APPLIED AND ENVIRONMENTAL MICROBIOLOGY, July 1994, p. 2431–2437
0099-2240/94/$04.00+0

Vol. 60, No. 7

Effect of Biosynthetic Manipulation of Heme on Insolubility of *Vitreoscilla* Hemoglobin in *Escherichia coli*

ROGER A. HART,† PAULI T. KALLIO,‡ AND JAMES E. BAILEY*

Division of Chemistry and Chemical Engineering, California Institute of Technology, Pasadena, California 91125

Received 15 December 1993/Accepted 10 May 1994

***Vitreoscilla* hemoglobin (VHb) is accumulated at high levels in both soluble and insoluble forms when expressed from its native promoter on a pUC19-derived plasmid in *Escherichia coli*. Examination by atomic absorption spectroscopy and electron paramagnetic resonance spectroscopy revealed that the insoluble form uniformly lacks the heme prosthetic group (apoVHb). The purified soluble form contains heme (holoVHb) and is spectroscopically indistinguishable from holoVHb produced by *Vitreoscilla* cells. This observation suggested that a relationship may exist between the insolubility of apoVHb and biosynthesis of heme. To examine this possibility, a series of experiments were conducted to chemically and genetically manipulate the formation and conversion of 5-aminolevulinic acid (ALA), a key intermediate in heme biosynthesis. Chemical perturbations involved supplementing the growth medium with the intermediate ALA and the competitive inhibitor levulinic acid which freely cross the cell barrier. Genetic manipulations involved amplifying the gene dosage for the enzymes ALA synthase and ALA dehydratase. Results from both levulinic acid and ALA supplementations indicate that the level of soluble holoVHb correlates with the heme level but that the level of insoluble apoVHb does not. The ratio of soluble to insoluble VHb also does not correlate with the level of total VHb accumulated. The effect of amplifying ALA synthase and ALA dehydratase gene dosage is complex and may involve secondary factors. Results indicate that the rate-limiting step of heme biosynthesis in cells overproducing VHb does not lie at ALA synthesis, as it reportedly does in wild-type *E. coli* (S. Hino and A. Ishida, Enzyme 16:42–49, 1973).**

Many heterologous proteins aggregate intracellularly when expressed in recombinant hosts (11, 21, 23). This phenomenon is best documented for protein expression in the gram-negative bacterial host *Escherichia coli*. However, it is not confined exclusively to this bacterium or for that matter to prokaryotic hosts (16, 25). The mechanisms underlying this intracellular aggregation are not well understood and may vary with protein, expression construct, and host. A common theme, however, appears to be malfunction or disruption of intracellular protein folding. Mitraki and King (21) have reviewed the ways that intracellular protein folding may malfunction and lead to insolubility.

Vitreoscilla hemoglobin (VHb) is a soluble homodimeric heme protein expressed by the obligate aerobic bacterium *Vitreoscilla* sp. in response to low oxygen concentration (24). This protein can be expressed from its native promoter in recombinant *E. coli* under similar hypoxic conditions (3, 12–14). VHb is accumulated concurrently in both soluble and insoluble forms when expressed in *E. coli* from a high-copy-number pUC19-derived plasmid under the control of its native promoter (7). The soluble form is found to partition between the cytoplasmic and periplasmic spaces despite the absence of a processed N-terminal signal peptide (15). Similar compartmentalization is observed in the native host *Vitreoscilla* sp. The insoluble form accumulated in *E. coli* is only found aggregated in cytoplasmic inclusion bodies (7). The principal contaminants of VHb inclusion body preparations are derived from cell wall debris which cosediment with inclusion bodies during centrifugation (7).

In their insightful review, Mitraki and King discussed the role of cofactors in protein folding and speculated that their absence during in vivo protein folding could lead to aggregation and inclusion body formation (21). Accordingly, one might expect apoprotein (protein lacking its necessary cofactor) aggregation whenever the ratio of net polypeptide synthetic rate to net cofactor synthetic rate exceeds their stoichiometric binding ratio. Such a state would arise if the cofactor biosynthetic pathway enzymes were saturated at maximal values and consequently unable to respond to a demand for increased flux. In the present case involving a heme protein, one would expect simultaneous accumulation of soluble and insoluble proteins with the two forms distinguishable by their heme contents.

Comparison between the VHb polypeptide accumulation rate and the reported enhanced *E. coli* heme accumulation rate (8, 10) suggests that the inadequate biosynthesis of heme may be involved in the insolubility of VHb in vivo. Soluble VHb accumulates during stationary phase when expressed in *E. coli* at a fairly constant rate of 0.2 μmol/g (dry wt)/h to a final concentration of 3.0 μmol/g (dry wt), which represents approximately 10% of total protein. This rate is consistent with previous analyses which indicate that under favorable conditions *E. coli* is capable of synthesizing approximately 0.2 μmol of heme per g (dry wt) per h during stationary phase (10). Accumulation of insoluble VHb during stationary phase varies but is generally highest, approximately 0.5 μmol/g (dry wt)/h, during phases exhibiting the accumulation of metabolic acids. In this study we investigate the biosynthetic limitation of heme as a possible mechanism for the in vivo aggregation of the insoluble form of VHb lacking the heme prosthetic group (apoVHb). The relationship between heme content and in vivo solubility was analyzed by physical characterization of the soluble and insoluble forms of VHb. The relationship between

* Corresponding author. Present address: Institut für Biotechnologie, ETH-Hönggerberg, CH-8093 Zürich, Switzerland. Phone: 411 633 3170. Fax: 411 371 06 58.

† Present address: Department of Recovery Process Research and Development, Genentech, Inc., South San Francisco, CA 94080.

‡ Present address: Institut für Biotechnologie, ETH-Hönggerberg, CH-8093 Zürich, Switzerland.

the biosynthetic activity of heme and the insolubility of VHb was investigated by genetic and chemical perturbation of the relative rates of polypeptide and heme syntheses. Such investigations may prove to be generally useful for recombinant expression of cofactor-requiring proteins.

MATERIALS AND METHODS

Bacterial strains and plasmids. *E. coli* JM101 with the genotype [*supE thi* Δ(*lac-proAB*) F′ *traD36 proAB lacI*^{q} Δ*lac*Δ*M15*] was used as the host strain throughout (26). Plasmid pRED2 was constructed from pUC19 (26) by insertion of a 2.2-kb *Hin*dIII fragment which contains the *vhb* gene under the control of its native promoter (12). Plasmid pVSP1 was constructed from pRED2 by insertion of a 2.9-kb *Bam*HI-*Hin*dIII fragment isolated from pJL68 (17, 18) which contains the *hemA* gene under the control of its native promoter. Plasmid pVDP1 was constructed from pRED2 by insertion of a 1.6-kb *Pst*I fragment isolated from pJL2 (20) which contains the *hemB* gene under the control of its native promoter. The *hemA* and *vhb* genes in pVSP1 were in the same orientation, while the *hemB* and *vhb* genes in pVDP1 were in the opposite orientation.

Media and growth conditions. Phosphate-buffered LB medium (10 g of Bacto Tryptone per liter, 5 g of Bacto Yeast Extract per liter, 5 g of NaCl per liter, 3 g of K_2HPO_4 per liter, 1 g of KH_2PO_4 per liter; pH 7) supplemented with 100 μg of ampicillin per ml was used as the basal medium in all cases. For atomic absorption spectroscopy, cells were grown by a fed-batch procedure, harvested, washed, lysed, and fractionated by differential centrifugation, using methods described previously (7). For heme perturbation studies, capped side-arm shake flasks (300 ml; Belco) containing 50 ml of medium were inoculated with 0.5 ml of a culture grown overnight and shaken at 250 rpm in a rotary shaker (New Brunswick) at 37°C. When the absorbance (Klett 54 filter) of the culture reached 150 Klett units, cells were harvested by centrifugation at 37°C and resuspended in prewarmed fresh medium to the same absorbance. The resulting stock culture was then aliquoted into culture tubes (Kimax; 16 by 150 mm) to a 5-ml volume and grown in a reciprocating water bath shaker (Reichert-Jung) at 150 cycles per min and 37°C. Culture tubes were used for VHb induction to ensure uniform microaerobic conditions necessary for controlled activation of the *vhb* promoter. All cells lacked VHb at the time of tube inoculation as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and difference absorption spectroscopy (<0.06 μmol/g [dry wt]). Culture tubes were supplemented as indicated with either levulinic acid or 5-aminolevulinic acid (ALA) from neutral-pH 1 M stock solutions and incubated for 12 h. All cultivations and analyses were conducted in duplicate. A Klett absorbance cell density of 150 is obtained in shake flask cultivation during late exponential growth of all cultures investigated. Test tube cultures grown from colony inoculation, however, reach a cell density of only 150 Klett units in late stationary phase, presumably because of oxygen transfer limitations. Cell growth in culture tubes inoculated with stock culture having a cell density of 150 Klett units is consequently considered microaerobic.

Atomic absorption spectroscopy sample preparation. Insoluble lysate fractions were obtained from late-stationary-phase cultures having the same optical density. Stocks were prepared from the insoluble lysate fraction by washing the fraction extensively with 50 mM potassium phosphate (pH 7.0)–10 mM EDTA followed by washing and resuspension in 50 mM potassium phosphate (pH 7.0). The resulting JM101::pRED2 debris stock contained approximately 0.5 mM VHb polypeptide, as determined by quantitative SDS-PAGE. Resulting *E. coli* JM101::pUC19 debris stock contained the same amount of cell wall material, as judged by the levels of the outer membrane proteins OmpA, OmpF, and OmpC. Inclusion body analogs were prepared by titrating purified VHb stock into JM101::pUC19 debris stock to achieve a protein composition comparable to that of JM101::pRED2 debris stock. VHb was purified from the soluble lysate fraction of JM101::pRED2 by previously described procedures (5). The concentration of VHb in purified stock solutions was determined by amino acid analysis and difference absorption spectroscopy.

Atomic absorption spectroscopy. Cell debris stocks, purified VHb stock, and inclusion body analogs were prepared for analysis by one of two methods. In the first method, samples were diluted with ultrapure HCl (Baker Ultrex Ultrapure HCl) and glass-distilled water to give a final HCl concentration of 1.2 N. In the second method, samples were hydrolyzed by vapor-phase hydrolysis in constantly boiling HCl at 165°C for 1 h, resuspended in 1.2 N Ultrapure HCl, and filtered through 0.22-μm-pore-size filters. Resulting samples were analyzed with an Instrumentation Laboratory Atomic Absorption Spectrometer following the procedures suggested by the Instrumentation Laboratory. Samples analyzed by the first method systematically gave lower measurements (typically by 20%) than samples analyzed by the second method. This error is attributed to a reduced flow rate through the needle orifice for samples containing particulate matter.

Heme perturbation sample preparation. Following cultivation, culture density was determined and 3-ml samples were collected. Cells were isolated by centrifugation, washed with a solution containing 100 mM Tris-HCl, 50 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, and 0.1 mM phenylmethylsulfonyl fluoride, and resuspended in 1 ml of the same solution. Cells were lysed by sonication on ice with a Heat Systems Ultrasonicator. Soluble and insoluble fractions were separated by centrifugation at 14,000 × *g* for 5 min. Soluble fractions were immediately assayed for the soluble form of VHb containing heme (holoVHb). The total proteins in soluble and insoluble fractions were separately isolated by 10% trichloroacetic acid precipitation. Residual trichloroacetic acid was removed with ethanol-ethyl ether (1:1) washes. Resulting samples were stored at −70°C for later electrophoresis analysis.

VHb and heme quantitation. Soluble holoVHb levels were determined by visible absorption spectroscopy of soluble lysate fractions. The VHb monomer visible difference (carbon monoxide-bound VHb minus reduced VHb) extinction coefficient [ε(VHbCO−VHb at 419 nm)−ε(VHbCO−VHb at 437 nm)] determined by amino acid analysis is $1.067 \times 10^5 (M\ cm)^{-1}$. Purified VHb exists naturally in the ferric state, presumably because of the absence of a reductase present in both *Vitreoscilla* sp. and *E. coli* (3). The Soret peak of VHb in examined samples had a maximum at 415 nm, indicating that the protein was present in the oxygenated state (22). Soluble and insoluble VHb polypeptide levels were determined by quantitative SDS-PAGE. Heme levels were determined by the pyridine hemochromogen assay using visible difference absorption spectroscopy (4).

Visible difference absorption spectroscopy. Samples were analyzed with a Shimadzu UV260 spectrophotometer interfaced to an IBM-XT computer. Matching 1-cm-path-length quartz microcuvettes were used with a temperature-controlled platform maintained at 25°C. Analyses were conducted in 50 mM potassium phosphate (pH 7.0). Reduced VHb was prepared by the addition of sodium dithionite. Carbon monoxide-bound VHb was prepared by reduction with sodium dithionite

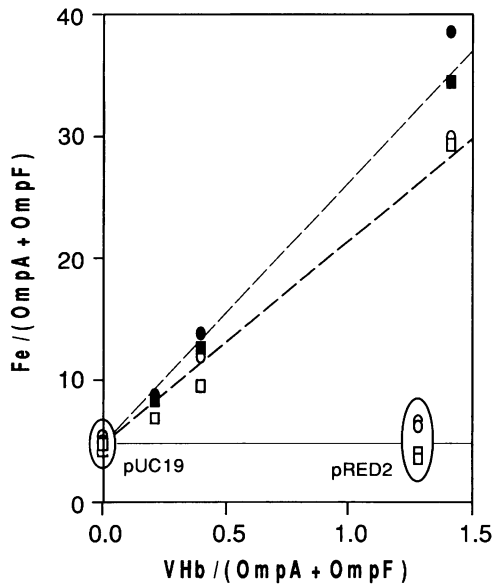

FIG. 1. Membrane-normalized iron contents of *E. coli* JM101::pUC19 and JM101::pRED2 insoluble lysate and inclusion body analogs. Open symbols represent values experimentally determined by iron analysis of the samples. Filled symbols represent values predicted from amino acid analysis (circle) and iron analysis (square) of purified VHb used for analog preparation. Samples were analyzed with (circle) and without (square) acid hydrolysis pretreatment as described in Materials and Methods. The units are relative units.

followed by incubation in carbon monoxide at 4 lb/in^2 for 30 min. Difference spectra relative to suitable buffer blanks were obtained in all cases. Serial dilution was used to ensure linearity of absorbance measurements.

Electrophoresis. SDS-PAGE was conducted with the Bio-Rad Protein II Multi Cell Electrophoresis system. Resolving gradient slab gels were composed from 10 to 20% acrylamide, 0.3 to 0.6% bisacrylamide, and 0.1% SDS with a discontinuous buffer stacking gel. Pellets from soluble and insoluble fractions were resuspended in sample buffer to a constant biomass concentration. Gels were run at 32.5 mA per gel and 4°C for 6 h and stained with Coomassie blue. Purified VHb was run as an internal standard on all gels to correct for variations in staining. Soluble and insoluble VHb polypeptide concentrations were determined by scanning Coomassie blue-stained electrophoresis gels with a Molecular Dynamics Computing Densitometer (Sunnyvale, Calif.). For quantitation, the band-integrated volume (absorbance intensity integrated over band area) was determined and the background-integrated volume (nonspecific background absorbance intensity integrated over an equal area) was subtracted to give the background-corrected band-integrated volume. The background-corrected band-integrated volume was then normalized by using the VHb internal standard to yield an absolute measure of VHb. All measurements were made within a background-corrected band-integrated volume range predetermined to vary linearly with VHb level.

RESULTS

Heme content of VHb inclusion bodies. To determine whether the soluble and insoluble forms of VHb produced in *E. coli* can be distinguished by heme content, the two forms were analyzed by atomic absorption spectroscopy and electron paramagnetic resonance spectroscopy. Isolates from the strain JM101::pRED2, which produces VHb from a high-copy-number pUC19-derived plasmid and the native *vhb* promoter, were used because their protein composition has been previously characterized (7). All *E. coli* insoluble lysate fractions are expected to contain iron because of the iron content of membrane-bound cytochromes. To account for the various amounts in the membrane among the different samples analyzed, measurements were normalized by the amount of the outer membrane proteins OmpA and OmpF. To determine how much iron would be present if all insoluble VHb contained iron, inclusion body analogs were prepared by titrating

TABLE 1. Effect of heme biosynthetic perturbation on heme accumulation

E. coli strain bearing plasmid	Amount (μmol/g [dry wt]) of heme accumulated					
	ALA (mM)			Levulinic acid (mM)		
	0	10	100	0	10	100
JM101::pUC19	<0.06	<0.06	0.17	<0.06	<0.06	<0.06
JM101::pRED2	0.98	1.17	0.68	0.98	1.02	0.66
JM101::pVDP1	0.19	0.31	0.24	0.19	0.17	0.14
JM101::pVSP1	1.00	0.82	0.49	1.00	0.62	0.22

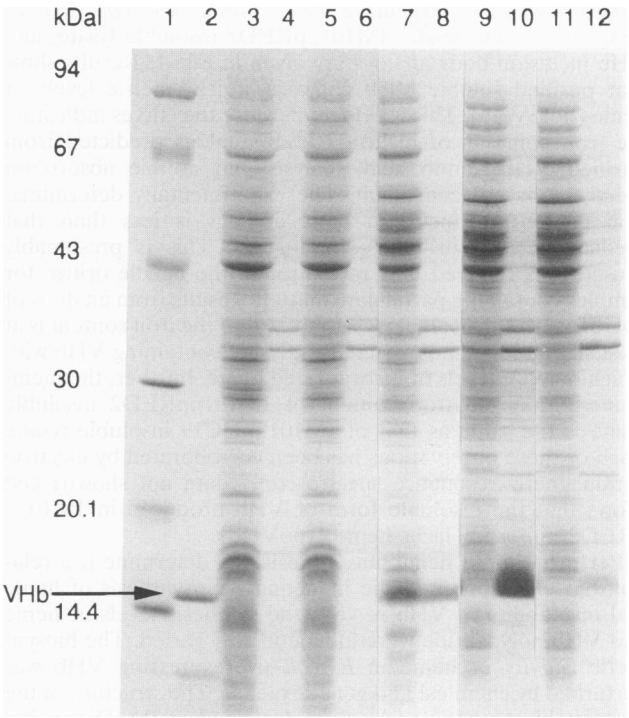

FIG. 2. Coomassie blue-stained SDS-polyacrylamide gel of lysate fractions from cells expressing VHb. Soluble (lanes 7, 9, and 11) and insoluble (lanes 8, 10, and 12) fractions of *E. coli* JM101::pRED2 (lanes 7 and 8), JM101::pVSP1 (lanes 9 and 10), and JM101::pVDP1 (lanes 11 and 12) following induction show the extent of VHb insolubilization. Comparisons with soluble (lanes 3 and 5) and insoluble (lanes 4 and 6) fractions of JM101::pUC19 (lanes 3 and 4) and JM101::pRED2 (lanes 5 and 6) prior to induction indicate protein pattern changes accompanying microaerobic incubation. Purified VHb standard (lane 2) and protein molecular mass markers (lane 1 [94, 67, 43, 30, 20.1, and 14.4 kDa]) are included for reference.

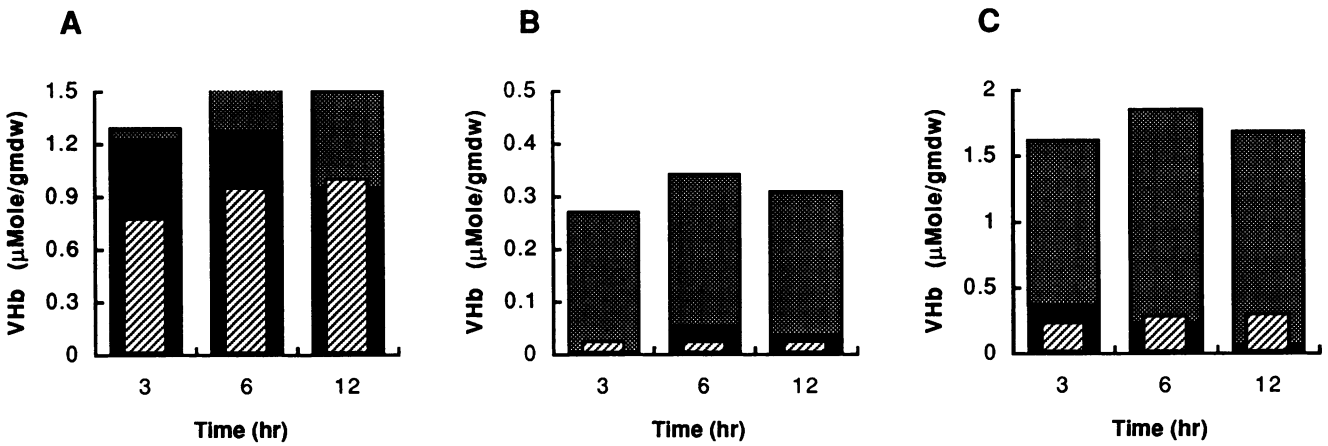

FIG. 3. Time course of VHb speciation following induction of *E. coli* JM101::pRED2 (A), JM101::pVDP1 (B) and JM101::pVSP1 (C). The outside stacked columns show soluble (dark shading) and insoluble (light shading) VHb polypeptide levels determined by SDS-PAGE. The inside columns show soluble holoVHb levels (stripes) determined by difference absorption spectroscopy.

purified VHb (5) into *E. coli* JM101::pUC19 insoluble lysate. The iron-to-polypeptide stoichiometry of the purified VHb stock was determined by amino acid analysis and atomic absorption spectroscopy to ensure that the titrated iron originated from VHb.

The membrane-normalized iron contents of *E. coli* JM101::pUC19 insoluble lysate, JM101::pRED2 insoluble lysate, and VHb inclusion body analogs are given in Fig. 1. Results show that purified soluble VHb contains stoichiometric levels of heme (holoVHb). This is evident because the curves indicating the iron contents of inclusion body analogs predicted from purified VHb amino acid analysis and atomic absorption spectroscopy are coincident. The experimentally determined iron content of inclusion body analogs is less than that predicted from purified VHb analysis. This is presumably caused by a reduced flow rate through the needle orifice for samples containing particulate matter. Results from analysis of JM101::pRED2 insoluble lysate show that the iron content is at least sixfold less than expected for lysate containing VHb with stoichiometric levels of incorporated heme. Further, the membrane-normalized iron content of JM101::pRED2 insoluble lysate is the same as that of JM101::pUC19 insoluble lysate. Each of these observations has been corroborated by electron paramagnetic resonance spectroscopy (data not shown) and shows that the insoluble form of VHb produced in JM101::pRED2 uniformly lacks heme (apoVHb).

Perturbation of heme biosynthesis. To determine if a relationship exists between the inadequate biosynthesis of heme and insolubility of VHb in vivo, the synthesis levels of heme and VHb polypeptide were independently varied. The biosynthetic activity of heme in *E. coli* overexpressing VHb was perturbed by chemical and genetic means. The structure of the putative biosynthetic pathway is described in the Discussion section of this article. Because the reported rate-limiting step in heme biosynthesis in *E. coli* lies at synthesis of ALA, ALA availability was increased by supplementing the medium. Levulinic acid, a competitive inhibitor of ALA dehydratase, was used for specific negative perturbation of heme biosynthesis. Additionally, the gene copy number for the enzyme ALA synthase, which catalyzes the formation of ALA from glutamate, was amplified by inserting the *hemA* structural gene under the control of its native promoter into the plasmid pRED2, yielding the plasmid pVSP1. To address a possible

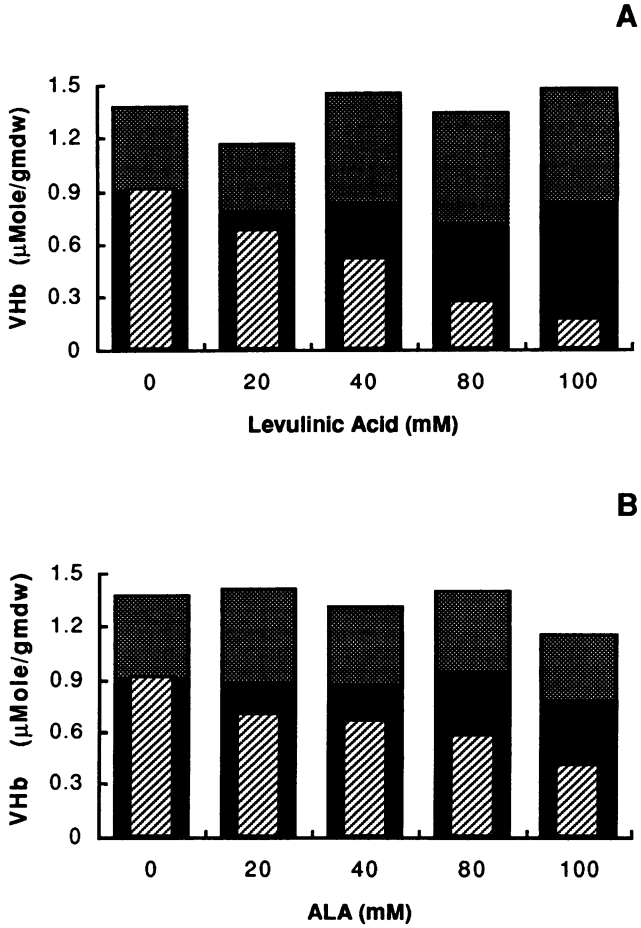

FIG. 4. Effect of levulinic acid (A) and ALA (B) supplementation on VHb accumulation by *E. coli* JM101::pRED2. The outside stacked columns show soluble (dark shading) and insoluble (light shading) VHb polypeptide levels determined by SDS-PAGE. The inside columns show soluble holoVHb levels (stripes) determined by difference absorption spectroscopy.

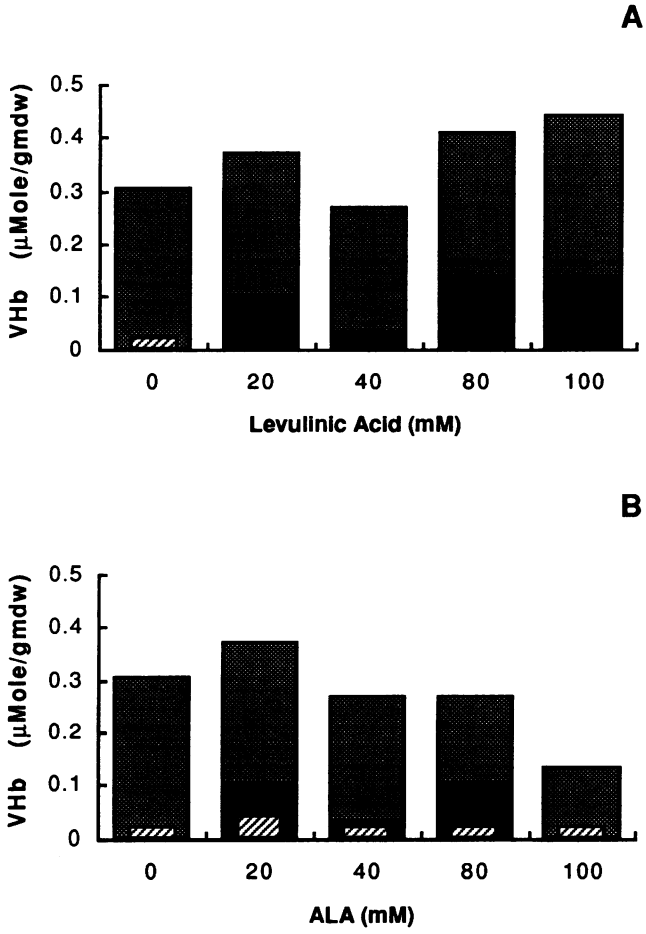

FIG. 5. Effect of levulinic acid (A) and ALA (B) supplementation on VHb accumulation by *E. coli* JM101::pVDP1. The outside stacked columns show soluble (dark shading) and insoluble (light shading) VHb polypeptide levels determined by SDS-PAGE. The inside columns show soluble holoVHb levels (striped) determined by difference absorption spectroscopy.

limitation in the synthetic activity of porphobilinogen, the copy number of the gene for the enzyme ALA dehydratase was amplified by inserting the *hemB* structural gene under the control of its native promoter into the plasmid pRED2, yielding the plasmid pVDP1.

Effect of perturbations on heme accumulation. The described perturbations have various effects on heme accumulation as shown in Table 1. High-level expression of VHb in *E. coli* itself leads to a dramatic increase (at least 20-fold) in the accumulation of heme, as evidenced by comparison of heme levels in cells bearing the plasmids pRED2 and pVSP1 with the heme level in cells bearing the parent plasmid pUC19. Heme levels in the former cells reach levels of 1 μmol/g (dry wt). Heme levels in cells bearing pVDP1, however, are significantly lower (approximately 0.2 times) than those in cells bearing either pRED2 or pVSP1 yet are higher than heme levels in cells bearing pUC19. This trend parallels relative VHb accumulation in these constructs (next section).

Supplementing ALA to cells bearing plasmids pUC19 and pVDP1 enhances heme accumulation. At moderate concentration (10 mM), ALA also enhances heme accumulation in pRED2; however, at higher concentrations (100 mM), it inhibits heme accumulation. ALA at all concentrations inhibits heme accumulation in cells bearing pVSP1.

Supplementing levulinic acid leads to inhibition of heme accumulation in all constructs which express VHb. Cells bearing pVSP1 are most susceptible, showing the highest inhibition at both moderate (10 mM) and high (100 mM) concentrations. A concentration of 5 mM levulinic acid is commonly used for ALA dehydratase inhibition in cell extracts from *E. coli* (19). In general, cells bearing pVSP1 behave similarly when the medium is supplemented with either ALA or levulinic acid, exhibiting increased inhibition with increasing concentration.

Effect of perturbations on VHb insolubilization. Cells bearing the plasmids pRED2, pVDP1, and pVSP1 accumulate very different levels of VHb, as shown in the SDS-polyacrylamide gel in Fig. 2. Prior to induction of the *vhb* promoter, the protein patterns of the soluble and insoluble fractions of the *E. coli* JM101::pRED2 (lanes 5 and 6) are very similar to those of its parent, JM101::pUC19 (lanes 3 and 4). Following transfer to microaerobic conditions, JM101::pRED2 accumulates large quantities of both soluble and insoluble VHb (lanes 7 and 8). By comparison, JM101::pVSP1 produces similar levels of VHb when induced (lanes 9 and 10) while JM101::pVDP1 produces much lower levels (lanes 11 and 12). The protein patterns of the different strains expressing VHb under microaerobic conditions are, otherwise, very similar.

The formation of different forms of expressed VHb, namely, soluble, insoluble, and holo forms, over time in cells bearing pRED2, pVDP1, and pVSP1 is very different, as shown in Fig. 3. *E. coli* JM101::pRED2 accumulates the majority of its VHb within 3 h of induction. At this time, most polypeptide is present in a soluble form, but only approximately 50% contains heme. Between 3 and 12 h, soluble apoVHb levels decline, leading to increases in both soluble holoVHb and insoluble apoVHb levels. After 12 h of cultivation, JM101::pRED2 contains approximately 1 μmol of soluble holoVHb per g (dry wt), no soluble apoVHb, and 0.5 μmol of insoluble apoVHb per g (dry wt). Both JM101::pVDP1 and JM101::pVSP1 also accumulate the majority of their VHb within 3 h of induction. Unlike JM101::pRED2, however, the formation of VHb species in these strains does not change significantly over the following 9 h of induction. After 12 h of cultivation, JM101::pVSP1 accumulates VHb to a level of approximately 1.7 μmol/g (dry wt), but only 25% contains heme and is soluble. In JM101::pVDP1, VHb is accumulated to a much lower level, approximately 0.3 μmol/g (dry wt) with an insignificant fraction being soluble and containing heme.

Response of VHb solubility to the competitive inhibitor levulinic acid is expected to be a good indicator of the sensitivity of insolubilization to heme availability. Levulinic acid supplementation does not affect the postinduction growth properties of either *E. coli* JM101::pRED2 or JM101::pVDP1 (data not shown). As shown in Fig. 4, however, levulinic acid does affect soluble holoVHb accumulation in JM101::pRED2. In particular, a monotonic decrease in the level of soluble holoVHb with increased levulinic acid concentration is observed when the concentration of levulinic acid exceeds 10 mM. Importantly, while the level of soluble holoVHb is decreased, the levels of soluble VHb and insoluble VHb are unaffected. Supplementing levulinic acid to JM101::pVDP1 cultures, shown in Fig. 5, slightly increases total VHb accumulation but does not affect the accumulation of insoluble VHb.

If the biosynthetic activity of heme in VHb-producing cells is limited by the ALA synthesis, supplementing this intermediate should increase flux through the pathway. ALA supplementation has previously been shown to increase heme biosynthesis in anaerobically or aerobically grown *E. coli* when incubated

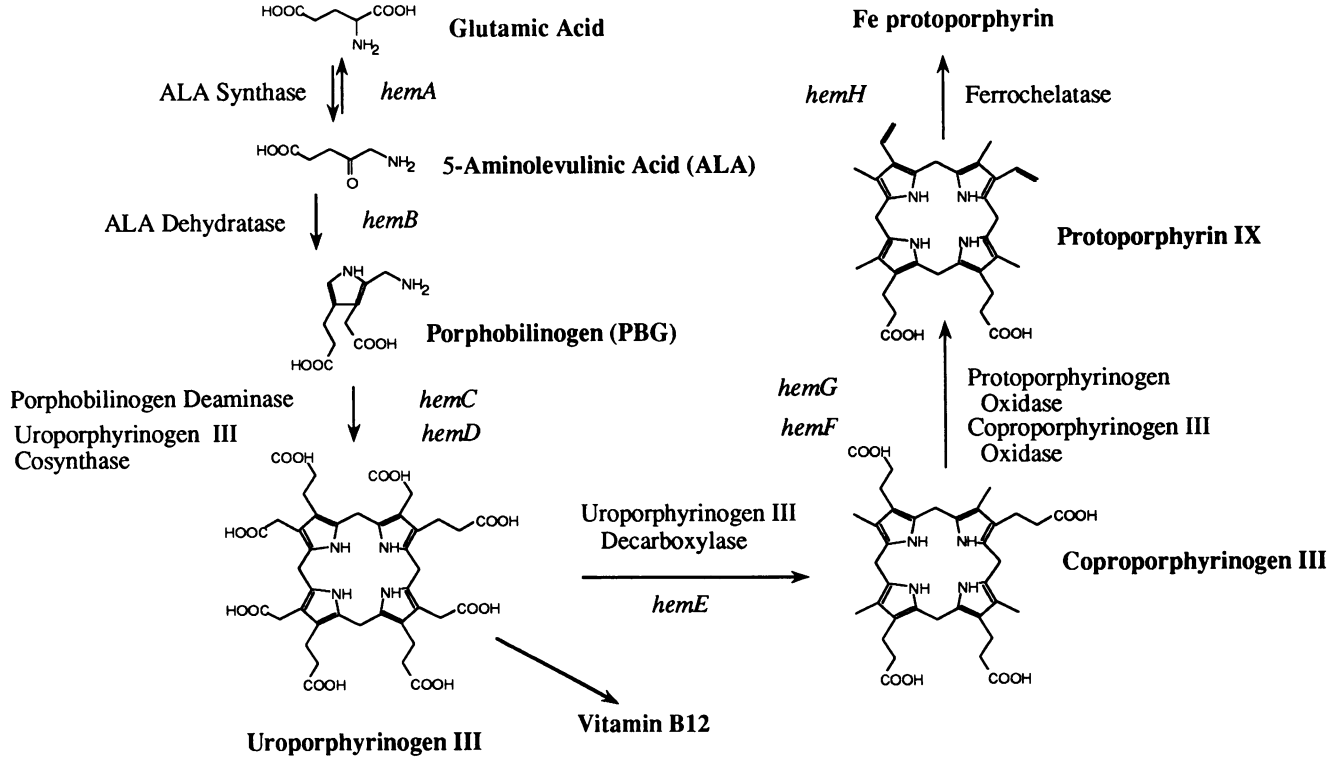

FIG. 6. Diagram of heme biosynthetic pathway of *E. coli*. The intermediates (in boldface type) and their structures, enzymes (in roman type), and genes (in italic type) required for the conversion of glutamate to heme are shown.

under aerobic conditions (10). As shown in Fig. 4, adding ALA to JM101::pRED2 cultures has qualitatively the same effect as adding levulinic acid. At concentrations above 10 mM, ALA is inhibitory to holoVHb production while it has little effect on total VHb level or partitioning between soluble and insoluble forms. Adding ALA to JM101::pVDP1 at concentrations in excess of 10 mM appears inhibitory to total VHb accumulation (Fig. 5). Results from supplementing with levulinic acid and ALA indicate that the level of holoVHb is dependent on heme levels but that the levels of insoluble apoVHb are not.

DISCUSSION

The pathway governing heme biosynthesis (Fig. 6) is complex and largely conserved among the plant, animal, and protista kingdoms (1). The most notable difference in the pathway between different organisms occurs in the synthesis of ALA. Most facultative aerobic bacteria, including *Vitreoscilla* species (2), synthesize ALA by the C_4 pathway from succinyl coenzyme A and glycine (19). *E. coli*, alternatively, synthesizes ALA by the C_5 pathway from the intact five-carbon chain of glutamate (19). The enzyme which catalyzes this transformation, ALA synthase, shows no amino acid homology with any other cloned ALA synthase (17). Previous studies on wild-type *E. coli* suggest that ALA synthesis is the rate-limiting step in the heme biosynthetic pathway (8, 10). Upon alleviating the limitation in ALA synthesis, the new limiting step may lie at porphobilinogen synthesis. ALA dehydratase, the enzyme responsible for this latter conversion, is competitively inhibited by levulinic acid (20).

Under hypoxic conditions, *Vitreoscilla* cells synthesize VHb polypeptide, NADH-methemoglobin reductase and ALA synthase (2). The increase in ALA synthase levels with VHb polypeptide synthesis presumably allows the heme biosynthetic pathway to respond to the greater demand for heme. Expression of VHb in *E. coli* leads to increased production of heme (3, 12). It is not known if this is accomplished by derepression of heme synthesis or by an amplification of ALA synthase levels as occurs in *Vitreoscilla* species.

The results described in this report suggest that ALA synthesis is not the rate-limiting step of heme biosynthesis in *E. coli* strains expressing high levels of VHb. This is evident since neither enhanced expression of ALA synthase or supplementing medium with ALA leads to significant increases in heme or soluble holoVHb levels. In fact, ALA supplementation resulted in decreased heme levels and ALA synthase amplification led to reduced levels of soluble holoVHb. The general similarity between these results and those observed with levulinic acid suggests that increased ALA levels actually inhibit heme synthesis in strains expressing high levels of VHb.

Spectroscopic analysis clearly shows insoluble VHb uniformly lacks heme. Perturbation of heme accumulation with analysis of VHb accumulation and solubility also shows that insolubilization is independent of heme availability. This condition is possible, because soluble VHb may or may not contain stoichiometric levels of heme, depending on the time postinduction and culture conditions. Given sufficient time, the recombinant cells can apparently synthesize sufficient heme to satisfy soluble VHb stoichiometric requirements. During this time delay, however, soluble apoVHb, which is known to be less thermodynamically stable than holoVHb (6), can be partly insolubilized. Soluble apoprotein was also observed during optimized expression of human hemoglobin in *E. coli* from a synthetic operon composed of α- and β-globin genes (9).

Further research is required to determine whether these soluble apoproteins accumulate because of inadequate heme biosynthesis during high-level globin expression in recombinant *E. coli.*

ACKNOWLEDGMENTS

We thank Sharon Cosloy for providing the genes for ALA synthase and ALA dehydratase and Alexander Sassarman for helpful comments regarding heme biosynthesis in *E. coli.*

This research was supported by the National Science Foundation (grant EET-8606179), by the Advanced Industrial Concepts Division of the U.S. Department of Energy, and by a grant for predoctoral training in biotechnology from the National Institute of General Medical Sciences (National Research Service Award 1 T32 GM 08346-01, Pharmacology Sciences Program).

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