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Identification of Membrane Anchor Polypeptides of *Escherichia coli* Fumarate Reductase

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Fumarate reductase of *Escherichia coli* has been shown to be a membrane-bound enzyme composed of a 69,000-dalton catalytic-flavin-containing subunit and a 27,000-dalton nonheme-iron-containing subunit. Using gene cloning and amplification techniques, we have observed two additional polypeptides encoded by the *frd* operon, with apparent molecular weights of 15,000 and 14,000, which are expressed when *E. coli* is grown anaerobically on glycerol plus fumarate. Expression of these two small polypeptides is necessary for the two large subunits to associate with the membrane. The four subunits remain associated in Triton X-100 extracts of the membrane, and a holoenzyme form of fumarate reductase containing one copy of each of the four polypeptides has been isolated. Unlike the well-characterized two-subunit form, the holoenzyme is not dependent on anions for activity and is not labile at alkaline pH. In these respects, it more closely resembles the membrane-bound activity.

Fumarate reductase serves as the terminal electron transfer enzyme when *Escherichia coli* is grown anaerobically on glycerol plus fumarate. We have purified this membrane-bound enzyme and extensively characterized its enzymatic, chemical, and physical properties (6, 15, 22). The enzyme we have described is an $\alpha\beta$ dimer of 69,000- and 27,000-dalton subunits. Recently, the nucleotide sequences (and thus the inferred amino acid sequences) of the *frdA* gene (69,000-dalton catalytic subunit) (3) and the *frdB* gene (27,000-dalton nonheme-iron-containing subunit) (4) have become available. Examination of the nucleotide sequence of the *Hin*dIII fragment coding for the *frdA* and *frdB* genes indicated the presence of two additional sequences coding for two small polypeptides of 15,000 and 13,000 daltons in the *frd* operon, originally termed G15 and G13 and now referred to as *frdC* and *frdD*, respectively. A portion of *frdD* appears to overlap the *ampC* gene (9). The amino acid compositions of the *frdC* and *frdD* polypeptides are extremely hydrophobic and relatively basic. It seemed possible that these two polypeptides were part of a fumarate reductase holoenzyme. In this paper, we show that *frdC* and *frdD* are expressed in concert with *frdA* and *frdB*, are components of the holoenzyme, and serve as the membrane anchors for the catalytic and iron-containing subunits.

MATERIALS AND METHODS

Strains and plasmids. *E. coli* HB101 is F^-, *hsdR*, *hsdM*, *pro*, *leu*, *gal*, *lac*, *thi*, *recA*, and *rpsL*, and was provided by G. McFadden of this department. The plasmid pLC16-43 has been described previously (12). pBR322 was isolated from HB101(pBR322) after chloramphenicol amplification (2, 10, 14).

Construction of pFRD63 and pFRD117. To construct pFRD63, 220 ng of pBR322 and 610 ng of pLC16-43 were digested with 5 U of the restriction endonuclease *Hin*dIII for 2 h at 37°C as described previously (19). The digested material was ligated with 1 U of T4 DNA ligase (17), and the DNA was isolated by ethanol precipitation at −70°C. For transformation, ligated DNA (44 ng of pBR322) was incubated with 0.3 ml of competent HB101 cells (17) and plated on Luria broth agar plates (17) containing 100 μg of ampicillin per ml. Colonies were screened for the appropriate plasmid by the miniscreening procedure of Birnboim and Doly (1), and the structure was confirmed by restriction endonuclease mapping (Fig. 1).

To construct pFRD117, we digested 690 ng of pFRD63 with restriction endonuclease *Sal*I, ligated it with 1 U of T4 DNA ligase, purified the ligated DNA, and transformed *E. coli* HB101 as described above. The appropriate plasmid was detected by miniscreening and confirmed by restriction endonuclease mapping (Fig. 1).

Preparation of cell envelope fraction, cytoplasmic fraction, and Triton X-100 extracts. Cells were grown aerobically or anaerobically on glycerol-fumarate medium (20) to late stationary phase, washed once with 50 mM Tris-hydrochloride (pH 8.0), resuspended to 0.2 g/ml, and lysed by two passages through a French pressure cell (American Instrument Co., Silver Spring, Md.) at 16,000 lb/in^2. The cell envelopes were obtained by centrifuging the crude lysate at 11,000 × *g* for 10 min at 4°C, and the supernatant was spun at 160,000 × *g* for 60 min at 4°C. The supernatant of the high-speed spin is referred to as the cytoplasmic fraction. The membranes were homogenized in 50 mM Tris-hydro-

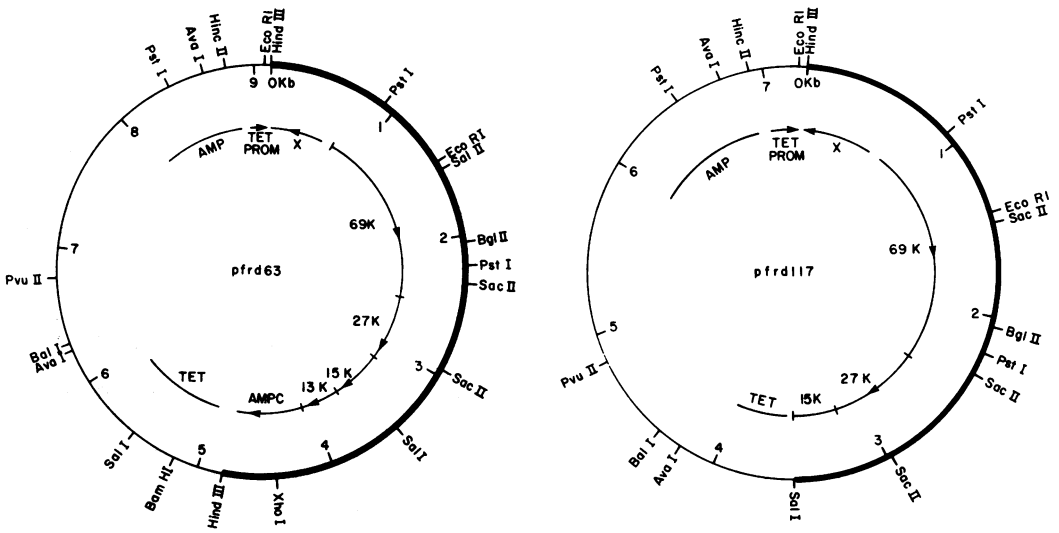

FIG. 1. Restriction endonuclease maps of pFRD63 and pFRD117. These maps were determined by multiple restriction enzyme digests as described previously (12, 19). pFRD63 is 9.1 kilobases, and pFRD117 is 7.2 kilobases. AMP refers to the ampicillin resistance gene of pBR322; AMPC refers to the *E. coli* chromosomal ampicillin-resistance gene adjacent to the *frd* operon.

chloride (pH 8.0) containing 0.15 M KCl, 0.25 M sucrose, and 10 mM EDTA, and spun at 160,000 × *g* for 60 min at 4°C. The membranes were homogenized in 50 mM Tris-hydrochloride and stored at a concentration of 7 mg of protein per ml at −70°C. To prepare the Triton X-100 extract, Triton was added to a final concentration of 1% (wt/vol) and incubated for 1 h at 4°C. The Triton-solubilized protein (4 mg/ml) was recovered after removal of insoluble debris by centrifugation at 48,000 × *g* for 60 min at 4°C.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Polyacrylamide gradient slab gels were run in the buffer systems described by Laemmli (11) or Weber and Osborn (21), as indicated in Results. Molecular weights were determined by using both the high- and low-molecular-weight marker mixes prepared by Bethesda Research Laboratories (BRL) (Bethesda, Md.). Electrophoresis was at 30 mA until the tracker dye, bromophenol blue, reached the bottom of the slab. Gels were stained in 0.1% Coomassie blue in 25% isopropanol–10% acetic acid–0.1% cupric acetate, destained in 25% methanol–10% acetic acid, and scanned on a Gilford model 2600 spectrophotometer equipped with a scanning attachment and integrator (Gilford Instrument Laboratories, Inc., Oberlin, Ohio).

Labeling cells with $^{35}SO_4^{2-}$. HB101(pFRD63) was grown anaerobically on sulfate-free glycerol-fumarate medium in the absence of added Casamino Acids to which was added 10 μCi of $^{35}SO_4^{2-}$. The final concentration of sulfate was adjusted to 120 μM with unlabeled sodium sulfate. Cells were harvested in late stationary phase and washed in 200 mM sodium phosphate (pH 6.8). The cells were lysed, and membranes were prepared as described above. Then 150,000 counts were applied to a 10 to 25% Laemmli gel. Destained gels were sliced into 1-mm slices; each slice was incubated in a scintillation vial for 4 h at 50°C with 0.5 ml of NCS solubilizer (Amersham Corp., Arlington Heights, Ill.) containing 6% water. The vials were cooled to room temperature, 17 μl of glacial acetic acid followed by 10 ml of Aquasol (New England Nuclear Corp., Boston, Mass.) was added, and the vials were shaken and counted.

Purification of fumarate reductase. The two-subunit form of fumarate reductase was purified as described previously (6). The holoenzyme form was purified by applying 100 μl (4 mg/ml) of the Triton X-100 extract (specific activity, 170 U/mg) to a 3.6-ml, 5 to 20% linear sucrose gradient prepared in 0.1 M potassium phosphate (pH 6.8) and 0.2% Triton X-100. The gradients were spun for 16 h at 50,000 rpm and 4°C in a Beckman SW56 rotor. Gradients were eluted from the bottom and assayed for activity. Recovery was routinely 80%, and the purity of the peak fractions (specific activity, 220 U/mg) was judged to be greater than 95% by polyacrylamide gel electrophoresis.

Assay of fumarate reductase. Fumarate reductase was assayed by following the oxidation of reduced benzyl viologen as previously described (6) in either 0.2 M potassium phosphate, pH 6.8 (anion buffer), or 50 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid), pH 6.8 (anion-free buffer), as detailed in Results. One unit of activity corresponds to 1 μmol of benzyl viologen oxidized per min at 24°C.

Stability of fumarate reductase to alkaline pH. The appropriate enzyme fraction was applied to a 2-ml column of Sephadex G-25 equilibrated with 20 mM Tris-hydrochloride (pH 8.6). The column was eluted with equilibration buffer, and active fractions were combined. This operation took 8 to 9 min. At 10 min, an aliquot was assayed for fumarate reductase, and this value was taken as 100%.

Sources of materials. $^{35}SO_4^{2-}$ sulfate (906 mCi/mmol) was from New England Nuclear. Restriction endonucleases were from BRL. T4 DNA ligase was from

Boehringer Mannheim Corp. (Montreal, Quebec, Canada). All other chemicals were from Sigma Chemical Co. (St. Louis, Mo.).

RESULTS

Expression of fumarate reductase in stains carrying pFRD63 and pFRD117. In a previous paper (12), we showed that the expression of the 69,000- and 27,000-dalton polypeptides of fumarate reductase correlated with enzyme activity and that these two bands were the major polypeptide components of the cytoplasmic membrane. The 15,000- and 13,000-dalton polypeptides were not visualized in those earlier experiments because they migrated with the tracker dye.

To examine the expression and role of *frdC* and *frdD*, we prepared French press membrane envelopes from cells carrying the plasmid pBR322 or the recombinant plasmid pFRD63 or pFRD117. pFRD63 carries a *Hin*dIII fragment coding for *frdA*, *frdB*, *frdC*, and *frdD* (3, 4). In pFRD117, we deleted the segment between the *Sal*I site in pBR322 and that within the 15K gene. Thus, pFRD117 carried only *frdA*, *frdB*, and a fragment of *frdC*. Table 1 summarizes the activity data we observed. The presence of pFRD63 resulted in a 20-fold amplification of activity when the cells were grown anaerobically. The appearance of activity in the soluble fraction was only observed after the membrane became saturated with fumarate reductase. This occurred when a specific activity of 60 to 70 U/mg was reached. During aerobic growth with a recombinant plasmid, we still observed significant activity, but it was 15-fold lower than under anaerobic conditions. The aerobic expression may have been due to the titration of a repressor protein (5).

A similar experiment was carried out with pFRD117, the recombinant plasmid which carries only the *frdA* and *frdB* genes. This plasmid also resulted in a substantial amplification of fumarate reductase activity, but, interestingly, the majority of the activity was in the soluble fraction. Thus, it appears that in the absence of the *frdC* and *frdD* polypeptides, the two large subunits do not associate with the membrane. Considering this in light of the very hydrophobic composition of both the 15,000- and 13,000-dalton polypeptides, we have proposed that these polypeptides serve as the membrane anchors for the catalytic part of the enzyme.

Expression and localization of *frdC* and *frdD*. The membrane envelopes from HB101(pFRD63) were examined by SDS-PAGE, using the Laemmli buffer system and 10 to 25% linear polyacrylamide gradients (11) (Fig. 2a). Four predominant bands at 69,000, 25,000, 15,000, and 14,000 can be seen. These molecular weights do not correspond exactly to the molecular weights estimated from the sequence. The 27,000-dalton polypeptide migrated at a position corresponding to 25,000, and the 13,000-dalton polypeptide migrated at 14,000. When the area under each stained peak was integrated, a mole ratio of 1:1.1:1.1:1.2 was obtained for these four bands. The corresponding mole ratio as determined with ^{35}S-labeled proteins was 1:1.3:1.2:1.0 (Table 2). When a Triton X-100 extract was prepared from these envelopes to remove outer membrane polypeptides (18) and subjected to SDS-PAGE, these four polypeptides were the predominant components of the extract. In fact, they comprised about 75% of the total protein in the fraction and were present in 1:1:0.8:1.1 mole ratios based on staining intensity (Fig. 2b). A similar experiment was done with cells grown aerobically, and all four bands were diminished about 10-fold in intensity (data not shown). If cells carrying pBR322 were used, these four polypeptides could barely be detected.

In a similar experiment, membranes and cytoplasm were prepared from HB101(pFRD117) which did not code for *frdC* and *frdD*. Very little of the polypeptides corresponding to fumarate reductase could be found associated with the membrane (Fig. 3a). However, when the cytoplasmic fraction was examined, the 69,000- and 27,000-dalton bands were clearly observed (Fig. 3b), in agreement with the activity measure-

TABLE 1. Aerobic and anaerobic expression of fumarate reductase[a]

Strain	Aerobic growth				Anaerobic growth			
	Membranes		Cytoplasm		Membranes		Cytoplasm	
	Sp act	Units	Sp act	Units	Sp act	Units	Sp act	Units
HB101(pBR322)	0.2	0.9	<0.1	<1.0	5.3	14.3	<0.1	<1.0
HB101(pFRD63)	3.7	15.2	<0.1	<1.0	66.8	294.0	9.9	128.0
HB101(pFRD117)	0.4	1.4	2.6	39.0	7.0	19.5	78.8	945.0

[a] Cells were grown either aerobically or anaerobically to stationary phase in glycerol-fumarate medium. Activity was assayed in 0.2 M potassium phosphate (pH 6.8) and is expressed as micromoles of reduced benzyl viologen oxidized per minute. Specific activity is expressed as units per milligram of protein.

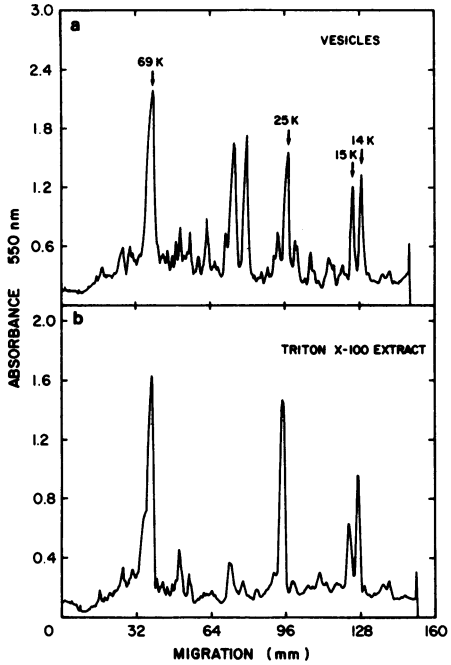

FIG. 2. Densitometric scans of the SDS-PAGE pattern of (a) French press membrane vesicles and (b) Triton X-100 extract of HB101(pFRD63). Membrane protein (200 μg) was applied to a 10 to 25% linear gradient polyacrylamide resolving gel in the Laemmli buffer system. Molecular weights were determined by using the following markers: alkaline phosphatase, 92,000; bovine serum albumin, 68,000; ovalbumin, 43,000; chymotrypsinogen, 25,000; β-lactoglobin, 18,400; cytochrome *c*, 12,300; bovine trypsin inhibitor, 6,200.

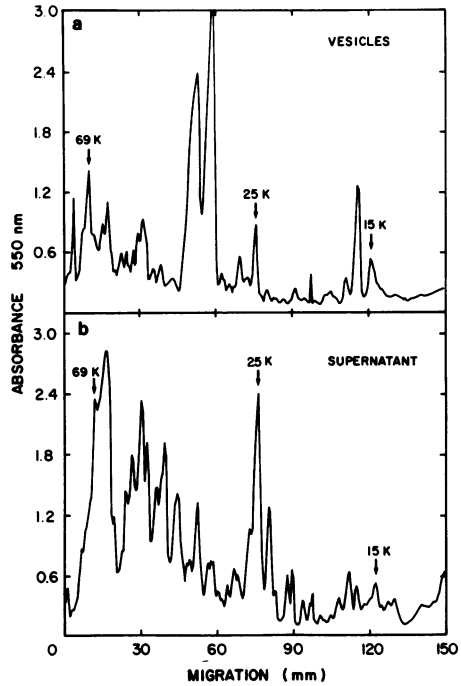

FIG. 3. Densitometric scans of the SDS-PAGE pattern of (a) French press membrane vesicles and (b) cytoplasmic fraction of HB101(pFRD117). Protein (200 μg) was applied to each well, and conditions were as described in the legend to Fig. 2.

ments. This experiment confirmed that expression of *frdC* and *frdD* was required for the 69,000- and 27,000-dalton polypeptides to bind to the membrane.

In agreement with the observation of Grundstrom and Jaurin (9), we observed anomalous behavior of *frdC* and *frdD* on different SDS-polyacrylamide gel systems. When the Weber and Osborn buffer system (21) was used, *frdC* and *frdD* could not be resolved.

TABLE 2. Determination of the fumarate reductase subunit mole ratio

Subunit	Number of cys + met[a]	cpm in subunit peak	cpm per cys + met	Mole ratio
69,000	30	6,044	201	1.0
27,000	20	5,331	267	1.3
15,000	4	950	238	1.2
13,000	7	1,403	200	1.0

[a] The number of sulfur-containing residues (cysteine + methionine) in each subunit was determined from the DNA sequence (3, 4, 9).

Properties of fumarate reductase holoenzyme. In a recent paper (15), we reported that the two-subunit catalytically active form of fumarate reductase was dependent on anions (e.g., phosphate) for optimal activity. Additionally, anions stabilized the enzyme against alkaline pH denaturation and thermal inactivation. Based on a comparison of these parameters for the membrane-bound versus purified forms, we proposed that anions converted the solubilized enzyme to a state which more closely resembled the membrane-bound state.

Interestingly, the four-subunit holoenzyme form of the enzyme was not dependent on anions for activity (Table 3) and was stable at alkaline pH (Fig. 4). It was, however, as thermolabile as the two-subunit form, and this thermo-

TABLE 3. Stimulation of fumarate reductase activity of potassium phosphate[a]

Enzyme	Assay A sp act	Assay B sp act	B/A ratio
Two-subunit form	100	550	5.50
Four-subunit holoenzyme	172	164	0.95
Membrane-bound form	62	68	1.09

[a] Enzyme was assayed in either (A) 25 mM HEPES (pH 6.8) or (B) 0.2 M potassium phosphate (pH 6.8).

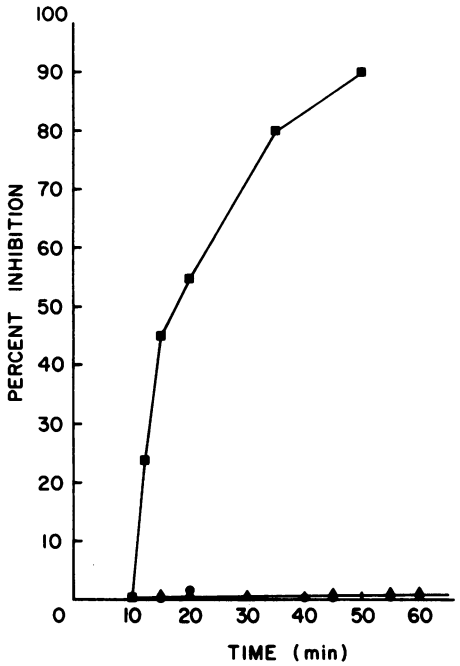

FIG. 4. Stability of fumarate reductase to alkaline pH. Incubations were at 24°C in 20 mM Tris-hydrochloride (pH 8.6). The membrane-bound enzyme (▲) was 6.6 mg/ml and had a specific activity of 59 U/mg. The four-subunit holoenzyme (Triton X-100 extract) (●) was 1.65 mg/ml; specific activity was 167 U/mg of protein. The two-subunit enzyme (■) was 2.8 mg/ml; specific activity was 550 U/mg of protein.

lability could be prevented by anions (Fig. 5). For these studies, we used either the Triton X-100 extract, which was estimated to be 75% pure by integration of Coomassie blue-stained polyacrylamide gels, or enzyme purified to 95% homogeneity by centrifugation through 5 to 20% sucrose gradients (see Methods). It should be noted that the specific activity of the holoenzyme was lower than expected, based on a comparison of staining intensity with our studies of the two-subunit form. The specific activity of the Triton X-100 extract was only 170 U/mg, whereas we would have expected a specific activity of about 400 U/mg based on the apparent purity. It may be that a fraction of the enzyme is catalytically inactive; this is currently under study.

Loss of the 15,000- and 13,000-dalton polypeptides during purification of catalytically active fumarate reductase. It is clear that the purified catalytically active fumarate reductase we have been characterizing is composed of only two subunits of 69,000 and 27,000 daltons and does not contain any material in the molecular weight range corresponding to *frdC* or *frdD* (16). As our purification involves only two steps—ammonium acetate precipitation and hydrophobic exchange chromatography on Phenyl Sepharose—we decided to see which of these steps removed the two small polypeptides. They were not dissociated by the ammonium acetate treatment. However, on a Phenyl Sepharose column, the 15,000- and 13,000-dalton polypeptides bound extremely tightly when applied in 50 mM Tris-hydrochloride (pH 6.8) containing 17% ammonium acetate and did not elute with the enzyme activity when the column was developed with a linear gradient of 17 to 2% ammonium acetate in 0.5% sodium cholate and 50 mM Tris-hydrochloride. They could be quantitatively eluted with a 1% Triton X-100 wash of the column. However, this fraction was heavily contaminated with other proteins retained by the Phenyl Sepharose column which coeluted.

DISCUSSION

In this paper, we have shown that the two small polypeptides of 15,000 and 13,000 daltons coded within the *frd* operon are part of a four-subunit holoenzyme form of fumarate reductase. This conclusion is based on the coexpression, coextraction, and copurification of all four subunits, as well as activity studies. The sequences of *frdC* and *frdD* show long stretches of hydrophobic amino acids (9), and we have proposed that they function, at least in part, to anchor the catalytic (69,000-dalton) subunit and the non-

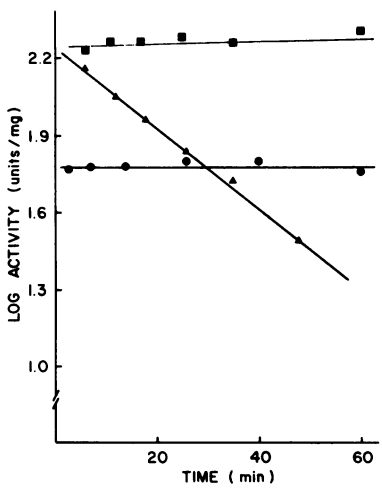

FIG. 5. Thermostability of membrane-bound and Triton X-100 (holoenzyme)-extracted fumarate reductase. The membrane vesicles in 25 mM HEPES (pH 6.8) (●) were 6.7 mg/ml, and the specific activity was 58 U/mg. The Triton X-100 extract in 25 mM HEPES (pH 6.8) (▲) was 1.4 mg/ml, and the specific activity was 180 U/mg of protein; in 200 mM sodium phosphate (pH 6.8) (■) it was 0.7 mg/ml, and the specific activity was 171 U/mg of protein. All incubations were at 45°C.

heme-iron-containing (27,000-dalton) subunit to the cytoplasmic membrane. This conclusion was drawn from studies with the plasmid pFRD117. This plasmid carries only the *frdA* and *frdB* genes, and fumarate reductase is found primarily in the cytoplasmic fraction in a strain harboring this plasmid.

These two small polypeptides also serve a role in maintaining the structure of the two large subunits. This was clearly shown by the anion independence and alkaline stability of the holoenzyme form of fumarate reductase compared with the two-subunit form. In these respects, the holoenzyme form closely resembles the membrane-bound activity. The picture is not complete however, as both the two-subunit and holoenzyme forms are thermolabile, in contrast to the membrane-bound form. It is interesting that anions stimulate and stabilize the two-subunit fumarate reductase, because both the 15,000- and 13,000-dalton polypeptides are rather basic proteins (9). The explanation for this dichotomy is not clear.

Fumarate reductase is not unique in having membrane anchor polypeptides. Beef heart succinate dehydrogenase appears to have two hydrophobic polypeptides which may serve a similar role (8, 13). The hydrophobic F_0 portion of F_0F_1ATPase appears to bind the F_1 portion to the membrane in addition to its role in proton translocation (7).

A more detailed study of the interactions between the anchor polypeptides and the catalytic subunits is presently being undertaken.

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