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Proteins of the Inner Membrane of *Escherichia coli*: Changes in Composition Associated with Anaerobic Growth and Fumarate Reductase Amber Mutation

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The inner membrane fractions of *Escherichia coli* grown anaerobically and aerobically were isolated, and their proteins were compared by electrophoresis in polyacrylamide gels. To maximimize the differences between the preparations, the anaerobic cultures were grown on complex medium with added glucose, but glucose was omitted from the aerobic cultures to prevent catabolite repression. The pattern of bands in the two types of preparation differed considerably, and changes in approximately 20 components were observed. In particular, the band identified as succinate dehydrogenase in aerobic preparations was greatly reduced in anaerobic preparations. Mutants lacking fumarate reductase were isolated, and inner membrane preparations of an *frd* amber mutant were deficient in a major component of 75,000 daltons and possibly a minor one of 87,500 daltons. The former was also present in greater amounts in anaerobic preparations and could represent a fumarate reductase subunit.

In a previous paper (19), the proteins of isolated inner membranes of aerobically grown *Escherichia coli* K-12 were analyzed by polyacrylamide gel electrophoresis, with a high degree of resolution. By comparing preparations from the parental strain with those of *sdh* amber mutants, the band which corresponded to a subunit of succinate dehydrogenase (EC 1.3.99.1) was identified.

Most of the previous studies of the membrane proteins have been restricted to aerobic preparations (6, 7, 16), although considerable differences between aerobic and anaerobic preparations might be expected. Many membrane-bound enzymes, such as the components of the respiratory electron transport chain, are predominantly aerobic, whereas others, such as the nitrate reductase system (17), are induced or derepressed anaerobically. Membrane proteins from mutants deficient in nitrate reductase (*chl* mutants) have been analyzed recently, and interesting pleiotropic effects were observed (10, 14).

The synthesis of fumarate reductase (EC 1.3.99.-) is derepressed under anaerobic conditions (5, 18) where it permits fumarate to function as a terminal electron acceptor, as well as providing an anaerobic route for succinate biosynthesis. The formation of a specific particulate complex coupling glycerol-1-phosphate oxidation to fumarate reduction in *E. coli* has been reported recently (13), and it seems likely that an anaerobic electron transport chain from glycerol-1-phosphate to fumarate, analagous to the nitrate reductase system, exists in *E. coli*. Such an electron transport system has already been reported in *Streptococcus faecalis* (2), *Bacillus megaterium* (8), and propionic acid bacteria (1).

In this work the inner membrane fraction of anaerobically grown *E. coli* has been isolated, and an analysis of the proteins by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis showed substantial differences compared with similar preparations from aerobic organisms. The membrane proteins of a fumarate reductase amber mutant showed two missing bands on polyacrylamide gels compared with the wild type.

MATERIALS AND METHODS

Organisms. *E. coli* K-12 strain WGAS, a spontaneous streptomycin-resistant derivative of WGA (W3110, F$^-$, *gal*, *trpA9761am*), was used as a source of wild-type membrane proteins and as a parental strain for the selection of mutants. The F′ donors: KLF17 (F117, *pyrB*$^+$)/KL132 (F$^-$, *thr*, *leu*, *proA*, *his*, *thy*, *thi*, *pyrB*, *lac*, *mal*, *xyl*, *mtl*, *recA*) and AW9 (F14, *supU*, *metB*$^+$)/AW1 (*metB*, *recA56*) were obtained from K. B. Low and J. Scaife, respectively.

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Media. The basal minimal medium contained (per liter): KH_2PO_4, 5.44 g; K_2HPO_4, 10.49 g; $(NH_4)_2SO_4$, 2 g; $MgSO_4 \cdot 7H_2O$, 0.05 g; $MnSO_4 \cdot 4H_2O$, 5 mg; $FeSO_4 \cdot 7H_2O$, 0.925 mg; and $CaCl_2$, 0.5 mg. Substrate additions were made, where appropriate, to give the following concentrations: glycerol, 0.04 M; and sodium fumarate, 0.04 M. The glycerol plus fumarate medium (GF medium) contained, in addition, Casamino Acids (0.5 g/liter). Anaerobic incubation of solid media was carried out under H_2/CO_2 (95:5, vol/vol). Minimal media were supplemented with L-tryptophan (30 μg/ml).

Growth of cultures. Cultures were grown anaerobically in stationary 1-liter Erlenmeyer flasks filled to the neck with medium (peptone, 0.4%; yeast extract, 0.4%; K_2HPO_4, 0.6%; glucose, 1%; pH 6.8), inoculated with 0.5 ml of an overnight broth culture, and harvested in log phase (approximately 5×10^8 cells/ml) for membrane preparations or in stationary phase, after overnight growth, for enzymology.

Isolation of inner membrane and gel electrophoresis. The inner membrane fraction was isolated on sucrose density gradients, and gel electrophoresis was carried out in SDS-polyacrylamide gels (8.5%) as described previously (19). Molecular weight calibrations with standard proteins (bovine serum albumin, fibrinogen, ovalbumin, pepsin, trypsin, lysozyme; 2 μg each) were run with each experiment.

Selection of mutants. Mutants of strain WGAS deficient in fumarate reductase (*frd*) were isolated, as described previously(18), by mutagenesis with *N-methyl-N'*-nitro-*N*-notrosoguanidine followed by aerobic expression on glycerol minimal medium and penicillin selection on GF medium anaerobically. Fumarate reductase was assayed in ultrasonic extracts by the method of Spencer and Guest (18), and conjugations were performed by the cross-streak method (4). Frd^+ recombinants were selected anaerobically on GF medium supplemented with L-tryptophan (30 μg/ml). After conjugation with AW9 (a strain carrying the *supU* amber-suppressor gene on an F-prime factor) and selection anaerobically on unsupplemented GF medium, positive results indicated the simultaneous suppression of *frd* and *trpA* amber mutations.

RESULTS

Electrophoresis of inner membrane proteins from E. coli grown anaerobically. The analysis of the inner membrane proteins of aerobic cultures of *E. coli* has been described previously (19). With exactly the same methods, the inner membrane fraction was isolated from anaerobically grown *E. coli* and subjected to analysis on SDS-polyacrylamide gels. Three different anaerobic preparations were studied and found to be reasonably reproducible, although more variability was found in anaerobic preparations than aerobic. A typical gel of an anaerobic preparation is shown in Fig. 1c with an aerobic preparation for comparison (Fig. 1a). Immediate inspection showed a radically different pattern from characteristic aerobic preparations, and corresponding bands could not readily be identified. However, by running anaerobic and aerobic preparations on a split gel, corresponding bands in the two gels could be matched (Fig. 1b). Scans of one aerobic and two anaerobic gels are shown in Fig. 2a, b, and c. The numbers given to the bands in the anaerobic preparations are based on their apparent equivalents in the aerobic preparation, as observed from the split gels, but the numbering system does not imply that the proteins are identical. The approximate intensities of the bands were compared by measuring the heights of the peaks, and the differences

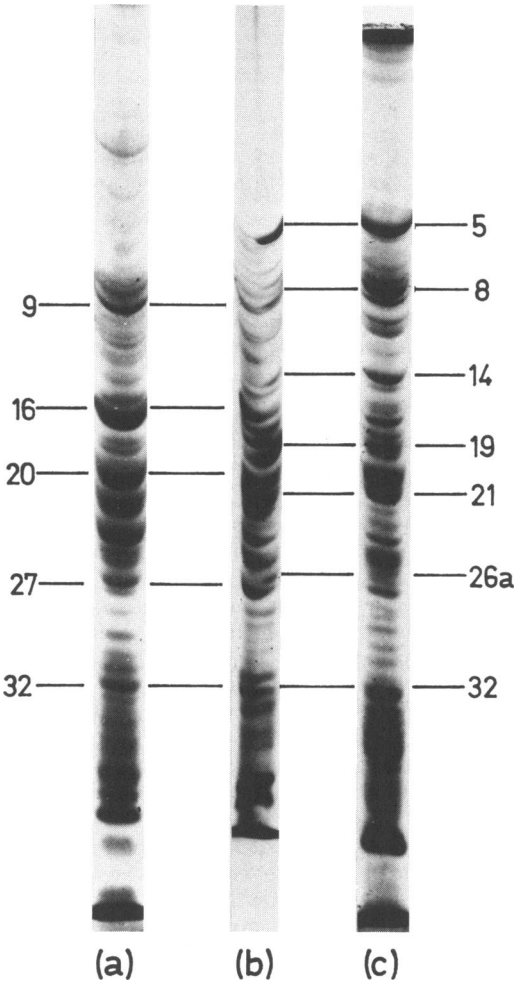

FIG. 1. *Polyacrylamide gels of the inner membrane proteins of the WGAS parental strain, grown aerobically and anaerobically. (a) WGAS, aerobic; (b) split gel: left-hand side, WGAS aerobic; right-hand side, WGAS anaerobic; (c) WGAS, anaerobic.*

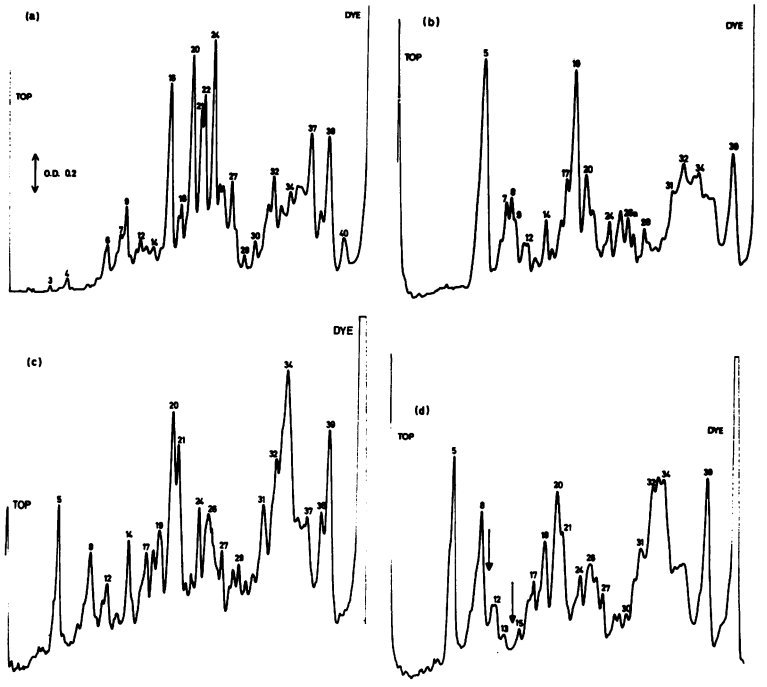

FIG. 2. *Profiles of the inner membrane proteins of the WGAS parental strain, grown aerobically and anaerobically, and an frd amber mutant, after separation on polyacrylamide gels. (a) WGAS, aerobic; (b and c) WGAS, anaerobic, two different preparations; (d) frd-3 (amber), anaerobic.*

observed, by comparing several gels from four aerobic and three anaerobic preparations, are summarized in Table 1. Quantitative comparisons are limited by the fact that no band can be assumed to remain constant between the preparations but, when a similar total loading of protein is used and the gels show a similar complexity in band distribution, the semiquantitative comparison used here can probably be justified. Amongst the notable differences anaerobically are the significant increase in three bands, 5, 14, and 19; the appearance of a new band, 26a; and the decrease in intensity of bands 16, 18, 22, 24, and 25. It is interesting to note that band 16, which exhibits a marked decrease in intensity in anaerobic preparations, has been shown to be a subunit of succinate dehydrogenase (19), an enzyme which is repressed in anaerobic growth (18).

Isolation of amber mutants deficient in fumarate reductase. Mutants lacking fumarate reductase are unable to utilize fumarate as an electron acceptor for anaerobic growth on glycerol, and the *frd* gene has been located at approximately 82 min on the *E. coli* linkage chromosome (18). Mutants with the desired nutritional phenotype were isolated from 10 independent mutagen-treated cultures. These were screened further by cross-streak conjugation tests with the F′ strains KLF17, which transfers the wild-type *frd* gene, to examine the location of the mutation, and with AW9 to test their sensitivity to the *supU* amber-suppressor gene. Of 58 mutants with the desired nutritional phenotype, 21 had lesions in the *frd* region, and 2 of these, from independent cultures, were suppressible. As a final confirmation that the mutants were deficient in fumarate reductase, the specific activity of fumarate reduction was measured in cell-free extracts of the two amber mutants, and six other mutants with lesions in the *frd* region (Table 2). Compared with the parental strain, all these mutants had low fumarate reduction activities typical of *frd* mutants. Of the other 37 mutants, which were unable to grow on GF medium anaerobically but mapped outside the *frd* region, 4 were suppressible. Cell-free extracts of three of these amber mutants showed about 35% of the parental fumarate reduction activity (Table 2).

Analysis of inner membrane proteins in an frd amber mutant. The inner membrane fraction of an *frd* amber mutant (*frd-3*) was isolated after anaerobic growth under the same conditions as the parental strain. Several gels of each of two different preparations were examined,

TABLE 1. *Changes, associated with anaerobic growth, in the intensities of the major bands of the inner membrane proteins on polyacrylamide gels*[a]

No. of band	Changes in intensity anaerobic vs. aerobic
5	++++
7	+
8	+
14	++
16	−−−
17	+
18	−−−−
19	+++
21	−−
22	−−−
24	−−
25	−−−
26[b]	++++
27	−−
28	−
29	+
32	(+)[b]
33	(+)
34	(+)
36	(+)
38	−
39	+
40	−

[a] +, Less than twofold increase; ++, two- to threefold increase; +++, three- to fourfold increase; ++++, more than fourfold increase; −, less than twofold decrease; −−, two- to threefold decrease; −−−, three- to fourfold decrease; −−−−, more than fourfold decrease.

[b] Brackets indicate variability.

and the gels of the *frd* amber mutant clearly lacked a prominent band in the wild-type preparation, as can be seen from a comparison of the photographs (Fig. 3a and c) and the scans (Fig. 2c and d). This band, number 14, was one of the bands which increased in intensity in the anaerobic preparations, and it corresponds to a protein of 75,000 daltons. In addition to this difference, the split gel preparation (Fig. 3b) revealed a possible decrease in intensity of a relatively minor band, number 9, in the *frd* amber mutant, and this difference can just be detected by a comparison of the scans. Band 9, which had an estimated molecular weight of 87,500 daltons, did not appear to vary appreciably in intensity between anaerobic and aerobic preparations.

DISCUSSION

The inner membrane proteins of aerobically and anaerobically grown *E. coli* were compared on polyacrylamide gels, and the patterns were found to be radically different. This is probably not unexpected in view of the fact that many membrane-bound enzymes, such as the components of the respiratory electron transport chain, are specifically aerobic enzymes, whereas others, such as the nitrate reductase system and fumarate reductase, are present only in anaerobic growth (3). Nevertheless, in the only published comparison between the membrane proteins of aerobic and anaerobic cultures of *E. coli*, few differences were found (15). There are probably two main reasons for this: first, the analyses were performed on samples of total envelope, resulting in a rather complex pattern which includes cell wall structural proteins and, second, the conditions of growth were not chosen to maximize the aerobic-anaerobic differences. Cultures grown aerobically and anaerobically on glucose minimal medium were compared, but glucose is well known to repress typically aerobic enzymes, such as those of the Krebs cycle and the respiratory electron transport chain (12), and it also partially derepresses the synthesis of fumarate reductase, a typically anaerobic enzyme.

In the work reported here, the differences between aerobic and anaerobic preparations were enhanced by the omission of glucose from the complex medium for aerobic growth, resulting in changes, many of them severalfold, in the intensity of approximately 20 bands. Among the changes observed was a great decrease in intensity in band 16, which has been identified previously as succinate dehydrogenase, an enzyme which is repressed by anaerobic growth.

TABLE 2. *Specific activities of fumarate reduction in cell-free extracts of the parental strain and mutants unable to grow on GF medium anaerobically*

Strain	Sp act[a]	Parental activity (%)
Parent		
WGAS	6.30	100
frd mutants		
frd-3 (amber)	0.06	1
frd-4	0.16	2
frd-5	0.04	<1
frd-6	0.03	<1
frd-7	0.13	2
frd-8	0.02	<1
frd-9	0.03	<1
frd-10 (amber)	0.04	<1
Other mutants		
810 (amber)	2.21	35
107 (amber)	1.18	30
118 (amber)	2.68	43

[a] Expressed as micromoles of substrate transformed per milligram of protein per hour.

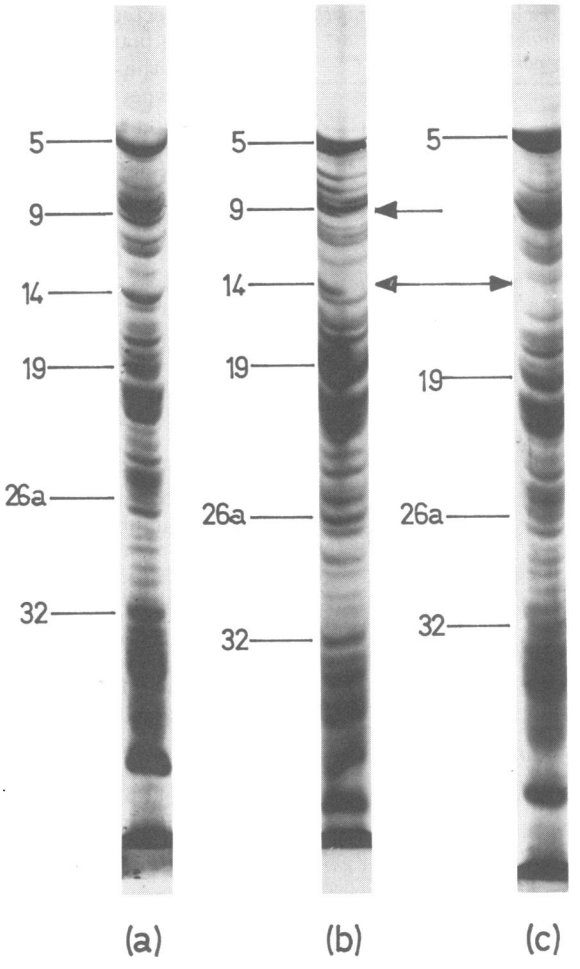

FIG. 3. *Polyacrylamide gels of the inner membrane proteins of the WGAS parental strain and an frd amber mutant, grown anaerobically. (a) WGAS anaerobic (b) split gel: left-hand side, WGAS, anaerobic; right-hand side, frd-3 (amber); (c) frd-3 (amber).*

Another major difference observed was the large decrease in band 5, which corresponds to a protein of 95,000 daltons and is possibly nitrate reductase. MacGregor and Schnaitman (10) have reported the identification of the nitrate reductase band on polyacrylamide gels of anaerobic membrane preparations, and shown that purified nitrate reductase runs in the same position near the top of the gels.

Gels of the inner membrane proteins of an *frd* amber mutant were found to lack two bands which were present in anaerobically grown wild-type preparations. The major band, number 14, had a molecular weight of 75,000 daltons, and the minor band, number 9, was 87,500 daltons. Of the two, band 14 is present in greater amounts in anaerobic preparations and is more likely to correspond to fumarate reductase, which is known to be derepressed anaerobically (18). The observed changes must be considered tentative since only one mutant was examined thoroughly, and a conclusive identification of fumarate reductase must await its purification.

The finding of more than one missing band is reminiscent of the pleiotropic effects seen in the nitrate reductase-less mutants (*chl* mutants) (10, 14). MacGregor and Schnaitman (10, 11) have recently analyzed mutants of the five different nitrate reductase loci and found some interesting pleiotropic effects in which most mutants lack more than one protein band. Altogether, differences in four different proteins are associated with nitrate reductase mutants, of which one has been identified as nitrate

reductase itself. This is clearly a useful system for studying the synthesis and incorporation of membrane-bound enzymes into the membrane structure.

Fumarate reductase should provide an analogous system for the study of membrane assembly especially if it proves to be a component in an anaerobic membrane-bound electron transport chain. When *E. coli* switches from aerobic to anaerobic growth, the components of this system must be synthesized and incorporated into membrane binding sites. The nature of these binding sites are little understood at present: Kung and Henning (9) have suggested that various membrane-bound enzymes, such as the flavoprotein dehydrogenases, may compete for common binding sites, and it would be interesting to know whether an anaerobic enzyme such as fumarate reductase shares a binding site with another, perhaps aerobic enzyme.

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