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Non-iron porphyrins cause tumbling to blue light by an *Escherichia coli* mutant defective in *hemG*

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ABSTRACT **Previously we showed that an *Escherichia coli hemH* mutant, defective in the ultimate step of heme synthesis, ferrochelatase, is somewhat better than 100-fold more sensitive than its wild-type parent in tumbling to blue light. Here we explore the effect of a *hemG* mutant, defective in the penultimate step, protoporphyrinogen oxidase. We found that a *hemG* mutant also is somewhat better than 100-fold more sensitive in tumbling to blue light compared to its wild-type parent. The amount of non-iron porphyrins accumulated in *hemG* or *hemH* mutants was more than 100-fold greater than in wild type. The nature of these accumulated porphyrins is described. When heme was present, as in the wild type, the non-iron (non-heme) porphyrins were maintained at a relatively low concentration and tumbling to blue light at an intensity effective for *hemG* or *hemH* did not occur. The function of tumbling to light is most likely to allow escape from the lethality of intense light.**

The last step in the biosynthesis of heme in *Escherichia coli* and other organisms is the incorporation of Fe^{2+} into protoporphyrin IX (1–6), as shown in Fig. 1 (7). This step is catalyzed by ferrochelatase, encoded by the *hemH* gene (1, 2, 4). Mutations in the *hemH* gene decrease or eliminate ferrochelatase and thereby cause accumulation of non-iron porphyrins; this accumulation reduces the viability of *E. coli* upon exposure to blue light (4). We have shown (11) that a *hemH* mutant tumbles to low intensities of blue light and that this tumbling occurs at an intensity of light lower than the intensity needed to kill, so the tumbling presumably has survival value.

The immediate precursor of protoporphyrin IX is protoporphyrinogen IX; this conversion is catalyzed by protoporphyrinogen oxidase, encoded by the *hemG* gene (Fig. 1). In the present work, we study light-induced tumbling by a *hemG* mutant. The mutant we used is a derivative of the *E. coli hemG* mutant SASX38, described by Săsărman *et al.* (12, 13). Recently another *hemG* mutant VSR751 was isolated from *E. coli* by Nishimura *et al.* (7).

We report that the *hemG* mutant AW823 tumbles to blue light at least as sensitively as the *hemH* mutant. In contrast, *E. coli* containing heme does not tumble to that intensity of blue light.

MATERIALS AND METHODS

Conditions of Growth. The bacteria were grown at 35°C in tryptone broth (1% Difco tryptone/0.5% NaCl), in Luria broth (1% Difco tryptone/0.5% Difco yeast extract/0.5% NaCl), in Difco brain-heart infusion (12), or in Vogel–Bonner minimal medium (14) containing 25 mM sodium DL-lactate and required amino acids (each at 1 mM).

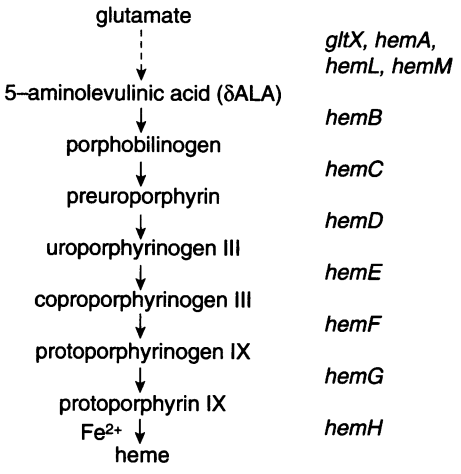

FIG. 1. Heme biosynthesis pathway in eubacteria and chloroplasts of algae and higher plants (modified from ref. 7). In yeast and in mammalian cells, the reactions leading to 5-aminolevulinic acid are different from those above, but the subsequent reactions are the same (7). For details of dashed line, see figure 1 of ref. 8 and ref. 9. Two additional genes have been found, *hemK* and *hemN* (10).

Bacterial Strains and Genetic Manipulations. All bacterial strains used are derivatives of *E. coli* K-12. We have described the *hemH* mutant AW804 (11) and its chemotactically wild-type parent AW405 (15).

Tight *hemG* mutants shut off the synthesis of porphyrins by inhibition of the synthesis of 5-aminolevulinic acid (T. Nakayashiki and H.I., unpublished results). The *hemG* mutant SASX38 (12) presumably is such a tight mutant. As such it was nonmotile because it grew too slowly (16), so a spontaneous fast-growing mutant, AW823, was selected by picking a large colony on a Luria-broth plate. When grown in brain-heart infusion, the doubling time of AW823 was 65 min, which was 1.8 times faster than SASX38. AW823 was capable of growing slowly in Vogel–Bonner minimal medium with sodium lactate and the required L-methionine, whereas SASX38 showed virtually no growth in this medium. Because AW823 grew quite slowly in tryptone broth or even in Luria broth, brain-heart infusion was used in the experiments reported in *Results*.

The growth of AW823 was sensitive to light (data not shown). This light-sensitive growth was rescued upon infection by λ phage carrying the *hemG* (ORF181) (7) gene. This phage is able to rescue the growth defect in the *hemG* mutants SASX38 and VSR751. Thus it appears clear that AW823 is a *hemG* mutant.

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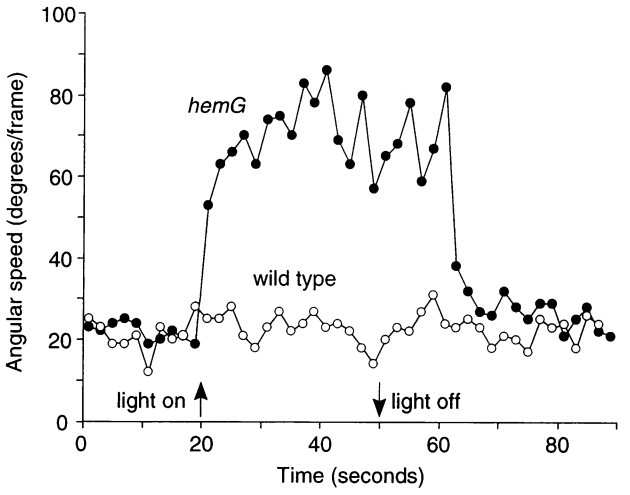

FIG. 2. Behavioral response to blue light by the *hemG* mutant AW823 and its revertant AW824. A blue-light stimulus (396–450 nm) at an intensity of 10 μmol of photons per m^2 per sec was given for 30 sec as indicated. Each point represents the average over a 2-sec period in two experiments for AW823 and one experiment for AW824.

The gene that could rescue the light-sensitive growth of AW823 had an 81% P1 (17) cotransduction rate (197 transductants were tested) with the *fadAB3165*::Tn*10*Kan marker of CAG18557 at 86.25 min on the *E. coli* chromosome (18). This cotransduction rate correlates with the linkage distance between the *fadAB3165*::Tn*10*Kan marker and the *hemG* gene, which is at 86 min (12). From this P1 transduction, we obtained a revertant AW824 (i.e., *hemG*$^+$) in the sense that its growth was resistant to light.

Porphyrins have a red fluorescence under UV light (19). Compared to SASX38, AW823 colonies on a Luria-broth plate or a brain-heart infusion plate had stronger red fluorescence when viewed under a UV lamp. This indicates that an additional mutation in AW823 (likely a second mutation in *hemG*) caused relatively fast growth by virtue of producing *hemG* with only a partial defect, and consequently more porphyrins accumulated in AW823 than in SASX38.

Study of Behavioral Response. Tumbling occurs when enteric bacteria swim toward increasing intensity of repellent or decreasing concentration of attractant (a spatial gradient) and running occurs when they swim toward decreasing intensity of repellent and increasing concentration of attractant (a spatial gradient). In the present study temporal gradients, rather than spatial gradients (20, 21), were used: The light was everywhere uniformly increased in intensity at once to produce tumbling or everywhere uniformly decreased at once to produce running.

The procedure for preparing cells for behavioral experiments, the apparatus for observing behavioral responses of cells to light (22), and the computerized motion analysis system (35) were as described (11). Values of an average over every 2-sec period were obtained in each experiment. Two experiments were done for each stimulus unless otherwise noted.

Assay for Porphyrins. For extraction of porphyrins, AW405 and AW804 were grown as described in ref. 11 in 100 ml of Vogel–Bonner minimal medium (14) containing 25 mM sodium DL-lactate and the required amino acids L-histidine, L-leucine, and L-threonine (each at 1 mM); AW823 and AW824 were grown in 100 ml of brain-heart infusion. Cells were harvested at an A_{590} of 0.4–0.6 unit and were washed once with 10^{-2} M potassium Hepes at pH 7.0 plus 10^{-4} M potassium EDTA (Hepes-buffer EDTA). The procedure for subsequent porphyrin extraction with acetone/NH_4OH has been described (5). The final volume for the extract was 1.5 ml. In each sample, the same amount of cells was used for extraction. The fluorescence spectra of porphyrin extracts were measured by use of an SLM 8000C spectrofluorometer (SLM Instruments, Rochester, NY).

At different excitation wavelengths, the intensity of the excitation light from the spectrofluorometer is different; the fluorescence intensity was adjusted to compensate for these differences, and consequently it is called a relative fluorescence intensity. This has no units because it represents the fluorescence intensity divided by the excitation intensity.

At different emission wavelengths, the fluorescence emission intensity is described as a relative fluorescence intensity. This value was derived from the fluorescence intensity divided by a constant intensity of excitation at 405 nm, and consequently it has no units.

Chemicals. Hemin is a stable ferric (Fe^{3+}) form of the labile ferrous (Fe^{2+})-containing heme. Stock solutions of hemin (bovine, type I, Sigma) at 1 mg/ml or protoporphyrin IX (disodium salt, Sigma) at 1 mg/ml were prepared in a mixture

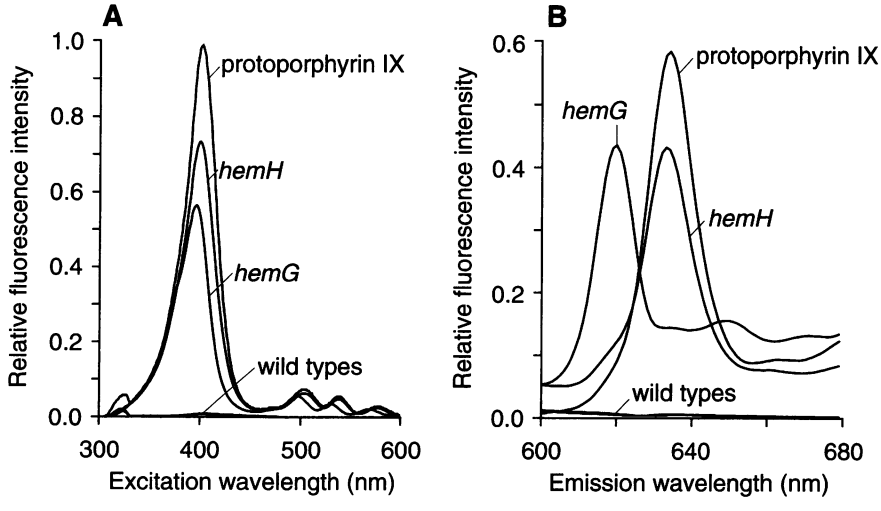

FIG. 3. Fluorescence spectra of acetone/NH_4OH extracts (5) of the *hemG* mutant AW823 and the *hemH* mutant AW804 and of the corresponding wild types AW824 and AW405, respectively. Curves for authentic protoporphyrin IX in acetone/NH_4OH are also included. (*A*) Fluorescence excitation spectra at 620 nm for AW823 and AW824 and at 633 nm for AW804, AW405, and authentic protoporphyrin IX. (*B*) Fluorescence emission spectra: the excitation wavelength for AW804, AW405, and protoporphyrin IX was 405 nm; the excitation wavelength for AW823 and AW824 was 395 nm.

Table 1. Accumulation of porphyrins

Strain	Genetic marker	Medium	Content of porphyrins, nmol/g (dry weight)		
			Protoporphyrin	Coproporphyrin	Uroporphyrin
AW405	Wild type	Vogel–Bonner minimal medium	<1.0*	<1.0*	<1.0*
AW804	*hemH*	Vogel–Bonner minimal medium	224.6	171.5	<1.0*
AW823	*hemG*	Brain-heart infusion	20.8	267.6	55.3
AW824	Revertant of AW823	Brain-heart infusion	<1.0*	<1.0*	<1.0*

Porphyrins were extracted and separated as described by Cox and Charles (1). Vogel–Bonner minimal medium contained 25 mM sodium DL-lactate, 1 mM L-histidine, 1 mM L-leucine, and 1 mM L-threonine. The results were dependent on the richness of the growth medium used; accumulation of porphyrins increased as conditions became poor. Thus for AW804 the total porphyrins were 15.0, 79.3, 196.9, and 449.4 nmol/g (dry weight) for brain-heart infusion, Luria broth, Vogel–Bonner D-glucose medium, and Vogel–Bonner sodium DL-lactate medium, respectively. *An entry of <1.0 means not detectable according to the limit of sensitivity of the technique used.

of acetone and NH_4OH (5). Hemin (final concentration, 15 μg/ml) or protoporphyrin IX (final concentration, 10 μg/ml) was used where indicated. Plates containing kanamycin (25 μg/ml) were prepared with Luria broth.

RESULTS

We have shown (11) that an *E. coli* mutant blocked in *hemH*, which encodes the ultimate step in the synthesis of heme, ferrochelatase (Fig. 1), tumbles to a low level (10 μmol of photons per m^2 per sec) of blue light (396–450 nm), while a strain capable of heme synthesis does not tumble to that intensity of light (11).

Mutant in *hemG* Is Repelled by Low-Level Blue Light. To investigate whether defects at steps other than *hemH* can cause increased behavioral response to blue light, we studied a mutant blocked in *hemG*, the penultimate step in the synthesis of heme (Fig. 1). *HemG* encodes protoporphyrinogen oxidase, which catalyzes the conversion of protoporphyrinogen IX to protoporphyrin IX (3).

The *hemG* mutant AW823 tumbles upon exposure to white light (data not shown). The blue range (396–450 nm) was used here because porphyrins typically absorb light at around 400 nm and because of the fluorescence excitation spectrum of porphyrin extract from *hemG* illustrated below.

The behavioral response of the *hemG* mutant AW823 was compared to that of its revertant (presumably heme-containing) AW824 upon irradiation with low-level blue light (Fig. 2). AW823 tumbled to blue light (396–450 nm) at an intensity of 10 μmol of photons per m^2 per sec, while AW824 had no response to the same blue light at the same intensity. The response by *hemG* terminated some 10 sec after the light was shut off; this indicates the production of some product that survives the light briefly (see *Discussion* for a possible mechanism).

Porphyrinogens undergo rapid oxidation under air to become porphyrins; a mutant that accumulates porphyrinogens consequently also contains corresponding porphyrins. In porphyrinogens the pyrrole rings are joined by saturated (—CH_2—) bridges, while in porphyrins they are joined instead by unsaturated ones (=CH—), which are part of a conjugation system (for example, see Fig. 5); consequently, porphyrinogens are colorless while porphyrins show light absorption, intensely around 400 nm (the Soret band) and weakly in the 450- to 650-nm region (3, 23). It must be this light absorption of consequent porphyrin that allows tumbling to light in strains accumulating porphyrinogen.

Measurement of Non-Iron Porphyrins in Mutants. When excited around 400 nm, porphyrins emit red fluorescence (19). Since porphyrinogens and metaloporphyrins, such as heme (Fe^{2+}-porphyrin) or hemin (Fe^{3+}-porphyrin), do not show this absorption around 400 nm, they do not show this fluorescence either (19). Fluorescence spectra are thus used as a way of assaying for non-iron (i.e., non-heme) porphyrins (19).

The fluorescence spectra of acetone/NH_4OH extracts (5) from *hemG* mutant AW823 and, for comparison, *hemH* mutant AW804 (Fig. 3) showed that the maximum excitation peak (Fig. 3*A*) of porphyrins in AW823, 395 nm, was different from that of AW804, 400 nm; the fluorescence emission spectrum (Fig. 3*B*) of AW823 showed a peak at 620 nm, which also was different from that of AW804, 633 nm. The curves for authentic protoporphyrin IX (Fig. 3) are close to the curves for AW804. These data indicate that the majority of porphyrins accumulated in an extract of AW823 is not protoporphyrin but other porphyrins, while the porphyrin in AW804 is largely protoporphyrin.

It has been reported that extracts of *hemG* mutants contain coproporphyrin, uroporphyrin, and a small amount of protoporphyrin, due to the autooxidation of coproporphyrinogen

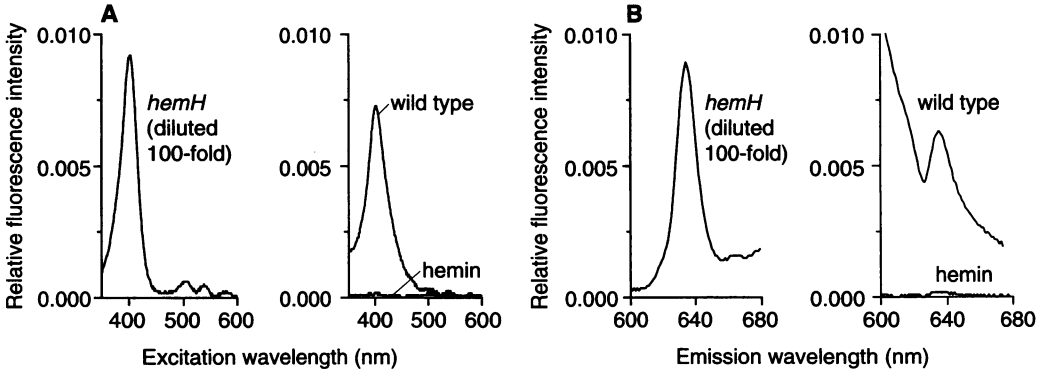

FIG. 4. Fluorescence spectra of acetone/NH_4OH extracts (5) of wild-type AW405 and *hemH* mutant AW804. The acetone/NH_4OH extract from AW804 was diluted 100-fold in acetone/NH_4OH. (*A*) Fluorescence excitation spectra at 633 nm. (*B*) Fluorescence emission spectra: The excitation wavelength was 405 nm for both AW405 and AW804. Hemin was used in acetone/NH_4OH at a final concentration of 15 μg/ml; note that hemin shows only weak fluorescence, as expected from ref. 19, and the fluorescent materials observed in Figs. 3 and 4 are, therefore, not due to hemin.

III, uroporphyrinogen III, and protoporphyrinogen IX, respectively (8, 12).

We determined the content of these various porphyrins in the strains we studied for phototaxis—namely, the *hemG* mutant AW823 and its revertant AW824, as well as in the *hemH* mutant AW804 (11) and its wild-type AW405 (11) (Table 1). An extract of the *hemG* AW823 contains protoporphyrin, coproporphyrin, and uroporphyrin, just like its *hemG* parent SASX38 (12), while an extract of the revertant AW824 lacks measurable quantities of these; and an extract of *hemH* AW804 contains protoporphyrin and coproporphyrin, while an extract of its wild-type AW405 lacks measurable quantities of these.

Thus the porphyrins corresponding to the porphyrinogens in *hemG*—namely, protoporphyrinogen IX, coproporphyrinogen III, and uroporphyrinogen III—apparently have effects similar to protoporphyrin IX and coproporphyrinogen III of *hemH*: They increase the sensitivity of the light-induced tumbling response relative to that found in heme-containing wild-type strains.

Measurement of Non-Iron Porphyrins in Wild Type. Clearly the amounts of non-iron porphyrins in AW823 and AW804 are much higher (somewhat more than 100-fold) than in the wild-type parents AW824 and AW405, respectively (Fig. 3). Do wild-type cells accumulate any non-iron porphyrins? As can be seen from Fig. 2, wild-type AW824 does not tumble to blue light at the intensity (10 μmol of photons per m^2 per sec) and wavelength (396–450 nm) that acts upon *hemG*. The same is true for wild-type AW405 compared to its *hemH* mutant AW804 (11).

The fluorescence spectra of acetone/NH_4OH extracts from wild-type AW824 and wild-type AW405 (Fig. 3) were analyzed at higher resolution. These enlarged spectra of AW405 (Fig. 4) and AW824 (data not shown) had similar patterns and sizes to the fluorescence spectra of porphyrins accumulated by the 100-fold diluted *hemH* mutant AW804 (Fig. 4) and by the 100-fold diluted *hemG* mutant AW823 (data not shown). This indicates that the wild-type cells do accumulate some relatively small amount of non-iron porphyrins. See similar indications of this in a study of *Salmonella typhimurium* (8). It should be noted that wild-type cells do tumble to intensely bright blue light (24–27).

DISCUSSION

Results in Fig. 2 show that a *hemG* mutant (Figs. 1 and 5), whose growth is light-sensitive, tumbles to blue light of low intensity (396–450 nm, 10 μmol of photons per m^2 per sec), while a normal relatively light-resistant heme-containing strain does not. We had shown (11) that a mutant in *hemH*, the gene for ferrochelatase (Figs. 1 and 5), also tumbles to such a low intensity of blue light.

Since light is lethal to bacteria, including *hemG* mutants (unpublished observation) and *hemH* mutants (4, 6), it would be advantageous for bacteria to escape from light at an intensity lower than that needed for killing.

The porphyrinogens and porphyrins that accumulate in the *hemG* mutant are for the most part different from those that accumulate in the *hemH* mutant (Fig. 3 and Table 1). It is known (8, 12) that *hemG* mutants accumulate largely coproporphyrinogen III (Fig. 1), uroporphyrinogen III (Fig. 1), and small amounts of protoporphyrinogen IX (Figs. 1 and 5), as well as their corresponding porphyrin analogs.

The amount of non-iron (non-heme) porphyrins in wild-type *E. coli* was at least 100-fold less than in the *hemG* or in the *hemH* mutants (Figs. 3 and 4); this correlates with the decreased sensitivity of the light-induced tumbling response in the wild type compared to the *hemG* mutant (Fig. 2) or the *hemH* mutant (11). Since some amount of non-iron porphyrins do accumulate in the wild type, however, this could possibly be responsible for some of the tumbling response of wild-type cells to very intense light (24–27), although that tumbling response has been attributed to perhaps flavoproteins (25).

We speculate on the mechanism of tumbling to blue light in the *hemG* mutant along the same lines indicated for *hemH* in ref. 11. Non-iron porphyrins are photosensitizers and produce an active species of oxygen that is harmful to the cell (ref. 28; for review, see ref. 11). Hydrogen peroxide, likely a conse-

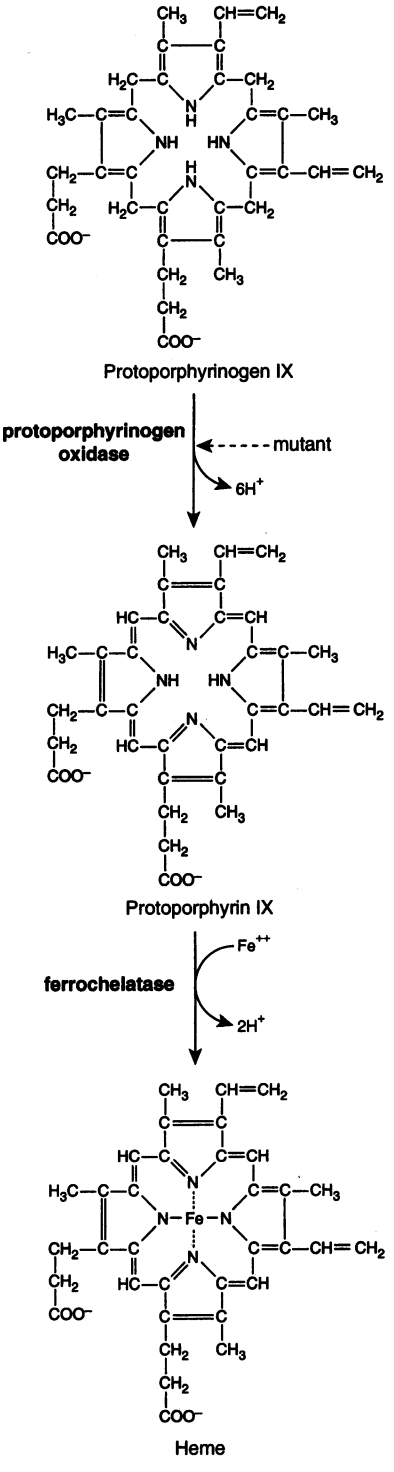

FIG. 5. Conversion of protoporphyrinogen IX to protoporphyrin IX is catalyzed by protoporphyrinogen oxidase. This reaction is deficient (dashed line) in human mutants having variegate porphyria and in bacterial *hemG* mutants. As a consequence of this mutation, the level of protoporphyrin IX and of heme is low in these mutants.

quence of these oxidative processes, has recently been shown by L. Benov and I. Fridovich (personal communication) to be a repellent for *E. coli*, so hydrogen peroxide could be the ultimate cause of tumbling to blue light in *E. coli*.

These light-induced responses are temporal; we have not yet shown any light-induced spatial responses. Until we do, the term "phototaxis" perhaps should not be used and perhaps should not have been used (11). For this kind of behavior the term "photophobic response" has been recommended (29).

The tumbling to low-intensity light in a *hemG* mutant of motile *E. coli* parallels the avoiding of light by human patients with severe cases of inherited variegate porphyria; these people have the same biochemical problem as bacterial *hemG* mutants, namely a defective enzyme for the oxidation of protoporphyrinogen IX to protoporphyrin IX (3) (Fig. 5). An example of a patient with variegate porphyria was described in this way (30): "On several occasions in the spring and summer the sun had produced a burning sensation of the exposed skin." We have compared (11) the avoidance of light in human patients with erythropoietic protoporphyria and in bacterial *hemH* mutants, where in both cases there is a defect in the ultimate step of heme synthesis, the enzymatic conversion of protoporphyrin IX to heme.

The biochemistry of heme biosynthesis is widely preserved, except for several preliminary steps leading to 5-aminolevulinic acid (see Fig. 1) (3, 8, 9). Two universally used early steps (*hemB* and *hemC* in Fig. 1) in heme synthesis precede the tetrapyrrole stage (3, 8); human diseases in which one or the other of these two steps are lacking are not accompanied by sensitivity to light (3), as expected if tetrapyrroles are needed for this sensitivity. In contrast, the five subsequent steps (*hemD* through *hemH*) in the synthesis of heme (Fig. 1) do involve tetrapyrrole or porphyrin structures (3, 8). A human deficiency is known in each of these five steps, and all five diseases (porphyrias) are marked by skin photosensitivity, among other symptoms (3). Extrapolating this information to *E. coli*, we would expect tumbling to light for mutants in each of these same five steps.

The amino acid sequences of the proteins that catalyze these terminal steps of heme biosynthesis (*hemD* through *hemH*) are quite similar in bacteria and humans insofar as these sequences have been determined: The similarities range from around 20% to 45% (31–33).

Related in structure to the phototaxis-involved closed-chain tetrapyrroles described in the present report are the phototropism-involved open-chain tetrapyrroles covalently bound to apoproteins: phycocyanin of cyanobacteria and phytochromes of plants (34).

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