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Ferrochelatase Activity and Protoporphyrin IX Utilization in *Haemophilus influenzae*

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Previous research showed that the heme-requiring human pathogen *Haemophilus influenzae* lacks the first six of the seven enzymes required for heme synthesis, starting with the precursor, 5-amino levulinic acid. In this study, I demonstrated either directly or by reasonable inference that all 57 strains of *H. influenzae* examined, including 2 unable to grow on protoporphyrin IX, possess ferrochelatase, which catalyzes heme formation by insertion of Fe^{2+} into the protoporphyrin IX nucleus and which is the last enzyme in the heme synthetic pathway. Further, I showed that this enzyme can also function in the reverse direction, releasing Fe^{2+} from heme.

Haemophilus influenzae, a gram-negative pathogen specific for humans, is unusual in that it is one of a small number of organisms that cannot synthesize heme (8). It does, however, require heme, at least for aerobic in vitro growth (6). *H. influenzae* has been shown to lack the first six of the seven enzymes in the heme synthetic pathway, but the last enzyme, ferrochelatase (protoheme ferrolyase; EC 4.99.1.1), which forms heme by catalyzing the insertion of Fe^{2+} into the immediate heme precursor, protoporphyrin IX, was assumed to be present since protoporphyrin IX could readily satisfy the *H. influenzae* heme requirement (8, 19). However, ferrochelatase activity per se has not heretofore been directly demonstrated to be present in *H. influenzae*.

Use of protoporphyrin IX to satisfy the heme requirement. Fifty-seven *H. influenzae* strains, including both type b encapsulated and nontypeable unencapsulated *H. influenzae* (NTHI), were tested for ability to grow from 2×10^7 CFU/ml to 5×10^9 CFU/ml at 37°C in aerated brain heart infusion medium supplemented with 2 μg of NAD per ml and 0.02 μg of protoporphyrin IX per ml. The latter is the lowest concentration of protoporphyrin IX (or heme) consistently supporting robust logarithmic growth, i.e., a doubling time of approximately 45 min, in our hands (data not shown). None of the strains grew more than a few divisions in the absence of a heme source, as determined by measurement of optical density at 600 nm. Of the 57 strains, all but 2, strains HE5 and 15, grew on protoporphyrin IX, showing that ability to use protoporphyrin IX as a heme source is common to most *H. influenzae* strains, as had been reported by others (10). The two strains unable to grow on protoporphyrin IX were among several conjunctivitis isolates from biogroup aegyptius; one (strain 15) had been described previously (19). Of interest, after continued incubation for 24 to 48 h in this medium both of the strains unable to grow on protoporphyrin IX yielded a turbid culture containing derivatives, single-colony isolates of which were as readily able to grow on protoporphyrin IX as were the other 55 strains tested.

Ferrochelatase activity. Ferrochelatase activity was assayed with cell envelope preparations of *H. influenzae. H. influenzae* was grown to 7.5×10^8 CFU/ml in brain heart infusion medium containing 2 μg of NAD per ml and a lysate of horse erythrocytes (1) (as a heme source) at a dilution of 1:50,000. The bacteria were harvested, washed with 0.05 M Tris (pH 7.8), and sonicated, and then the membranes were collected by ultracentrifugation as described elsewhere (12). The membrane pellets were suspended in 0.05 M Tris (pH 7.8) and stored at −20°C. The assay used was adapted from that of Dailey (4) and was set up and performed under anaerobic conditions provided by an anaerobe chamber (Coy Laboratory Products Inc., Grass Lake, Mich.). The 0.83-ml reaction mixture contained 50 mM Tris (pH 7.8), 5 mM dithiothreitol (DTT), 160 μM ferrous ammonium sulfate, 80 μM deuteroporphyrin, 0.33% Tween 80, and about 1 mg of envelope protein, as determined by the method of Lowry et al. (13). After incubation of the mixture at 37°C for 45 min, the reaction was stopped by addition of 0.1 ml of 0.4 M iodoacetamide. The concentration of deuteroheme formed was determined by the pyridine hemochromogen assay (4).

Ferrochelatase activity was observed in all strains examined (Table 1). There was little or no activity (less than 2% of that observed in the complete system) in the absence of Fe^{2+}, DTT, enzyme, or deuteroporphyrin IX. Heat-treated (65°C, 15 min) enzyme was inactive. Deuteroheme formation in the assay as performed was linear with time and proportional to the amount of enzyme; activity was confined to the cell envelope (data not shown). The variation in specific activity among strains was reproducible (data not shown). The two strains unable to grow on protoporphyrin IX both had activity at levels equal to those of their respective derivatives. Thus, the assumption (19) that these strains lack ferrochelatase is refuted. Outer membranes prepared by Triton X-100 extraction and analyzed on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels (12) revealed that both parent strains differ most noticeably from the derivatives in having increased amounts of a 57-kDa protein (Fig. 1). Inner membranes, concentrated from Triton X-100 extracts by precipitation with 10% cold trichloroacetic acid, showed no difference in protein composition upon SDS-PAGE (data not shown).

Reverse ferrochelatase activity. To assay for reverse activity, envelope extracts were prepared as for the forward reaction, except that 1 mM DTT was included in all steps, beginning with the washing of the bacterial cell pellet. The mixture for the reverse reaction, performed as was the forward reaction under anaerobic conditions, consisted of 50 mM Tris (pH 7.8), 5 mM DTT, 2.5 μM hemin, 0.24 mM ferrozine [3-(2-pyridyl)-

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TABLE 1. Ferrochelatase activity of *H. influenzae* strains

Source of enzyme (strain)	Expt no.	ET[a]	Biotype	Anatomic or other source	Sp act[b]
Eag (type b)	1	6	I	CSF[c]	11.0 ± 1.0
3219B (NTHI)	1	11	II	Ear	13.1 ± 2.5
1161B (NTHI)	1	45	III	Blood	8.5 ± 1.9
820 (NTHI)	1	80	III	CSF	3.6 ± 0.3
HE5 (NTHI)	2		III (aegyptius)	Eye	13.6 ± 0.0
HE5 derivative	2		III (aegyptius)	Laboratory	13.9 ± 1.6
15 (NTHI)	2		III (aegyptius)	Eye	13.7 ± 1.8
15 derivative	2		III (aegyptius)	Laboratory	13.4 ± 0.5
3219B (NTHI)	2	11	II	Ear	10.7 ± 3.5

[a] ET, electrophoretic type. ETs were determined for type b (15) and NTHI (14) strains as a means of determining their genetic similarity. The NTHI strains are spread out on a scale of increasing genetic diversity, ranging from ET 1 to ET 94.

[b] Specific activity is measured as nanomoles of deuteroheme formed per milligram of protein per hour and is the average of duplicate determinations.

[c] CSF, cerebrospinal fluid.

5,6-bis(4-phenyl sulfonic acid)-1,2,4-triazine], 5 μM NADH, 1% Tween 80, and about 1 mg of protein in a volume of 0.83 ml. The reducing agents in the reaction mixture would convert the hemin to heme. Ferrozine, a chelator of Fe^{2+} (18), was present to serve as a sink for the Fe^{2+} released from heme. The samples were incubated for 90 min, and the reaction was stopped with iodoacetamide as described above. The samples were centrifuged (8,000 × *g* for 5 min) to decrease turbidity due to the enzyme preparation, and the amount of product formed was determined by assaying for protoporphyrin IX by using a Perkin-Elmer (lambda 3) fluorimeter with both excitation and emission slit widths set at 4 nm. The excitation wavelength was 410 nm. The emission spectrum was taken from 615 to 645 nm, with the protoporphyrin IX peak being at 632 nm (2). To convert the fluorescence units to the absolute concentration of product, fluorimetry was performed with a standard solution of protoporphyrin IX of known concentration, as determined spectrophotometrically in 2.7 N HCl by using the millimolar extinction coefficient of 13.5 at 554 nm (7). With an extract from strain 3219B, 2.4 ± 0.2 pmol of protoporphyrin IX per mg of protein (results of duplicate determination) was formed, with 0.15% of the substrate being converted. There was little or no reaction (2% of that observed in the complete system) in the absence of enzyme, hemin, or ferrozine. Activity was proportional to enzyme concentration, and heat-treated enzyme was inactive (data not shown).

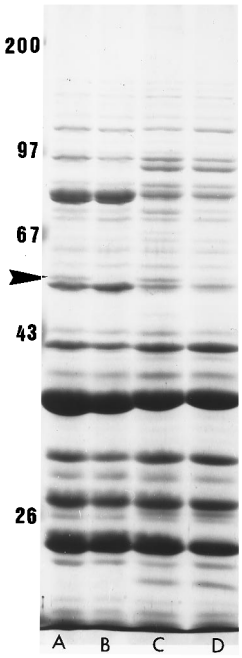

FIG. 1. Coomassie blue stain of SDS-PAGE gel of outer membrane preparation of parent strains HE5 (lane A) and 15 (lane C) and their respective derivatives (lanes B and D). Molecular masses of protein markers appear on the left in kilodaltons. The arrow points to the 57-kDa protein.

Rhodobacter sphaeroides (ATCC 17023), whose ferrochelatase is present at high levels and has been well characterized (4), also has reverse activity (1.3 ± 0.0 nmol/mg of protein in 90 min), as determined by using a reaction mixture as described for *H. influenzae* except for omission of NADH and presence of protein and Tween 80 at concentrations of 0.032 μg/ml and 0.04%, respectively, with the protein/Tween 80 ratio being approximately the same as in the *H. influenzae* system. The major product was Zn-protoporphyrin IX, as determined by fluorescence at 590 nm upon excitation at 420 nm (2). The ratio of the Zn-protoporphyrin IX to protoporphyrin IX, 3.5:1, was increased to 11:1 upon addition of 5 mM $ZnCl_2$ to the reaction mix, and the yield of total porphyrin was increased by 1.7-fold. The Zn^{2+} in this case acted as another sink in addition to ferrozine, which is absolutely required for the reaction. We assume that in the *R. sphaeroides* extract the protoporphyrin IX formed in the reverse reaction was then converted to Zn-protoporphyrin IX by the forward reaction. Attempts to identify Zn-protoporphyrin IX, formed either by endogenous or added Zn^{2+}, as a product in the *H. influenzae* reverse reaction were unsuccessful because the high concentration of enzyme used produced too great a fluorescence background in the 590-nm region.

Discussion and conclusions. The ability of 55 of 57 strains of *H. influenzae* to grow at the same rate on very low levels of protoporphyrin IX and the herein-demonstrated presence of ferrochelatase in some of the 55 strains, as well as in the 2 strains unable to utilize this substrate as a heme source, strongly imply the presence of the enzyme in all 57 strains of *H. influenzae*, where it functions to convert protoporphyrin IX to heme.

The presence in *H. influenzae* of ferrochelatase as the only enzyme in the heme synthetic pathway is unusual. This raises the issues of the origin of the enzyme, i.e., whether it has been acquired from another organism, and of the role of the enzyme. Regarding the latter, in the only well-studied human systems, erythrocytes and serum, heme is greatly preponderant over protoporphyrin IX (5, 9, 11, 17). This suggests that ferrochelatase functions in vivo in the reverse reaction, enabling *H. influenzae* to obtain Fe^{2+} from the host. I have shown that the reverse reaction can occur; further, in vitro heme can serve as a source of Fe^{2+} (3, 16). However, there are gaps in our knowledge. *H. influenzae* may have some other enzyme that releases Fe^{2+} from heme; heme may be bound much more tightly than protoporphyrin IX to serum proteins, obviating the ratio that appears to favor heme; protoporphyrin IX may be more available than heme in certain biological niches; and lastly, *H. influenzae* may have specific and efficient mechanisms for uptake of protoporphyrin IX.

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