FUMARATE AS ALTERNATE ELECTRON ACCEPTOR FOR THE LATE STEPS OF ANAEROBIC HEME SYNTHESIS IN ESCHERICHIA COLI

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SUMMARY

The anaerobic formation of protoheme from added excess delta amino levulinic acid was markedly reduced if fumarate was omitted from an anaerobic incubation mixture containing resting suspensions of E. coli grown anaerobically on a medium containing fumarate. However, the formation of coproporphyrinogen did not require fumarate, suggesting a role for fumarate at some step between coproporphyrinogen and heme in the anaerobic heme biosynthetic pathway. The appearance of fluorescence during the anaerobic incubation of these cell suspensions was also dependent upon the presence of fumarate, suggesting that fumarate could anaerobically oxidize protoporphyrinogen to protoporphyrin. This was confirmed by demonstrating that fumarate could serve as an alternate electron acceptor to replace oxygen in the oxidation of chemically reduced protoporphyrinogen under anaerobic conditions in extracts of these cells. This was the first demonstration of the enzymatic oxidation of protoporphyrinogen to protoporphyrin by a physiological electron acceptor other than oxygen in any type of cell.

INTRODUCTION

In mammalian and plant tissue, the two oxidative reactions in the late steps of heme biosynthesis (Figure 1), the oxidations of coproporphyrinogen to protoporphyrinogen and of protoporphyrinogen to protoporphrin, 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 this reaction in most heterotrophic bacteria is not

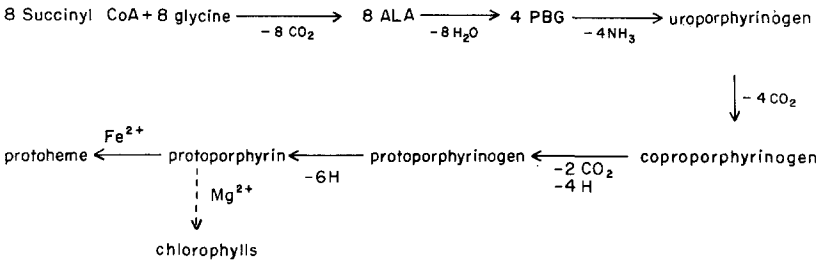

Figure 1. Pathway of heme biosynthesis.

known. The specific oxidation of protoporphyrinogen to protoporphyrin by a physiological electron acceptor other than oxygen has not been previously demonstrated in any type of cell. In this communication, we report that fumarate can anaerobically oxidize protoporphyrinogen to protoporphyrin in extracts of fumarate-grown E. coli, and we demonstrate that fumarate is a physiologically important electron acceptor in anaerobic heme synthesis in this organism.

METHODS

E. coli W was grown anaerobically in completely filled, stoppered bottles (8) in a peptone medium containing 0.2% glucose (9) for 15 hr at 37°C. Where indicated, 0.6% potassium fumarate and 0.28 mM delta amino levulinic acid (ALA) were added. Following centrifugation at 0°C under a nitrogen atmosphere, the porphyrins and heme were determined after extraction of the cells with acidified acetone or the supernatant fluid with ethyl acetate (8,9). The porphyrin concentrations (Table 1) are expressed as URO, COPRO and PROTO and represent the porphyrin and porphyrinogen concentration determined as uroporphyrin, coproporphyrin and protoporphyrin respectively, although the analytical procedures used did not distinguish between the porphyrinogen and porphyrin forms.

Resting cell suspensions were prepared from cells grown anaerobically in medium with fumarate but without ALA. After centrifugation at 0°C, the cells were washed and resuspended in one-tenth of the culture volume of 50 mM phosphate buffer pH 6.3 under an atmosphere of prepurified nitrogen gas. For anaerobic incubation of resting suspensions, all reagents were gassed with nitrogen at 0°C and placed in completely filled, tightly stoppered, 60 ml bottles at 37°C. The incubation mixture contained: glucose, 22 mM; potassium fumarate, 48 mM; $FeSO_4$, 0.23 mM; phosphate buffer, pH 6.3, 80 mM; ALA, 1.2 mM; and 150 mg (dry weight) of cells. The presence of fluorescence during the incubation was observed with a long wave U.V. lamp. After incubation, the bottle was chilled to 0°C, the cells were rapidly removed by centrifugation, and the cells and supernatant fluid were extracted with solvents as indicated above.

Cell-free extracts were prepared by washing cells in one-tenth culture volume and finally resuspending in one-fiftieth the culture volume with 50 mM Tris buffer pH 7.4, disrupting by sonic oscillation (10) and centrifuging at 23,000 x g for 7 min to remove cell debris.

The oxidation of protoporphyrinogen, prepared from protoporphyrin (Porphyrin Products, Logan, Utah) by reduction with sodium amalgam (1), was measured directly in extracts. The appearance of the typical four-banded protoporphyrin spectrum during the incubation was followed by rapidly scanning the visible spectrum with a Cary 14 spectrophotometer, using the procedure described for determining this enzyme activity in yeast mitochondria (11). The reaction mixture, in an anaerobic Thunberg cuvette, contained: EDTA, 1 mM; glutathione, 5 mM; Tris buffer, pH 7.6, 50 mM; enzyme, 72 mg protein; and water to a volume of 3.0 ml. Potassium fumarate (43 mM) was added where indicated. This tube was thoroughly bubbled with deoxygenated H_2 gas at 0°C and taken through three cycles of evacuation and replacement with hydrogen gas. To start the reaction, protoporphyrinogen (5 mM) was added from the sidearm and the cuvette was incubated at 37°C and scanned at the time intervals indicated.

RESULTS

To determine the optimal conditions for anaerobic heme synthesis, *E. coli* was grown under a variety of anaerobic conditions in the presence of excess amounts of the early heme precursor, ALA, and the porphyrin and heme content was determined. In medium with 0.2% glucose as principal energy source, ALA was converted largely to coproporphyrin (850 p moles/mg cells) and only small amounts of heme (50 p moles/mg cells) were formed. However, when this medium was supplemented with 0.6% fumarate, coproporphyrin production was only slightly stimulated, while heme accumulation increased 16 fold (to 900 p moles/mg cells). These results suggested a possible role for fumarate at some step beyond coproporphyrinogen in the heme biosynthetic pathway.

Additional evidence was obtained by incubating ALA under anaerobic conditions with resting suspensions of cells grown anaerobically in media containing fumarate. In the presence of added iron, the maximal accumulation of heme from ALA was obtained upon addition of glucose and fumarate (Table 1, line 1). Heme was markedly decreased if fumarate was omitted, although coproporphyrin or coproporphyrinogen production was undiminished (line 2). (The analytical procedures used do not distinguish between the presence of porphyrinogens or porphyrins since the former are converted to the latter prior to quantitative

TABLE 1. Anaerobic accumulation of porphyrins or porphyrinogens and heme from ALA in resting suspensions of fumarate grown cells

	Additions to anaerobic suspensions[1]				Picomoles per milligram					Fluorescence observed during incubation
					URO	COPRO	PROTO	HEME	Δ HEME[2]	
1.	ALA	glu	fum	Fe	200	880	8	273	205	-
2.	ALA	glu	-	Fe	153	1,120	6	121	63	-
3.	ALA	glu	fum	-	200	830	29	193	125	++
4.	ALA	glu	-	-	200	1,160	21	68	0	-
5.	-	glu	fum	-	0	13	1	68	0	-

[1] incubated anaerobically at 37°C for 5 1/2 hr, in the presence of ALA, glucose (glu), fumarate (fum) or ferrous sulfate (Fe) as indicated in "Methods".
[2] to obtain the amount of heme formed specifically from ALA during the incubation (Δ heme), the amount present in cells incubated without ALA (line 5) was subtracted. The amount of heme present in cells incubated without ALA (line 5) was approximately the same as that present in cells prior to incubation.

measurement.) Upon omission of iron (lines 3 and 4), heme accumulation was somewhat depressed in the presence of fumarate, (line 3) but was markedly inhibited if fumarate was also omitted (line 4). As expected, omission of iron caused a markedly increased accumulation of protoporphyrin or protoporphyrinogen. Although this accumulation was not markedly diminished by omission of fumarate (lines 3 and 4), we visually observed that the cells incubated with fumarate exhibited fluorescence *in vivo* (line 3), while the cells incubated without fumarate were not fluorescent (line 4). These observations suggested that fumarate was required for anaerobic heme synthesis since it caused the oxidation of the nonfluorescent protoporphyrinogen to the fluorescent protoporphyrin, thereby allowing heme synthesis to proceed.

Further confirmation of this role for fumarate came from studies with

cell-free extracts of these cells, incubated with protoporphyrinogen. As indicated in Figure 2, the colorless protoporphyrinogen spectrum was oxidized to the typical 4-banded protoporphyrin spectrum upon exposure of the cuvette to oxygen. This enzymatic oxidation with oxygen as acceptor is presumably the same as that recently characterized in yeast mitochondria (11) and demonstrated in animal mitochondria (1,2) and aerobically grown E. coli (12). We found that fumarate could replace this effect of oxygen in oxidizing protoporphyrinogen in these extracts (Figure 2). This activity with fumarate was

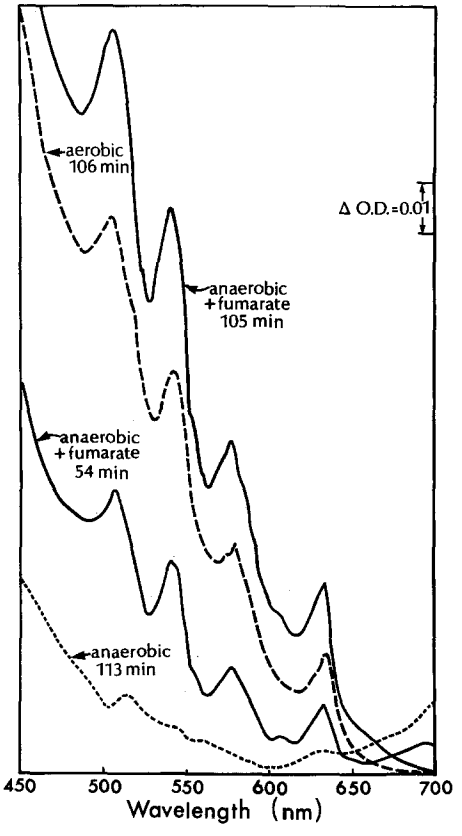

Figure 2. Effect of fumarate and oxygen on the oxidation of protoporphyrinogen to protoporphyrin. Following addition of protoporphyrinogen, the cuvettes were scanned at the time intervals indicated, using an enzyme mixture containing no protoporphyrinogen as a blank.

markedly inhibited by incubation of the extract with trypsin, indicating its enzymatic nature. Succinate could not replace the effect of fumarate in this reaction.

DISCUSSION

These physiological and enzymatic findings establish a role for fumarate as an alternate electron acceptor in the late step of heme synthesis involving the oxidation of protoporphyrinogen to protoporphyrin in E. coli. This is the first demonstration of a physiological electron acceptor able to replace oxygen in this reaction in any tissue. In yeast, coproporphyrinogen was converted to protoporphyrinogen in the absence of oxygen if extracts were supplemented with ATP, methionine and nicotinamide adenine dinucleotides (7), but only molecular oxygen could serve as electron acceptor for protoporphyrinogen oxidation (13). In extracts of photosynthetic and denitrifying bacteria, the anaerobic oxidation of coproporphyrinogen was demonstrated in the presence of these same three supplements, but it is not known if they participated in protoporphyrinogen oxidation (6,14). The anaerobic oxidation of coproporphyrinogen was reported in extracts of an aerobically grown Pseudomonad, but no requirement for an alternate electron acceptor was demonstrated (15). The anaerobic conversion of coproporphyrinogen to protoporphyrin has not been demonstrable in extracts of E. coli or several other facultative or anaerobic heterotrophic bacteria (16,17) although physiological studies clearly showed that this conversion occurred anaerobically in growing cultures of E. coli (18).

It seems likely that a role for fumarate in anaerobic heme synthesis will be demonstrable in several other cytochrome-containing anaerobic or facultative bacteria, since the process of fumarate reduction to succinate is an important metabolic reaction in many of these organisms. Fumarate is also known to be the anaerobic electron acceptor able to replace oxygen in an oxidative step in pyrimidine biosynthesis in E. coli growing anaerobically (19,20).

Further biochemical studies will be required to elucidate the mechanisms of the anaerobic oxidation of protoporphyrinogen in the presence of fumarate in extracts of E. coli. Presumably, electrons are passed from the protoporphyrinogen oxidizing enzyme to the fumarate reductase enzyme through the mediation of anaerobic electron transport carriers such as quinones or flavins, but a more indirect role for fumarate has not been ruled out.

ACKNOWLEDGEMENT

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