability might be due to different rotor speeds at a particular setting during the spun column treatment, since erratic accelerations and surging of the motor was sometimes observed with the clinical table-top centrifuge used for these experiments.

Nevertheless, the data support the conclusion that PHMB treatment does not remove the dipyrromethane cofactor from PBG D. The mean PBG D activity value for the ME and PHMB-treated sample (Tube 4) before and after G-25 are similar and both are similar to the before and after values for the untreated sample (Tube 1). If the cofactor had been removed by the PHMB treatment, the after G-25 value should have been lower.

3c Control of Heme Biosynthesis

The effects of hemin on the enzyme activities of the early steps in heme biosynthesis was studied *in vivo*

3c.1 Effects of hemin on ALA synthesis *in vivo*

Hemin is an important regulator of ALA S in the C4 pathway. When extracts of *E. coli* were assayed for ALA synthase activity from succinyl-CoA and glycine as substrates, no activity was found. Therefore an indirect approach was taken to assess the effect of hemin on ALA accumulation. The possible regulatory role of heme in the synthesis of ALA in *E. coli* was investigated by growing parallel cultures containing increasing hemin concentration. It was thought that a higher hemin concentration outside the cell would result in a higher intracellular hemin concentration during the growing period. ALA accumulation was quantitated in selected strains when grown under increasing hemin concentrations.

When HU1029 was grown in the absence and presence of hemin, ALA accumulated in the pellet and in the medium (Table 7a). No clear trend for ALA accumulation in cell extracts and/or media with increasing hemin concentration was observed, however. The value given in Table 7a for total ALA accumulation for HU1029 at a hemin concentration of 7.0 μg/ml in the growth medium, 96.4 nanomoles ALA formed in the extract plus medium per mg protein, is thought to be erroneous due to an unusually low protein concentration from this extract.

Similarly, when HU108 was grown in the presence of hemin at increasing concentrations, ALA accumulated in the cell extract (the medium was not tested) (Table 7b), and to a higher level than for HU1029 extracts. However, once again, no clear relationship between increasing hemin concentration in

Table 7a. The effect of hemin concentration in the growth medium on ALA accumulation in HU1029.

		Hemin (ug/ml)						
Medium[a]	Fraction[b]	0.0	0.5	1.0	5.0	7.0	10.0	20.0
LB	Extract[c]	0.872	28.0	3.50	6.59	30.1	11.2	29.5
	Medium[d]	164	285	252	223	289	391	332
	Total[e]	6.60	21.3	9.77	9.20	96.4	9.76	12.8
Minimal	Extract[c]	1.35	15.1	12.9	1.55	7.66	11.8	21.7
	Medium[d]	168		343	318	263	248	106
	Total[e]	9.23		23.0	19.4	15.1	14.0	9.08

[a] 50 ml cultures were grown aerobically in either LB or minimal media to early stationary phase at 37 °C in a rotary shaker bath.

[b] After growth the cells were harvested by centrifugation. The pellet was washed with and resuspended in 2.0 ml of 0.1 M Tris, pH 7.6 before sonication. The sonicate was centrifuged at 10,000 rpm for 20 min. Both the medium and the sonicate supernatants were assayed for ALA as described in Materials and Methods.

[c] All values are in units of nanomoles ALA formed in the extract.

[d] All values are in units of nanomoles of ALA formed in the medium.

[e] All values are in units of nanomoles of ALA formed in the extract plus the medium, per mg protein.

Table 7b. The effect of hemin concentration on ALA accumulation in extracts of HU108.

	Hemin (µg/ml)					
Experiment	**0.5**	**1.0**	**5.0**	**7.0**	**10.0**	**20.0**
1	1.58	2.20	2.39		1.11	0.401
2	1.90	2.14	2.04	1.78	1.63	1.50
3	2.07	2.30	1.88	1.67	2.46	2.40

50 ml cultures were grown aerobically in LB to early stationary phase at 37 °C in a rotary shaker bath. Cells were harvested and crude extracts prepared from the sonicate. ALA was determined as described in Materials and Methods and is reported as nanomoles of ALA formed per mg protein. The ALA pyrrole-containing samples in experiment 3 were extracted with dichloromethane.

the medium and ALA accumulation in the extract emerged. These results suggest that hemin does not regulate the synthesis of ALA in *E. coli*, unlike results found for *R. spheroides* (Lascelles, 1964) and *M. denitrificans* (Granick and Beale, 1978).

Considerable variability in ALA accumulaion between the conditions of increasing hemin concentration within an experiment in Table 7a were observed, as well as in Table 7b, although to a lesser extent.

3c.2 Effect of hemin in the growth medium on ALA D activity

HU227, HU2001, HU1029, and C600 as a positive control were grown in rich and minimal media in the presence and absence of hemin. Results from several experiments indicated that ALA D activity in crude extracts was not inhibited by hemin (data not shown).

3c.3 Effect of hemin in the growth medium on PBG D activity

C600, and the hemin-permeable transductants HU1029(*hemB*$^+$) and HU2001(*hemA*$^+$) were grown aerobically in minimal media in hemin-supplemented and unsupplemented cultures. Crude extracts prepared from cells grown under each condition were assayed for PBG D activity and no differences were found (Table 8). Therefore hemin did not appear to regulate *E. coli* PBG D activity *in vivo*.

The effect of hemin on ALA D and PBG D activities was studied *in vitro*

Table 8. Effect of hemin in the growth medium on PBG D activity in selected wild-type hemin-permeable and impermeable strains.

Strain	Genotype or phenotype	Hemin permeability	Growth[a] supplement	Specific activity[b]	
				Expt.1	Expt.2
C600	Hem^+	-	None	1.06	0.804
			Hemin	1.05	0.781
HU1029	$hemB^+$	+	None	1.02	0.73
			Hemin	1.11	0.693
HU2001	$hemA^+$	+	None	1.03	0.581
			Hemin	1.03	0.595

[a]Cells were grown in 100 ml minimal media under the same conditions described in Table 4 and were either unsupplemented or supplemented with hemin at 4 μg/ml.

[b]Specific activities are in units of A_{405} per mg protein per incubation period.

3c.4 Effect of hemin on purified ALA D

It was shown that hemin does not inhibit purified ALA D at levels normally used for growth of *E. coli* heme mutants., although at 1.39 x 10^{-3} M ALA D activity is inhibited 45% (Braithwaite and Russell, unpublished results).

3c.5 Effect of hemin on partially-purified PBG D activity

PBG D was partially purified from a crude *E. coli* extract by heat treatment, DEAE-cellulose chromatography, and Sephadex G-100 gel filtration, as described in Materials and Methods. An aliquot of the enzyme preparation was incubated without or with hemin in increasing hemin concentrations. No effect of hemin on PBG D activity *in vitro* was observed (Table 9).

3c.6 Effect of oxygen and glucose in the growth medium on PBG D and ALA D activities

When C600 was grown aerobically in rich media in the presence of glucose at 0.2% final concentration, PBG D activity was reduced almost two-fold as compared to activity obtained in the absence of glucose, while ALA D activity was not affected by glucose (Table 10).

When grown aerobically compared to anaerobically, PBG D activity was marginally lower in the presence of oxygen while ALA D activity was not affected (Table 10). (The data were analyzed using a one-way ANOVA with Statview 512^{+} which indicated that all means for PBG D specific activities were significantly different, while no differences were found for the means for ALA D specific activities.)

Table 9. Effect of hemin concentration on the specific activity of partially purified PBG D.

	Hemin (μM)				
	0.0	0.766	1.53	7.66	15.3
Exp 1	2053	1769	1963	1919	1924
Exp 2	1955	2200	1990	1938	1917
mean	2004	1985	1977	1929	1921

PBGD was prepared from 10 L of TB1 (pBG101) grown aerobically in LB, as described in Materials and Methods. The extract was heat- treated and processed through DEAE-cellulose and Sephadex G-100 as described in Materials and Methods. Aliquots of the enzyme preparation were preincubated with hemin at the indicated final assay concentrations before addition of substrate to initiate the reactions. Specific activities are in units of A_{405} per mg protein per 1.5 hr. incubation at 37 °C.

Table 10. The effect of oxygen and glucose in the growth medium on PBG D and ALA D activities in extracts of C600.

Condition		PBG D		ALA D	
glucose	oxygen	Expt.1	Expt. 2	Expt.1	Expt.2
+	+	0.27	0.26	0.21	0.15
-	+	0.47	0.46	0.14	0.11
+	-	0.36	0.34	0.10	0.10

200 ml aerobic and 550 ml anaerobic LB cultures were grown for 20 hrs. at 37 °C with reciprocal shaking. PBG D activity was determined as defined in Materials and Methods and is expressed as A_{405} per mg protein. ALA D activity was determined as defined in Materials and Methods and is expressed as A_{552} per mg protein. The absence of oxygen was maintained in an anaerobic chamber generating H_2 and CO_2. Glucose was supplemented to 0.2% final concentration.

3c.7 Effect of nalidixic acid on PBG D gene expression in C600 and HU1029

DNA gyrase imparts negative supercoils into DNA (Gellert et. al., 1986) which are necessary for DNA replication and for the binding of several regulatory proteins, while topoisomerase I relaxes negatively supercoiled DNA. A fairly constant level of supercoiling is thought to be maintained *in vivo* by these activities (Herrin and Bennett, 1986). Gyrase consists of an A subunit, associated with a double-strand break and repair mechanism, and a B subunit, associated with an ATPase activity (Cozzarelli, 1980). Nalidixic acid is an inhibitor of the A subunit.

The addition of nalidixic acid to growing cultures of *E. coli* is known to affect the activities of many different gene products at the level of transcription, and different promoters respond differently (Herrin, 1986). Some promoters are stimulated while others are repressed.

C600 and HU1029 showed different responses in PBG D activity in the extract with respect to an increase in nalidixic acid concentration in the growth medium. When grown in the absence of nalidixic acid, PBG D specific activities for C600 and HU1029 were comparable. At a nalidixic acid concentration of 20 µg/ml or higher, however, PBG D activity for C600 increased by 70% whereas for HU1029 it decreased by 40% from their respective control activities (Fig. 10). These changes were maintained at the higher nalidixic acid concentrations of 40, 60, and 80 µg/ml.

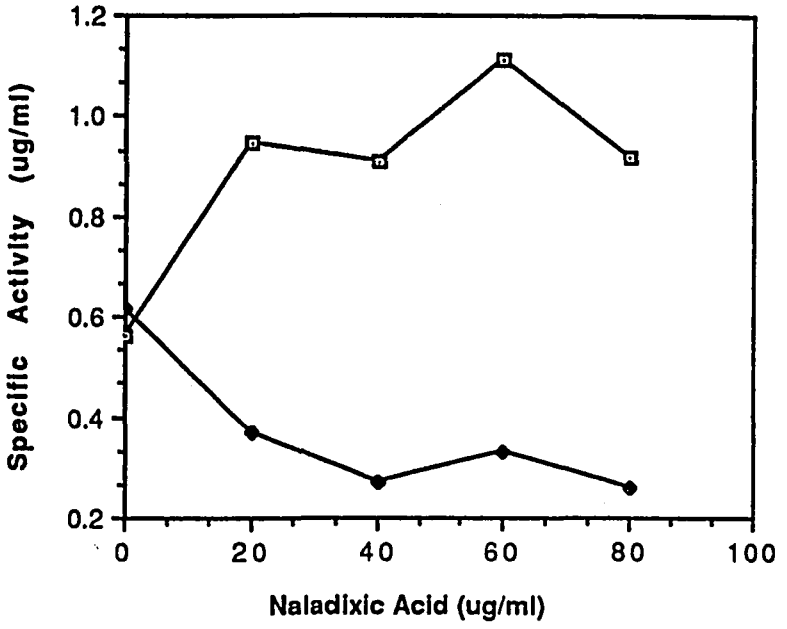


Fig. 10 Graph of PBG D specific activity vs. nalidixic acid concentration for C600 and HU1029 (*HemB*$^+$, phHU16). Cultures of were grown in the absence and presence of nalidixic acid. Cells were harvested, extracts prepared, and PBG D activity assayed in C600 (▫) and HU1029 (♦) as described in Materials and Methods.

Both C600 and HU1029 are *hemC*$^+$ and *hemB*$^+$. Both genes are located at their normal chromosomal positions in C600 as is the *hemC* gene in HU1029; however the chromosomally defective *hemB* gene in HU1029 is complemented by a functional *hemB* gene residing on a circular, extrachromosomal phasmid DNA molecule. A possible explanation of the different PBG D specific activities found in these two strains in the presence of nalidixic acid could be the differential expression of the *hemB* gene depending on its topology (see discussion for the effect of nalidixic acid).

3c.8 *In vitro* Transcription-Translation of *hemA*, *hemB*, and *hemC* Genes

3c.8a *Analysis of the protein products*

Examination of proteins expressed by pJL68(*HemA*$^+$) (see Fig. 11) on SDS-polyacrylamide gels showed three bands unique to the plasmid when compared to vector DNA, pTZ19U, lacking the insert (see Fig. 12): a narrow band approximately 51,000 in molecular weight (MW), a wider band of MW 47,500, and a fairly wide band of approximately 23,000 MW corresponding to a truncated release factor 1 protein. The 47,500 MW band corresponds to what is thought to be the ALA dehydrogenase protein and the 51,000 MW band may represent an alternate form of the same protein. Only one start site was identified in the open reading frame of the hemA gene and only one band corresponding to the hemA gene product was seen on autoradiographs obtained from maxicell preparations of pJL69, however (Li et. al., 1989c).

A similar examination of proteins expressed by pJL2(*HemB* $^+$) (Fig. 13) showed two bands unique to the hybrid plasmid relative to bands expressed from the vector, pTZ18U, lacking an insert: a wide band of MW 39,000 and a narrow band of MW 37,500 (Fig. 14). The heavier band corresponded to the molecular weight of purified ALA D on SDS-polyacrylamide gels; the narrower band may represent an alternate form of this protein. These bands coincide in size with products obtained from two possible start sites identified in the hemB open reading frame (Li et. al., 1989a).

Examination of proteins expressed by pBG101(*HemC* $^+$) using the *in vitro* transcription-translation system revealed a protein of MW 36,000 unique to the hybrid relative to the vector, pUC8, lacking an insert (Fig. 15). This protein corresponded to the known MW of PBG D when determined by SDS-PAGE. A second unique band of MW 24,000 was thought to represent a truncated

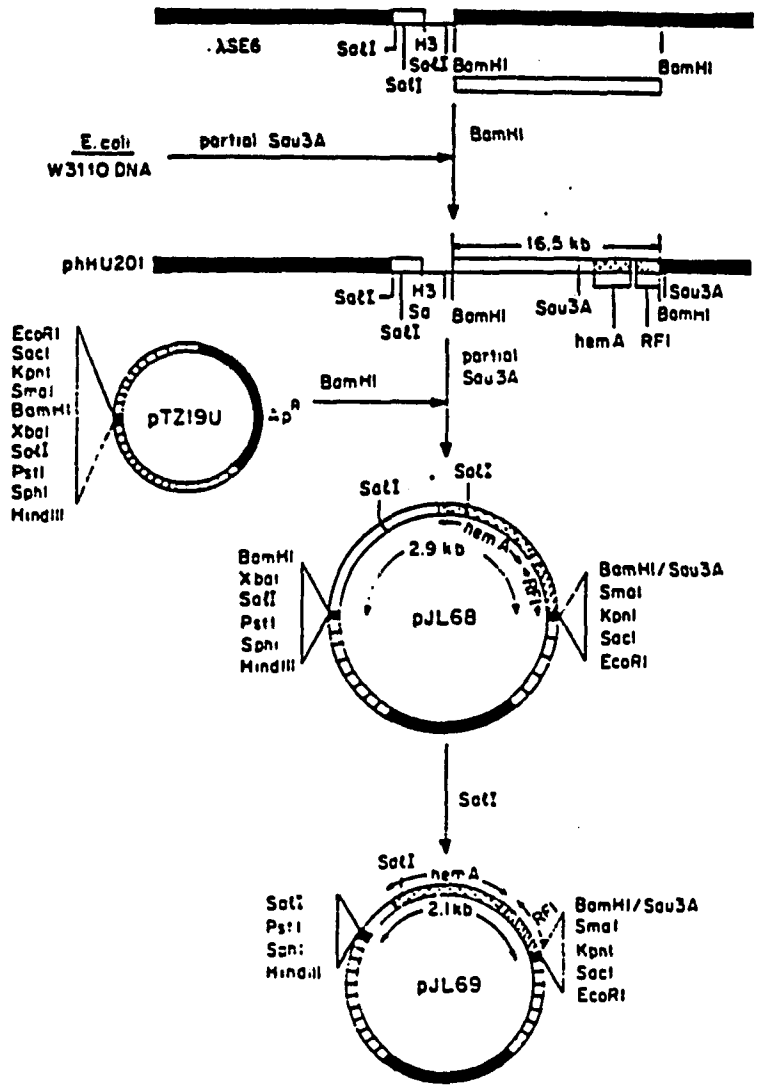


Fig. 11 Cloning of the *E. coli hemA* gene (Li et. al., 1989c).

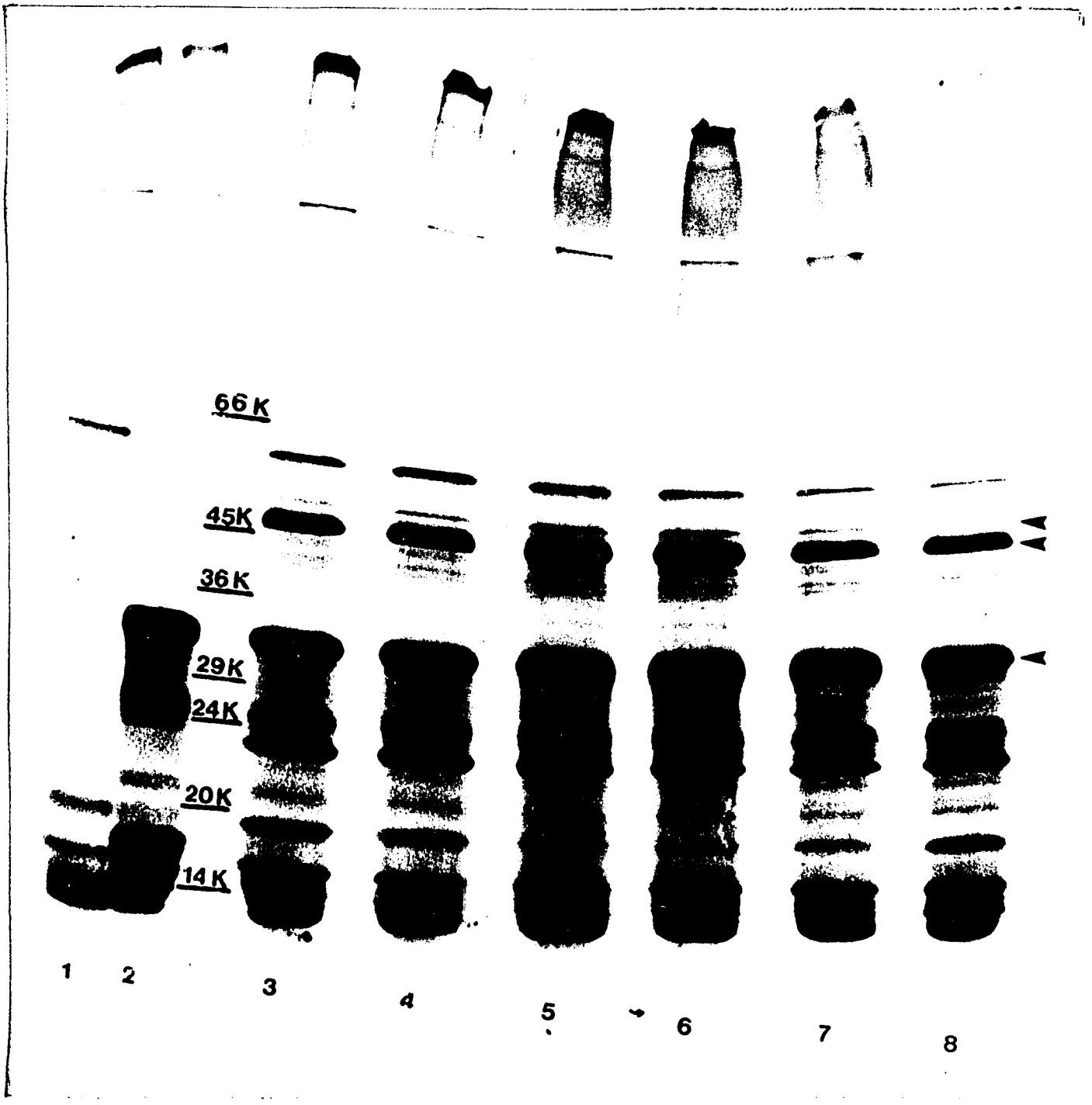


Fig. 12 Autoradiograph of SDS-PAGE of ^{35}S-met labelled proteins synthesized during *in vitro* transcription-translation of pJL68(*hemA*$^{+}$). Reaction mixtures contained no DNA (Lane 1), pTZ19U (Lane 2), or pJL68 (Lanes 3-8). Mixtures also contained either no supplement (Lanes 1, 2, and 3), or glutamate (Lane 4), ALA (Lane 5), PBG (Lane 6), protoporphyrin IX (Lane 7), or hemin (Lane 8). Upper arrows indicate *hemA* gene products. Lower arrow indicates β-lactamase. See Results for analysis of protein bands.

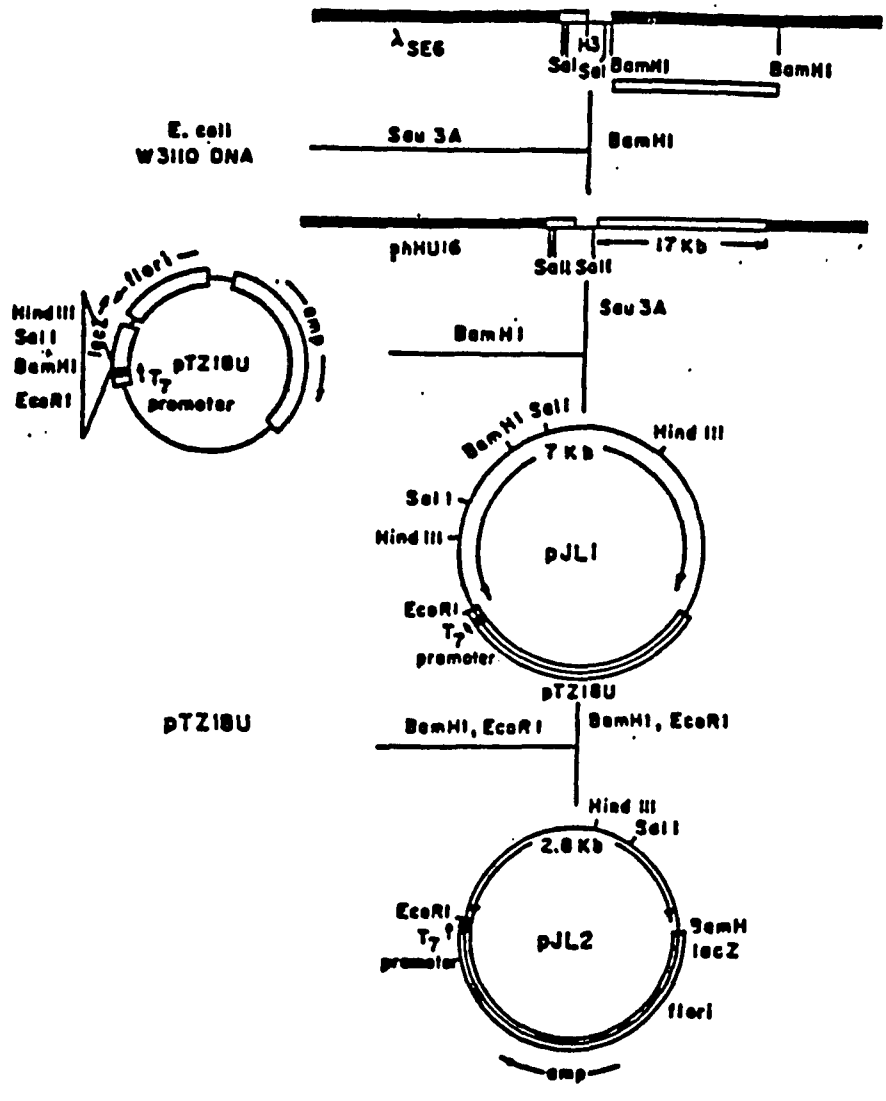


Fig. 13 Cloning of the *E. coli hemB* gene (Li et. al., 1988).

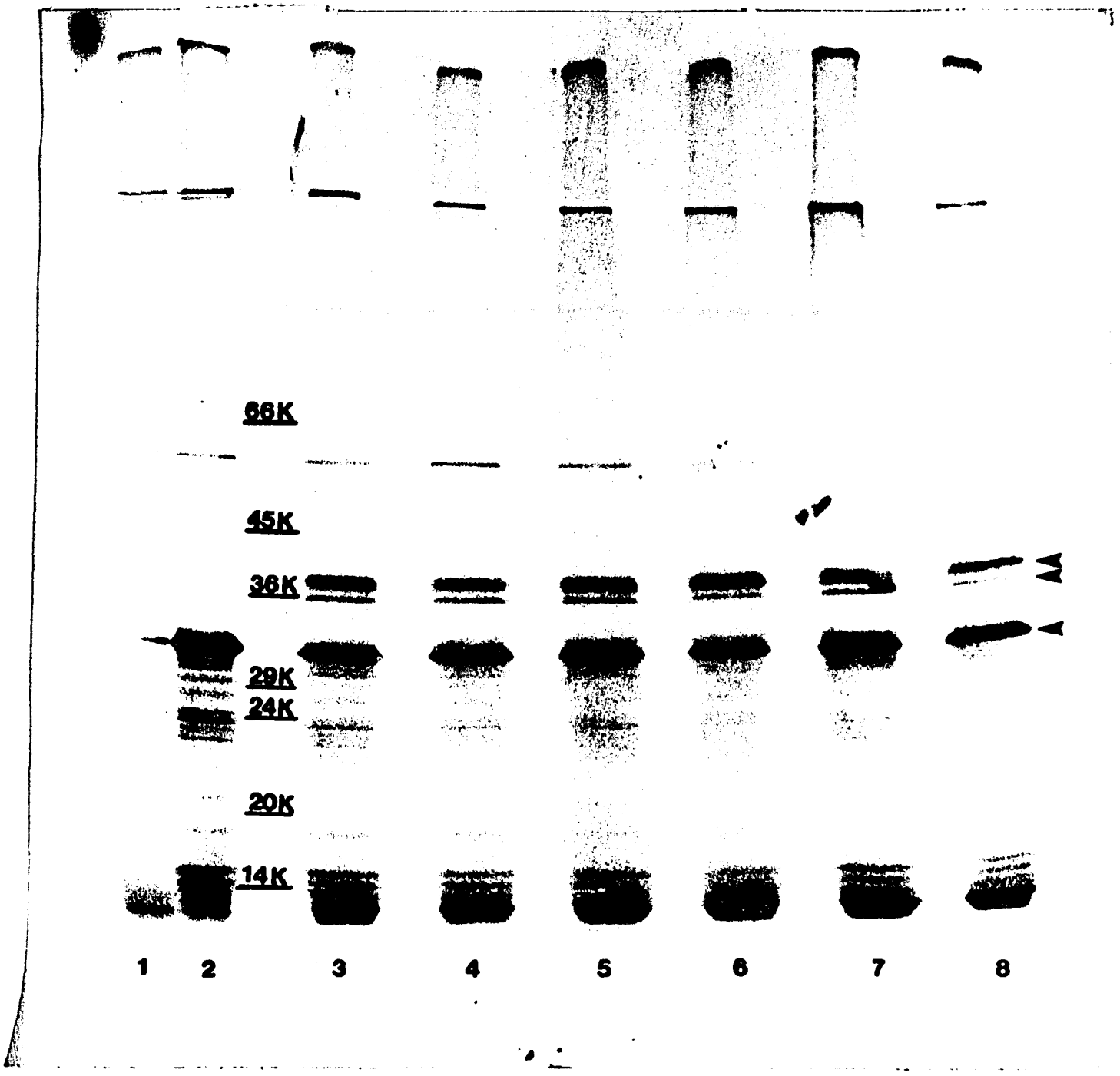


Fig. 14 Autoradiograph of SDS-PAGE of ^{35}S-met labelled proteins synthesized during *in vitro* transcription-translation of pJL2(*hemB*$^{+}$). Reaction mixtures contained no DNA (Lane 1), pTZ18U (Lane 2), or pJL2 (Lanes 3-8). Mixtures also contained either no supplement (Lanes 1, 2, and 3), or glutamate (Lane 4), ALA (Lane 5), PBG (Lane 6), protoporphyrin IX (Lane 7), or hemin (Lane 8). Upper arrows indicate *hemB* gene products. Lower arrow indicates β-lactamase. See Results for analysis of protein bands.

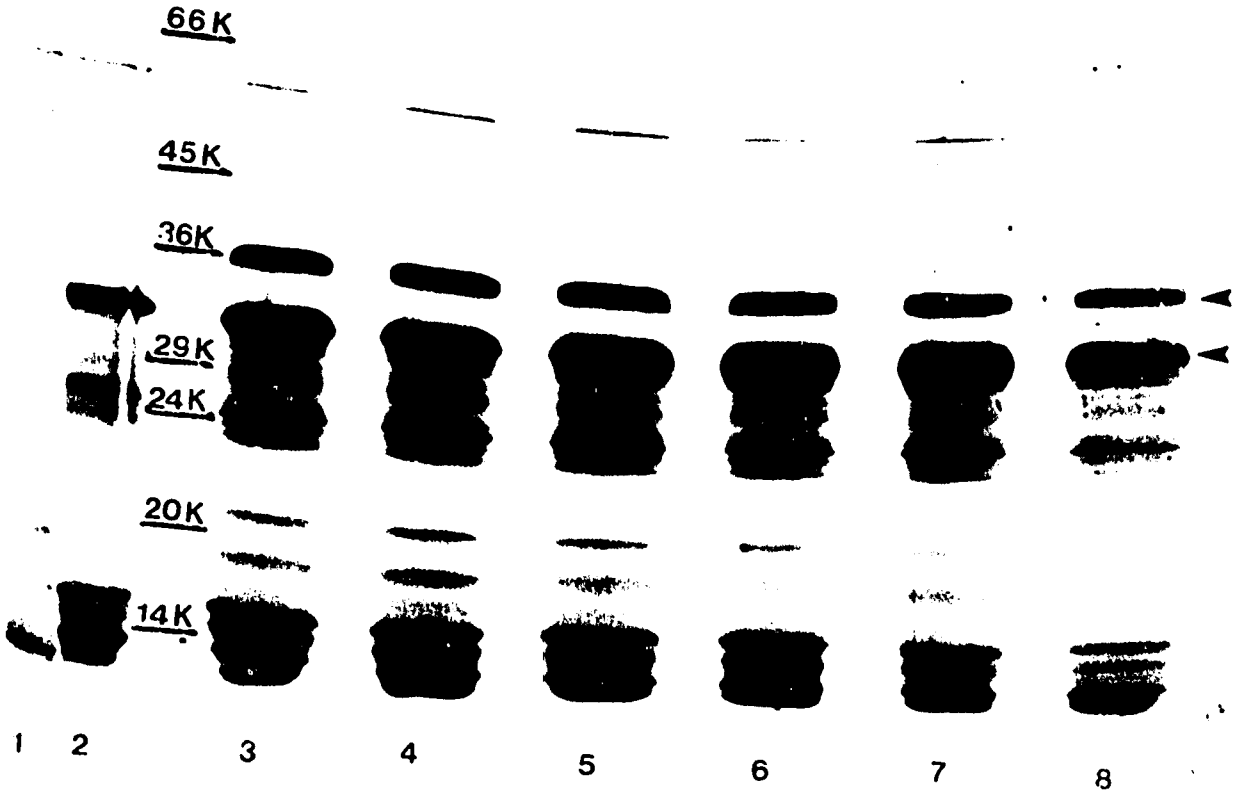


Fig. 15 Autoradiograph of SDS-PAGE of ^{35}S-met labelled proteins synthesized during *in vitro* transcription-translation of pBG101(*hemC*$^{+}$). Reaction mixtures contained no DNA (Lane 1), pUC8 (Lane 2), or pBG101 (Lanes 3-8). Mixtures also contained either no supplement (Lanes 1, 2, and 3), or glutamate (Lane 4), ALA (Lane 5), PBG (Lane 6), protoporphyrin IX (Lane 7), or hemin (Lane 8). Upper arrow indicates *hemC* gene product. Lower arrow indicates β-lactamase. See Results for analysis of protein bands.

uroporphyrinogen III cosynthase transcribed from the *hemC* promoter; truncated because only part of its coding sequence is contained on the pBG101 insert.

3c.8b *DNA concentrations used for in vitro transcription-translation reactions*

Adjusting the template DNA concentrations used in the *in vitro* transcription-translation reactions was important for the validity of any regulatory effects which might have been seen. Regulatory proteins are often present at very low intracellular concentrations (Zubay, 1973). It is possible to include an excess of DNA template in the reaction which could titrate out a regulatory protein. As a result, transcription-translation of the excess DNA in this reaction (escape synthesis) would be unaffected by normal regulatory mechanisms. Therefore a series of experiments were carried out in order to determine a minimal amount of DNA for each plasmid which could generate newly synthesized proteins detectable under the conditions used. For the *hemA*, *hemB*, and *hemC* template DNAs used in the *in vitro* transcription-translation reactions, 122 ng, 300 ng, and 330 ng were used, respectively.

3c.8b *Effect of potential regulators on gene expression*

Five potential effectors on the expression of three early enzymes of heme biosynthesis was studied using template DNA containing separate cloned inserts for the *E. coli hemA*, *hemB*, and *hemC* genes, respectively, and an *E. coli in vitro* transcription-translation system. Glutamate, ALA, PBG, protoporphyrin IX, and hemin were added to separate reactions including *hemA*, *hemB*, or *hemC* DNA, an *E. coli* S-30 extract, and all necessary transcription and translation factors. Newly synthesized proteins from each reaction were labelled with ^{35}S-met, separated on SDS-polyacrylamide gels,

and quantitated with respect to newly synthesized ß-lactamase by scintillation counting. None of the potential effectors were found to affect the expression of the *E. coli hemA*, *hemB*, or *hemC* genes based on statistical analysis of the means of the ratio of counts per minute obtained from experiments done in triplicate (Table 11) as well as on visual inspection of autoradiographs of the dried gels.

3c.8c *Variability in results from quantitating the effect of potential regulators between replicate experiments*

The data reported in Table 11 are ratios of counts per minute for each *hem* gene protein band excised from the dried gel divided by counts per minute for the internal control β-lactamase band present in the same lane. The use of the internal control was meant to control for any errors in pipetting which might have occurred during experimental manipulations. If more reaction mixture were inadvertantly loaded into one lane than from a second reaction mixture on another lane, or if more DNA were loaded into a reaction with one effector than for a different effector, then an inflated number of counts could result from the *hem* gene protein band from those reaction tubes in which the error was made. But any increase in the amount of heme enzyme loaded should be accompanied by the same increase in β-lactamase and so the ratio of their counts should not change. It was assumed that the β-lactamase gene would not respond to any of the effectors added to each reaction.

An examination of the results of these experiments in Table 11 will reveal a wide range of variability between replicate experiments. It remains to be determined what the main sources of this variability are.

In one preliminary experiment the variability in the ratio of counts obtained from samples of equal volume from identical reaction tubes loaded

Table 11. Effect of heme pathway-related compounds on the *in vitro* transcription-translation of pJL68 (*HemA*$^+$), pJL2 (*HemB*$^+$), and pBG101 (*HemC*$^+$) template DNA.

hemA

Effector	0	glu	ala	pbg	proto	heme
Exp1	0.250	0.213	0.280	0.227	0.246	0.209
Exp2	0.591	0.223	0.384	0.262	0.357	0.306
Exp3	0.209	0.408	0.345	0.213	0.409	0.607
mean	0.350	0.281	0.336	0.234	0.337	0.374
std. error	0.121	0.0634	0.0303	0.0146	0.0481	0.120

hemB

Effector	0	glu	ala	pbg	proto	heme
Exp1	0.970	0.743	1.240	1.050	1.360	8.340
Exp2	0.327	0.279	0.447	0.642	0.532	0.320
Exp3	0.467	1.010	0.580	1.410	0.682	0.487
mean	0.588	0.677	0.756	1.034	0.858	3.049
std. error	0.195	0.214	0.245	0.222	0.255	2.650

hemC

Effector	0	glu	ala	pbg	proto	heme
Exp1	0.091	0.161	0.123	0.095	0.126	0.114
Exp2	0.254	0.113	0.235	0.224	0.153	0.126
Exp3	0.0489	0.161	0.177	0.133	0.144	0.215
mean	0.131	0.145	0.178	0.151	0.141	0.152
std. error	0.0625	0.0160	0.0323	0.0383	0.00794	0.0319

Plasmid DNA was prepared from strains JL1268 (*HemA*$^+$), JL1102 (*HemB*$^+$), and TB1 (*HemC*$^+$) as described in Materials and Methods. *In vitro* transcription-translation reactions were carried out as described in Materials and Methods according to a modified Amersham procedure which was based on that of Zubay (1973). SDS-PAGE, autoradiography, and scintillation counting were carried out as described in Materials and Methods. For each lane on thedried gel both the protein, synthesized from the cloned gene on the plasmid, and the ß-lactamase band, as an internal control, were excised and counted. Each value represents a ratio of counts per minute obtained from the cloned gene product band divided by counts per minute obtained from the ß-lactamase band in the same lane. A one-way ANOVA was performed using the SAS General Linear Models program to evaluate the effect of each effector on the expression of the *hemA*, *hemB*, and *hemC* genes. 0, no effector added; glu, glutamate; ala, 5-aminolevulinate; pbg, porphobilinogen; proto, protoporphyrin IX. Final effector concentrations are given in Materials and Methods.

onto lanes in the same gel was no more than 5%. Another preliminary experiment was carried out to test the proportionality between counts per minute obtained from each *hem* gene protein band (and β-lactamase band) and the volume of reaction mixture loaded into the well. Aliquots of 5.0, 8.0, 10.0, 13.0, 20.0, and 30.0 µl from one reaction mixture were loaded into separate lanes on a gel. Counts per minute obtained from the heme enzyme band and from the beta-lactamase band were approximately linearly proportional to the volume added. However the ratio of counts per minute for the aliquots were .371, .493, .306, .749, .338, and .250, with respect to increasing volume. Theoretically, these values should have been same, since the ratio of counts per minute was thought to be independent of the volume of reaction added.

This result suggests several possible problems in the methodology used. There may be considerable variability in the efficiency of transcription and/or translation which was not controlled during the reactions. The proteins removed from each reaction for loading onto the gel may not have been homogenously dispersed, despite mixing each tube by hand before removing an aliquot. The efficiency of protein extraction from the hydrated gel strip containing the band of interest into the scintillation fluid is another possible source of variability from one gel to the next.

4 DISCUSSION AND CONCLUSIONS

4a Mutant Construction and Characterization

Based on the results characterizing the hemin-permeability mutations in HU227, RP523 and their derivative strains (section 3a.2), the permeability mutation in RP523 is distinct from the mutation in HU227. The mutation in HU227 and its derivative HU2001 is more restrictive to the passage of molecules (such as PBG, citrate, and crystal violet) through the membrane than the mutation in RP523 and its derivative HU1029. Once complemented for the *hemA* or *hemB* mutation the transductant was able to grow without hemin supplementation and the positive selection for the hemin-permeability mutation was lost. The inability of HU1029 to grow in the presence of actinomycin D and crystal violet, and the inability of HU2001 to grow in the presence of actinomycin D and its impaired growth with crystal violet were considered proof that the permeability mutations in the parent strains were retained after transduction with the complementing λSE_6.

4b Effects of supplements in the growth medium on PBG D activity

When HU227 a *hemA*, hemin-permeable mutant was grown in complete minimal media supplemented with hemin, no PBG D activity was obtained.from the crude extract. When grown in the same medium supplemented with ALA in place of hemin, PBG D activity was restored to levels comparable to those obtained from the *hemA*$^+$ transductant, HU2001. Wild-type *E. coli* is freely permeable to ALA. Growth of HU227 in PBG-supplemented medium resulted in only low levels of PBG D activity because of restricted PBG-permeability of this strain (see discussion of mutant characterization).

When RP523, a *hemB* mutant, was grown in complete minimal media supplemented with hemin, no PBG D activity was detected in the crude extract. Growth in the same medium with PBG (40 µg/ml) substituting for hemin resulted in restored PBG D activity, at a level comparable to that obtained from the *hemA*$^{+}$ transductant, HU1029.

ALA can be converted to PBG by HU227 (*hemA*) but not by RP523 (*hemB*). Therefore RP523 was unable to grow in ALA-supplemented media and showed no PBG D activity in extracts from cells grown in this media. PBG availability, therefore, is required by *E. coli* for PBG D activity.

PBG D specific activities for C600 were approximately twice that found for HU1029 (Table 4). Even though both strains are Hem^{+}, HU1029 harbors a functional *hemB* gene on a phasmid and a mutant *hemB* allele on its chromosome. The differences in PBG D specific activity between the two strains may be due to abnormal regulation of *hemB* on the phasmid DNA compared to normal chromosomal regulation. Lowered ALA D activity would result in lower endogenous PBG levels resulting in lowered PBG D activity due to the cofactor requirement for PBG by PBG D. This hypothesis is supported by the experimental observation that ALA accumulates in HU1029, but not in C600. Less ALA would be converted to PBG in HU1029 than in C600, therefore the accumulation of ALA would be greater.

While it is clear from these experiments (Table 4) that the availability of PBG is required for PBG D activity and from the work of others that PBG is incorporated into a unique dipyrromethane cofactor (Jordan and Warren, 1987; Miller et. al., 1988; Scott et. al., 1988b), the significance of this regulatory feature for heme biosynthesis is not as clear. If the control of levels of PBG is part of the regulatory apparatus for heme biosynthesis, then it would seem likely that some

step (or steps) before the PBG D step would be subject to regulation, thereby indirectly controlling PBG D activity and levels of heme synthesis.

4c Effect of PHMB on the Dipyrromethane Cofactor of PBGD

The determination of the significance of PBG in the cofactor of PBG D led to a natural question. Could the inhibition of PBG D activity by PHMB be due to release of the cofactor from the enzyme caused by the mercury-containing reagent? This could be answered by a comparison of enzyme activities and porphyrin levels obtained from partially-purified PBG D samples in the presence and absence of PHMB, since the presence of the cofactor is required both for enzymatic activity and is also a source for porphyrin formation after release from the enzyme upon incubation with acid.

PHMB appeared to bind covalently to PBG D since the PHMB-treated enzyme was much less active before and after G-25 than was the untreated enzyme (Table 6). However, some of the binding of PHMB to the enzyme may be reversible since partial activity of the PHMB-treated enzyme was recovered after G-25. Another possible, interpretation of these results is that PHMB was not fully removed by the spun column and that if all PHMB had been removed, full activity would have been restored after G-25 for Tube 3.

The fact that PHMB-induced inhibition of PBG D activity was reversed by incubation of the treated enzyme with ME was expected since ME was at a concentration of 50 mM in the reaction tube while PHMB was at 1mM.

The data indicate that the cofactor was not released by PHMB: Although PBG D activity is low after G-25 for the PHMB-treated enzyme (Tube 3) compared to levels after G-25 for all other treatments, which suggests that some of the cofactor might have been released, porphyrin levels obtained from the

same sample after G-25 (Tube 3) were about the same as those obtained with ME or both PHMB and ME treatments. If cofactor were released by PHMB, then it was expected that porphyrin levels should also be reduced.

Furthermore, ME treatment (Tube 2) certainly did not release cofactor since PBG D activities before and after G-25 are comparable. Also, treatment with both PHMB and ME (Tube 4) did not release cofactor for the same reason. Therefore, there is no reason to conclude that PHMB treatment released cofactor. Although it certainly inhibited PBG D activity, the most likely explanation for the lower PBG D activity after G-25 for the PHMB-treated enzyme (Tube 3), is that PHMB bound covalently to the enzyme at two different sites. Binding at one site may be reversible by the spun column treatment whereas binding at the other site might be irreversible. Another possible explanation is that some PHMB remained in the sample following G-25 treatment, thus inhibiting the enzyme.

A more difficult question to answer is why porphyrin levels were lower after G-25 than before, even for the untreated enzyme. If losing PBG D on the G-25 column were the reason, then enzyme activity should have been reduced after G-25 as well, but that wasn't found for any of the treatment groups. One possible explanation is that only one, rare enzyme species is responsible for the generation of porphyrin. For instance, the species with 4 PBG's bound to the dipyrromethane cofactor might be in low concentration and the main source for porphyrin formation, since a linear tetrapyrrole released from it would quickly condense to form porphyrin. If this species were selectively absorbed on the G-25 column, porphyrin levels would be reduced although overall enzyme activity would not be greatly affected.

4d Lack of effect of hemin on PBG D activity *in vivo* and *in vitro*

Heme is an important allosteric regulator of heme biosynthesis in the C4 pathway of the mammalian liver as well as in *R. spheroides* and *M. denitrificans*. Since hemin was found to have no effect of ALA synthesis in *E. coli*, its possible role as a regulator for PBG D was investigated.

If hemin inhibited PBG D activity in *E. coli* , the specific activity of PBG D from hemin-supplemented cultures should be lower than for the corresponding unsupplemented culture. When hemin was added to the growth medium of C600 and two hemin-permeable transductants, HU1029 (*hemB*$^+$) and HU2001 (*hemA*$^+$), no effect on PBG D specific activity in the extract was observed for any of the strains (Table 8). And when partially-purified PBG D was preincubated with hemin at increasing concentrations, no effect on PBG D activity was found (Table 9).

Neither hemin nor any of the potential effectors studied was found to affect the expression of the *hemA*, *hemB*, or *hemC* gene (Table 11). The lack of effect on the expression of the *hem* genes studied probably reflects the lack of *in vivo* regulation of expression by these effectors in *E. coli*, certainly in the case of hemin since other results from other experiments, *in vivo* and *in vitro*, show no effect by hemin on various aspects of regulation. In this transcription-translation system, however, it is possible that a necessary but unknown regulatory protein(s) did not survive the preparation of the S-30 extract, as has been known for the *E. coli araC* protein (Zubay, 1973).

4e Effect of Oxygen and Glucose in the Growth Medium on PBG D and ALA D Activities

In aerobically grown LB cultures with glucose, PBG D activity was approximately half of that obtained from cultures grown without glucose (Table

10). This effect is probably indirect since glucose is known to affect the expression of many genes that are catabolite repressible. There is also the possibility that a more direct effect is occurring to regulate *hemC* expression. The start codon of the adenylate cyclase (*cyaA*) gene is 386 base pairs away from the start codon of *hemC*, but is transcribed in the opposite direction . In this intervening region are several stop codons in all reading frames (Thomas and Jordan, 1986). Perhaps protein-DNA interaction inhibitory to *cyaA* transcription, which might be expected to occur in the presence of glucose (when cAMP levels are low), hinders promotion at the *hemC* promoter.

In anaerobically grown LB cultures PBG D levels were minimally higher (the difference was statistically significant, however) than for aerobically grown cultures (Table 10) (both cultures had glucose present, since anaerobic cultures of *E. coli* require a fermentative carbon source). This effect may be related to similar effects seen for ALA D, showing higher specific activities for anaerobic cultures than for aerobic cultures (Ishida and Hino, 1972). However, in this study no such stimulatory effect on ALA D activity was seen. It is not clear why early heme pathway enzyme activities should be stimulated by anaerobic growth, but this may reflect a real increase in cytochrome synthesis to counteract less efficient ATP production from anaerobic respiration compared to aerobic respiration.

4f How is Heme Biosynthesis in *E. coli* Regulated?

Unlike sugars and amino acids, heme has no transport system in *E. coli.* Therefore it is synthesized from basic precursors whether it is present in the growth medium or not. *E. coli* is a facultative anaerobe able to synthesize ATP via aerobic or anaerobic respiratory chains. In light of the possibility in *E. coli* for heme synthesis under both aerobic and anaerobic growth conditions, it

seems possible that heme synthesis is constitutive. Levels of heme production may be fine-tuned rather than shut off.

Based on work done in this study and by others, several aspects of the regulation of heme biosynthesis in *E. coli* stand out. Studies on heme synthesis from ALA in resting cultures showed a dramatic increase in synthesis when anaerobically grown cultures were incubated aerobically (Ishida and Hino, 1972). Oxygen, therefore is involved in the fine-tuning of heme synthesis in the coproporphyrinogen and protoporphyrinogen oxidase reactions, and possibly at other steps and levels of regulation as well.

The reactions for the synthesis of ALA, the first committed intermediate, are the likely candidates for regulation of the pathway, however this has not been demonstrated. It is possible that glutamyl-tRNA synthase (ligase) regulates heme synthesis since this would be the first enzymatic step if the *E. coli* pathway is identical to the better characterized C5 pathway of plants.

The dipyrromethane cofactor of PBG D points to an enzyme level regulatory mechanism and suggests that levels of PBG may indirectly regulate levels of heme synthesis, but the real relevance of this regulatory feature for *E. coli* is not yet clear. That PBG is required for PBG D activity is one reason for believing that PBG levels should be regulated and probably so prior to the PBG D enzymatic step.

Since the heme biosynthetic pathway in *E. coli* has a branch at uroporphyrinogen III, this compound is a possible effector. Its role as a potential regulator needs to be studied further since branch-point compounds are known to modulate regulatory steps in biosynthetic pathways.

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