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NITRATE, FUMARATE, AND OXYGEN AS ELECTRON ACCEPTORS FOR A LATE STEP IN MICROBIAL HEME SYNTHESIS

N. J. JACOBS and J. M. JACOBS

Department of Microbiology, Dartmouth Medical School, Hanover, N. H. 03755 (U.S.A.)

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SUMMARY

Nitrate can serve as anaerobic electron acceptor for the oxidation of protoporphyrinogen to protoporphyrin in cell-free extracts of *Escherichia coli* grown anaerobically in the presence of nitrate. Two kinds of experiments indicated this: anaerobic protoporphyrin formation from protoporphyrinogen, followed spectrophotometrically, was markedly stimulated by addition of nitrate; and anaerobic protoheme formation from protoporphyrinogen, determined by extraction procedures, was markedly stimulated by addition of nitrate. In contrast, anaerobic protoheme formation from protoporphyrin was not dependent upon addition of nitrate. This was the first demonstration of the ability of nitrate to serve as electron acceptor for this late step of heme synthesis. Previous studies with mammalian and yeast mitochondria had indicated an obligatory requirement for molecular oxygen at this step.

In confirmation of our previous preliminary report, fumarate was also shown to be an electron acceptor for anaerobic protoporphyrinogen oxidation in extracts of *E. coli* grown anaerobically on fumarate. For the first time, anaerobic protoheme formation from protoporphyrinogen, but not from protoporphyrin, was shown to be dependent upon the addition of fumarate.

The importance of these findings is 2-fold. First, they establish that enzymatic protoporphyrinogen oxidation can occur in the absence of molecular oxygen, in contrast to previous observations using mammalian and yeast mitochondria. Secondly, these findings help explain the ability of some facultative and anaerobic bacteria to form very large amounts of heme compounds, such as cytochrome pigments, when grown anaerobically in the presence of nitrate or fumarate. In fact, denitrifying bacteria are known to form more cytochromes when grown anaerobically than during aerobic growth.

An unexpected finding was that extracts of another bacterium, *Staphylococcus epidermidis*, exhibited very little ability to oxidize protoporphyrinogen to protoporphyrin as compared to *E. coli* extracts. This finding suggests some fundamental differences in these two organisms in this key step in heme synthesis. It is known that these two facultative organisms also differ in that *E. coli* synthesizes cytochrome during both aerobic and anaerobic growth, while *Staphylococcus* only synthesizes cytochromes when grown aerobically.

INTRODUCTION

In mammalian and plant tissue, the two oxidative reactions in the late steps of heme biosynthesis (Fig. 1), the oxidations of coproporphyrinogen to protoporphyrinogen and of protoporphyrinogen to protoporphyrin, are known to require molecular oxygen [1–5]. Since some obligately anaerobic and facultative bacteria can form cytochromes during growth in the absence of oxygen, physiological electron acceptors capable of replacing oxygen in these oxidative steps must exist. Although the anaerobic oxidation of coproporphyrinogen to protoporphyrinogen has recently been demonstrated to be dependent upon ATP, methionine and nicotinamide adenine dinucleotides in some photosynthetic bacteria and yeast [6, 7], the exact nature of the anaerobic electron acceptor for coproporphyrinogen oxidation in other heterotrophic bacteria remains to be determined [8].

The next enzymatic step in the pathway, the oxidation of protoporphyrinogen to protoporphyrin, has not been shown to use electron acceptors other than oxygen in mammalian mitochondria [1]. In yeast, only molecular oxygen can serve this function [9]. A recent report from our laboratory demonstrated, for the first time in any tissue, that an alternate electron acceptor, fumarate, could replace oxygen as a physiological electron acceptor for protoporphyrinogen oxidation. We used extracts of *Escherichia coli* grown anaerobically with fumarate as electron acceptor [10].

In the present communication, we have extended these findings to demonstrate that nitrate can also serve as alternate electron acceptor for protoporphyrinogen oxidation in extracts of *E. coli* cells grown anaerobically on nitrate. In addition, we have confirmed the physiological significance of these observations by demonstrating that anaerobic protoheme synthesis from protoporphyrinogen (but not from protoporphyrin) is markedly stimulated by the presence of fumarate or nitrate in extracts of anaerobically grown *E. coli*. We have also made the unexpected observation that extracts of another cytochrome-containing facultative bacterium, *Staphylococcus epidermidis*, exhibited little capacity for the oxidation of protoporphyrinogen, as compared to *E. coli* extracts.

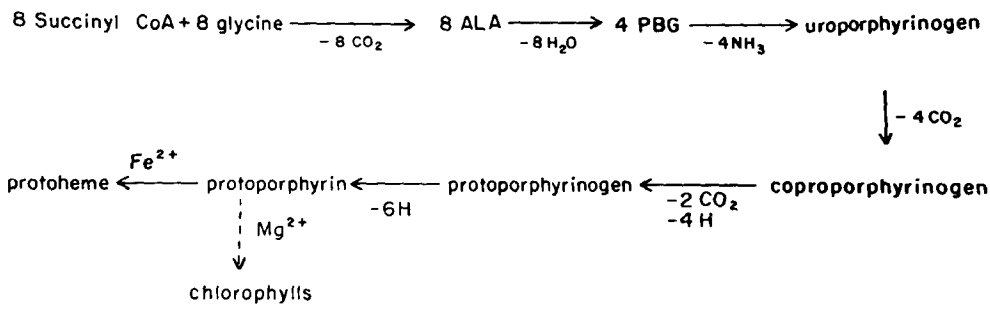

Fig. 1. Pathway of heme biosynthesis.

MATERIALS AND METHODS

Chemicals. Protoporphyrinogen was prepared by reduction of protoporphyrin with sodium amalgam [10]. All other chemicals were of reagent grade and are readily available.

Cultures, growth conditions, and extract preparation. E. coli was grown anaerobically in completely filled, stoppered bottles in a peptone medium containing 0.2 % glucose for 15 h at 37 °C as previously described [10]. Either 0.6 % potassium fumarate or 0.2 % KNO_3 were added to the medium as anaerobic electron acceptor. *S. epidermidis*, strain AT2, was grown aerobically on a rotary shaker for 10 h at 37 °C as previously described [11]. Cell-free extracts were prepared by sonic oscillation [10]. Extracts of *E. coli* were centrifuged at 23 000 $\times g$ for 7 min to remove cell debris and obtain a relatively clear extract suitable for direct spectrophotometric observation. For experiments measuring protoheme formation, a particulate, reddish layer above the bottom cellular layer was carefully removed and added to the supernatant solution to yield a turbid extract. Extracts of *S. epidermidis* were centrifuged at 12 000 $\times g$ for 10 min, and a portion of the grey layer above the cells was added to the supernatant to obtain a slightly turbid extract, which was nevertheless suitable for direct spectrophotometric studies.

Direct spectrophotometric assay of protoporphyrinogen oxidation to protoporphyrin. The appearance of the four-banded protoporphyrin spectra during the incubation of extracts in the presence of protoporphyrinogen and EDTA was followed by rapidly scanning the visible spectrum with a Cary 14 spectrophotometer at room temperature as previously described [10, 12]. Protoporphyrinogen (5 mM) was added directly to the reaction mixture held at 0 °C. The anaerobic Thunberg cuvette was then taken through two additional cycles of evacuation and replacement with deoxygenated H_2 gas at 0 °C. The reaction was then initiated by placing the cuvette in an incubator and slowly warming at 37 °C. In most experiments, there was no, or only slight, fluorescence detectable immediately after adding protoporphyrinogen to the extract at 0 °C, and the slow development of fluorescence (followed visually with a long wave ultraviolet lamp) during the first 30 min after placing the cuvette at 37 °C seemed well coordinated with the appearance of the four-banded spectrum of protoporphyrin, followed spectrophotometrically.

Due to the turbidity of these crude extracts, it was not possible to estimate the rate of conversion of protoporphyrinogen to protoporphyrin by simply measuring the absorbance at various maxima and calculating concentrations by using known extinction coefficients of protoporphyrin standards. Instead, a slight modification of the assay introduced by Poulson and Polglase [7] was employed. In the difference spectrum, the absorbancy increment between the maxima at the α, β and γ peaks at 633, 578 and 540 nm and a line drawn between the minima at either side of each peak (see Fig. 2 in ref. 10) was determined. These three absorbancy increments were converted to concentration of protoporphyrin formed by using the following extinction values determined by adding known quantities of pure protoporphyrin to these same extracts, and measuring the absorbancy increment at the three maxima by the same procedure. The millimolar extinction coefficients (ε_{mM}) determined were: α (633 nm) ε_{mM}, 2.56 $mM^{-1} \cdot cm^{-1}$; β (578 nm) ε_{mM}, 2.23 $mM^{-1} \cdot cm^{-1}$; γ (540 nm) ε_{mM}, 3.08 $mM^{-1} \cdot cm^{-1}$.

Anaerobic incubation conditions were obtained in anaerobic Thunberg cuvettes with repeated cycles of evacuation and replacement with deoxygenated H_2 gas [10]. Aerobic conditions were obtained by occasional gentle stirring of the reaction mixture in an open cuvette. Since these crude extracts doubtlessly contain considerable endogenous reducing power, these conditions were probably not fully aerobic. Where indicated, 43 mM potassium fumarate or 150 mM potassium nitrate were added as electron acceptors to the anaerobic cuvettes.

Assay of protoheme formation using protoporphyrinogen or protoporphyrin as substrate. All incubation conditions were as described above, except that EDTA was omitted, and iron salts were added, where indicated. The reaction was terminated by rapidly introducing 10 ml of cold acidified acetone (1 % concentrated HCl in acetone). This was stirred for at least 1 h at 0 °C to quantitatively extract protoheme bound to protein [13]. After centrifugation, the residue was re-extracted with a second 10-ml aliquot of acidified acetone. The protoheme was taken into ether by dilution with ether and 1 % HCl by a procedure previously described [14] except that protoporphyrin was exhaustively extracted from the ether layer by 3–6 extractions with 3 ml volumes of 1.5 M HCl. By this procedure, protoheme, in the ether layer, is separated from protoporphyrin, in the 1.5 M HCl layer, and the latter is directly assayed by the height of the peak at 406 nm using extinction values [13]. During the extraction procedure, which involves overnight storage in 1.5 M HCl at 0 °C in the dark, the residual protoporphyrinogen was oxidized to the porphyrin form. The recovery of substrate either as protoporphyrin or protoheme can be calculated for each experimental condition employed. Protoheme was determined by evaporating the ether to dryness under a stream of prepurified N_2 gas, adding 5 ml of cold 0.25 M NaOH and 1.4 ml of pyridine and by measuring the difference spectra between the oxidized and reduced form of the pyridine hemochromogen as previously described [13, 14].

Protein and nitrite were determined as described previously [7, 15].

RESULTS

Nitrate and fumarate as electron acceptors for anaerobic oxidation of protoporphyrinogen in extracts of E. coli grown anaerobically. In extracts of nitrate-grown cells, addition of nitrate considerably enhances protoporphyrinogen oxidation (Fig. 2). Oxygen also enhances protoporphyrinogen oxidation, to a lesser degree. However, there is considerable oxidation of protoporphyrinogen in these extracts in the absence of any added electron acceptors. Although further studies will be needed to clarify the nature of this endogenous oxidation, we did not find a significant rate of endogenous oxidation in extracts of cells grown under different conditions, with fumarate as electron acceptor (Fig. 2). This may indicate some endogenous electron acceptors in the crude extracts of nitrate-grown cells.

As we previously documented [10], extracts of fumarate-grown cells are readily able to oxidize protoporphyrinogen with either fumarate or oxygen as electron acceptor (Fig. 2).

Heme formation from protoporphyrinogen and protoporphyrin in extracts of nitrate-grown E. coli. To confirm the ability of nitrate to serve as a physiological anaerobic electron acceptor for protoporphyrinogen oxidation, we followed heme formation from protoporphyrinogen in the presence of nitrate. Since protoporphyrin,

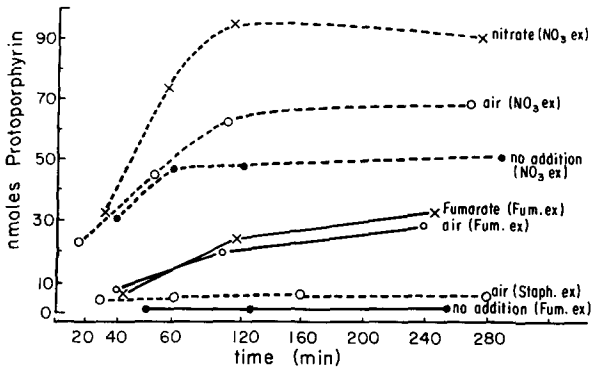

Fig. 2. Oxidation of protoporphyrinogen to protoporphyrin by extracts of *E. coli* and *S. epidermidis*. The reaction mixture, described in Materials and Methods, contained 130 nmol protoporphyrinogen, and the following amounts of extract: nitrate-grown *E. coli*, 54 mg protein; fumarate-grown *E. coli*, 72 mg protein; *S. epidermidis*, 41 mg protein. The extract of nitrate-grown *E. coli* (NO_3 ex) and aerobically grown *S. epidermidis* (Staph ex) are illustrated by dashed lines. The extract of fumarate-grown *E. coli* (Fum. ex) is indicated by a solid line. The use of various electron acceptors is indicated by the following: ×, nitrate or fumarate; ○, air; ●, no electron acceptors added. In addition, the reaction mixture contained: EDTA, 1 mM; glutathione, 5 mM; Tris buffer, pH 7.6, 50 mM; and water to a volume of 3.3 ml. Where indicated, the following was added: KNO_3 nitrate, 500 μmol; and potassium fumarate, 260 μmol.

TABLE I

EFFECT OF NITRATE ON HEME FORMATION FROM PROTOPORPHYRINOGEN AND PROTOPORPHYRIN IN EXTRACTS OF NITRATE-GROWN *E. COLI*

Substrate	nmol	Additions to reaction mixture[a]	Total proto-heme (nmol)	Increase in proto-heme[b] (nmol)	Protopor-phyrin remaining (nmol)
Protoporphyrinogen	165	none	22	10	89
Protoporphyrinogen	165	nitrate, iron	69	57	42
Protoporphyrinogen	165	nitrate	42	30	80
Protoporphyrinogen	165	iron	18	6	93
None	0	none	12	0	0
Protoporphyrin	83	none	42	32	38
Protoporphyrin	83	nitrate, iron	54	44	30
Protoporphyrin	83	nitrate	45	35	44
Protoporphyrin	83	iron	42	32	39
None	0	none	10	0	0

[a] Incubation was as described in Materials and Methods, at 37 °C for a period of 100 min under anaerobic condition. The reaction mixture was as described in Fig. 1, except that EDTA was omitted and 3 μmol of freshly prepared $FeSO_4$ was added, where indicated. 80 mg of extract protein were present.

[b] The basal amount of protoheme present in the extract before incubation with substrate was subtracted from the total protoheme present after incubation to obtain the increase in protoheme.

but not protoporphyrinogen, is the substrate for the ferrochelatase enzyme, anaerobic heme formation should be dependent upon the presence of nitrate with protoporphyrinogen, but not with protoporphyrin, as substrate. Table I illustrates that heme formation from protoporphyrinogen is markedly stimulated by the addition of nitrate, confirming the physiological significance of nitrate as anaerobic electron acceptor. During the incubation period, 1.5 μmol of nitrite were formed from the nitrate added. As indicated, nitrate does not markedly stimulate heme formation with protoporphyrin as substrate (Table I), although a slight stimulation by nitrate remains to be explained.

Of separate interest is the fact that iron salts are not required for heme formation from protoporphyrin in these experiments (Table I). Probably sufficient quantities of endogenous iron are present in the large amounts of crude extract used in these experiments to supply the needs of the ferrochelatase enzyme.

Effect of fumarate on protoheme formation from protoporphyrinogen in extracts of E. coli grown anaerobically on fumarate. We have previously documented that fumarate can anaerobically oxidize protoporphyrinogen [10]. Table II confirms the physiological significance of this observation. As indicated, anaerobic protoheme formation from protoporphyrinogen is markedly dependent upon the addition of fumarate, with very little heme formation occurring in the absence of added fumarate. Fumarate is not stimulatory when protoporphyrin is substrate. Again, heme formation is not dependent upon addition of exogenous iron in these crude extracts.

Attempts to demonstrate a protoporphyrinogen-oxidizing enzyme in extracts of S. epidermidis. As compared to *E. coli* extracts, extracts of aerobically grown *S. epidermidis* exhibited little activity in the assay for aerobic oxidation of protoporphyrinogen using the spectrophotometric assay for protoporphyrin formation (Fig. 2). Addition of either fumarate or nitrate to the reaction mixture did not increase this activity.

Lack of protoporphyrinogen-oxidizing activity in extracts of *S. epidermidis* was also confirmed by measuring protoheme formation from protoporphyrinogen and

TABLE II

EFFECT OF FUMARATE ON HEME FORMATION FROM PROTOPORPHYRINOGEN AND PROTOPORPHYRIN IN EXTRACTS OF FUMARATE-GROWN *E. COLI*

Substrate	nmol	Additions to reaction mixture[a]	Total proto-heme (nmol)	Increase in proto-heme (nmol)	Protopor-phyrin remaining (nmol)
Protoporphyrinogen	219	none	6	1	174
Protoporphyrinogen	219	fumarate	39	35	98
Protoporphyrinogen	219	iron	14	9	139
None	0	none	5	0	0
Protoporphyrin	165	none	53	49	85
Protoporphyrin	165	fumarate	59	55	75
Protoporphyrin	165	iron	40	36	100
None	0	none	4	0	0

[a] Conditions were as described in Table I. 60 mg of extract protein were present.

TABLE III

HEME FORMATION FROM PROTOPORPHYRINOGEN AND PROTOPORPHYRIN IN EXTRACTS OF *S. EPIDERMIDIS*

Substrate	nmol	Additions to reaction mixture[a]	Total proto-heme (nmol)	Increase in proto-heme (nmol)	Protopor-phyrin remaining (nmol)
Protoporphyrinogen	165	none	4.4	0.2	118
Protoporphyrinogen	165	fumarate	5.5	1.3	118
Protoporphyrinogen	165	nitrate	5.5	1.3	92
Protoporphyrinogen	165	air	5.6	1.4	120
None	0	none	4.2	0	0
Protoporphyrin	165	none	14.3	11.5	139
Protoporphyrin	165	omit iron	5.7	2.9	151
None	0	none	2.9	0	0

[a] Incubation was as in Table I, except that 3 μmol of $FeSO_4$ were added to each tube. 41 mg of extract protein was present.

protoporphyrin. As indicated (Table III), heme formation from protoporphyrin (ferrochelatase activity) is demonstrable in these extracts, although they are less active in ferrochelatase activity than *E. coli* extracts (compare to Tables I and II). However, much less heme is formed when protoporphyrinogen, rather than protoporphyrin is substrate. Addition of either fumarate or nitrate did not enhance protoporphyrinogen conversion to protoheme (Table III).

DISCUSSION

Previous biochemical studies on enzymatic protoporphyrinogen oxidation in mammalian or yeast cells [1–3, 12] indicated that molecular oxygen was the only physiological oxidant which could serve as electron acceptor [8]. Since our present study shows that both nitrate and fumarate can serve as electron acceptors, a mechanism requiring molecular oxygen has been ruled out, at least in *E. coli*. Our study also confirms, by a separate method, that protoporphyrinogen cannot serve as a substrate for the final step of heme synthesis, the ferrochelatase enzyme, but must first be oxidized to protoporphyrin.

The mechanism of the anaerobic coupling between the presumed protoporphyrinogen-oxidizing enzyme and the nitrate or fumarate reductase remains to be discovered. In analogy with other microbial anaerobic electron transport systems, it seems reasonable to predict that menaquinones, flavoproteins or non-heme iron carriers may be involved.

With regard to microbial physiology, these observations partially explain, for the first time, how some facultative and anaerobic bacteria can synthesize cytochromes when grown in the absence of oxygen. This question has been of interest ever since it was shown that the two oxidative steps in heme synthesis in mammalian tissue, the oxidations of coproporphyrinogen and of protoporphyrinogen, required molecular oxygen. It is pertinent that both fumarate and nitrate serve as anaerobic electron

acceptors for protoporphyrinogen oxidation, since several cytochrome-containing anaerobes are able to reduce these compounds. For instance, denitrifying bacteria are known to produce more cytochromes when grown anaerobically in the presence of nitrate than when grown aerobically. In this regard, it is of interest that we found nitrate to be a better electron acceptor for protoporphyrinogen oxidation than was oxygen in extracts of *E. coli* grown anaerobically on nitrate (Fig. 2). *Propionibacterium pentosaceum* is an example of a fumarate-reducing facultative anaerobe which synthesizes more cytochromes during anaerobic than during aerobic growth [16]. Active fumarate reductases are also known to occur in the anaerobic, cytochrome-containing organisms *Vibrio succinogenes* [17], *Bacteroides* [18], and *Desulfovibrio* species [19].

It seems pertinent to comment on the advantage of an anaerobic mechanism for the late steps in the heme biosynthetic pathway. Since the final step in this pathway, the ferrochelatase enzyme, is known to be inhibited by oxygen, a mechanism for oxidizing protoporphyrinogen in the absence of molecular oxygen seems desirable. This may partially explain why some facultative bacteria actually synthesize more cytochromes during anaerobic than during aerobic growth [8].

One problem which remains unresolved in *E. coli* and other heterotrophic bacteria is the mechanism of anaerobic coproporphyrinogen oxidation [21, 22]. In the photosynthetic *Rhodopseudomonas spheroides*, the denitrifying *Micrococcus denitrificans* [6, 20] and in yeast [7], this oxidation can occur anaerobically provided that ATP, methionine and magnesium are present.

Of further interest is our observation that extracts of *S. epidermidis* are quite deficient in ability to oxidize protoporphyrinogen as compared to extracts of *E. coli*. One unresolved question is why some facultative bacteria, such as *E. coli* and some denitrifying bacteria, can synthesize cytochromes when grown under either aerobic or anaerobic conditions, while other facultative bacteria, such as Staphylococci, will only synthesize cytochromes when grown aerobically [8]. Perhaps Staphylococci cannot make heme anaerobically since they lack an enzyme system for oxidizing protoporphyrinogen. Under aerobic conditions, the non-enzymatic autooxidation of protoporphyrinogen may be sufficient to account for aerobic heme synthesis in this organism. Obviously, further studies to test this hypothesis are needed. In previous studies [21, 22], we found an oxygen-dependent coproporphyrinogen-oxidizing enzyme in *E. coli*, but not in Staphylococci.

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