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Structure of Fumarate Reductase on the Cytoplasmic Membrane of *Escherichia coli*

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The terminal electron transfer enzyme fumarate reductase has been shown to be composed of a membrane-extrinsic catalytic dimer of 69- and 27-kilodalton (kd) subunits and a membrane-intrinsic anchor portion of 15- and 13-kd subunits. We prepared inverted membrane vesicles from a strain carrying the *frd* operon on a multicopy plasmid. When grown anaerobically on fumarate-containing medium, the membranes of this strain are highly enriched in fumarate reductase. When negatively stained preparations of these vesicles were examined with an electron microscope, they appeared to be covered with knob-like structures about 4 nm in diameter attached to the membrane by short stalks. Treatment of the membranes with chymotrypsin destroyed the 69-kd subunit, leaving the 27-, 15-, and 13-kd subunits bound to the membrane; these membranes appeared to retain remnants of the structure. Treatment of the membranes with 6 M urea removed the 69- and 27-kd subunits, leaving the anchor polypeptides intact. These vesicles appeared smooth and structureless. A functional four-subunit enzyme and the knob-like structure could be reconstituted by the addition of soluble catalytic subunits to the urea-stripped membranes. In addition to the vesicular structures, we observed unusual tubular structures which were covered with a helical array of fumarate reductase knobs.

Fumarate reductase serves as the terminal electron transfer enzyme in a very simple electron transport chain induced when *Escherichia coli* is grown anaerobically on glycerol and fumarate (6). This enzyme is a membrane-bound tetramer consisting of four nonidentical subunits: a 69-kilodalton (kd) subunit, *frdA*, which contains a covalently bound flavin adenine dinucleotide cofactor (12); a 27-kd subunit, *frdB*, containing a non-heme iron sulfur center (2); and two small very hydrophobic subunits, *frdC* and *frdD*, of 15 and 13 kd (8). The latter two subunits have been shown to anchor the catalytic 69- and 27-kd subunits to the cytoplasmic membrane (8).

Fumarate reductase can be purified either as a catalytically active dimer composed of the larger two subunits or as a tetrameric holoenzyme (3, 9). The properties of the holoenzyme and the two-subunit enzyme differ in a number of respects. The dimer requires anions for optimal activity, whereas the holoenzyme is anion independent (10). Additionally, the dimer is considerably more heat and alkaline labile than the holoenzyme (8).

As reported in this paper, we have extended our understanding of the structure of fumarate reductase and have shown that it can be visualized on the inner surface of cytoplasmic membranes containing greatly amplified levels of the enzyme, which were isolated from a strain which carries a multicopy recombinant plasmid coding for the fumarate reductase operon. The holoenzyme is composed of a membrane-extrinsic catalytic domain and a membrane-intrinsic anchor domain. These domains can be dissociated and functionally reassociated in everted membrane vesicles.

MATERIALS AND METHODS

Strains and plasmids. *E. coli* HB101 is F⁻ *hsdR hsdM pro leu gal lac thi recA rpsL*. The plasmids pBR322, pFRD63, and pFRD117 have previously been described (8).

Preparation of everted inner membranes and cytoplasmic fractions. Cells were grown anaerobically on glycerol-fumarate medium to late stationary phase (11). Purified everted inner membranes prepared by French pressure cell lysis (Aminco Instruments, Rockville, Md.) were isolated by the procedure of Yamato et al. (13), except that the dialysis step and final wash were omitted. Preparation of the cytoplasmic fraction from HB101/pFRD117 has been described previously (8).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Electrophoresis on 10 to 25% acrylamide gradient gels was carried out by using the Laemmli buffer system (7).

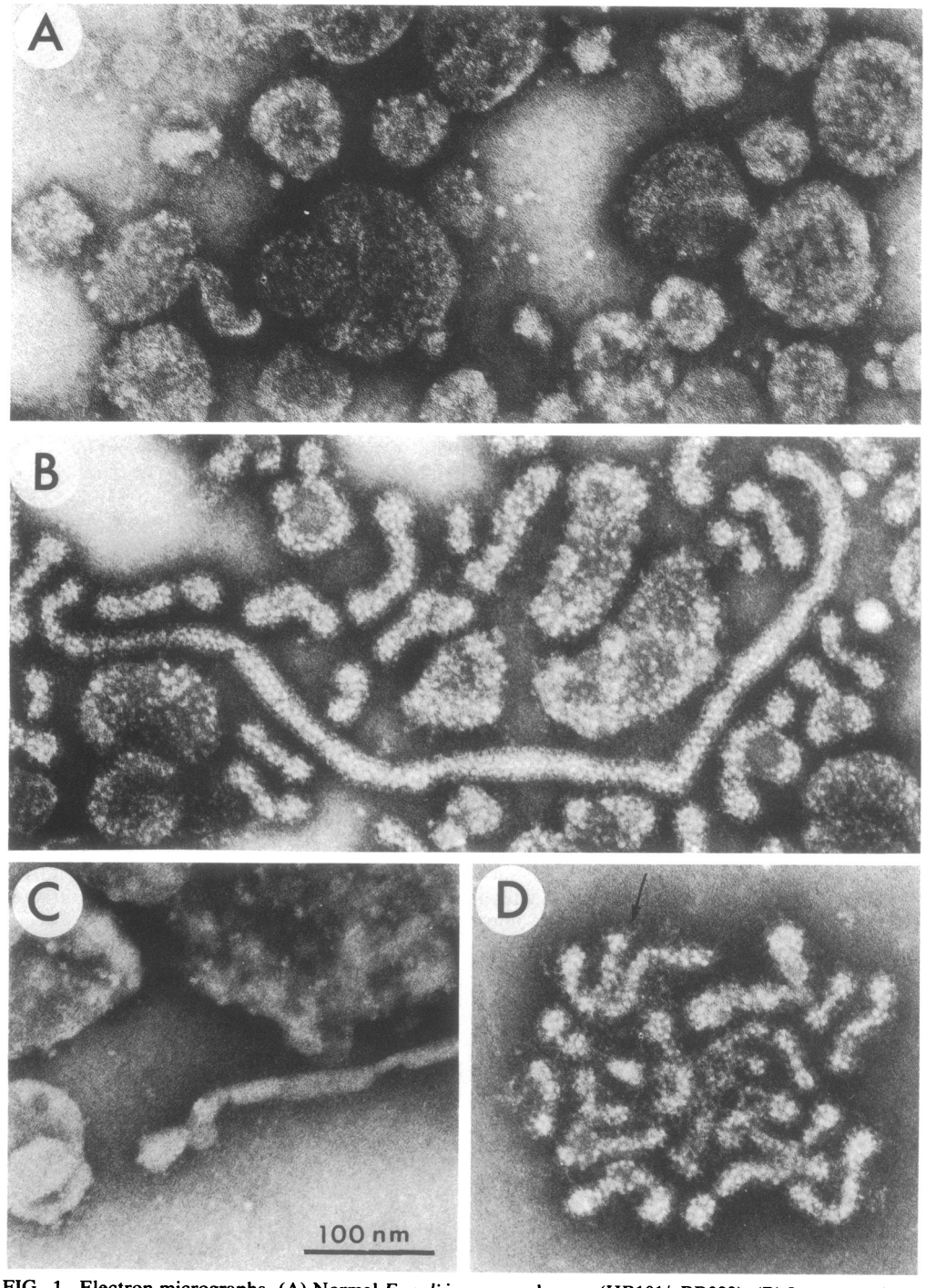

FIG. 1. Electron micrographs. (A) Normal *E. coli* inner membranes (HB101/pBR322). (B) Inner membranes from *E. coli* carrying plasmid pFRD63. (C) Same as (B) but after overnight incubation at room temperature with proteinase K. (D) Same as (B) but after the addition of antibody (IgG fraction) against the catalytic dimer of fumarate reductase. The arrow shows an IgG molecule.

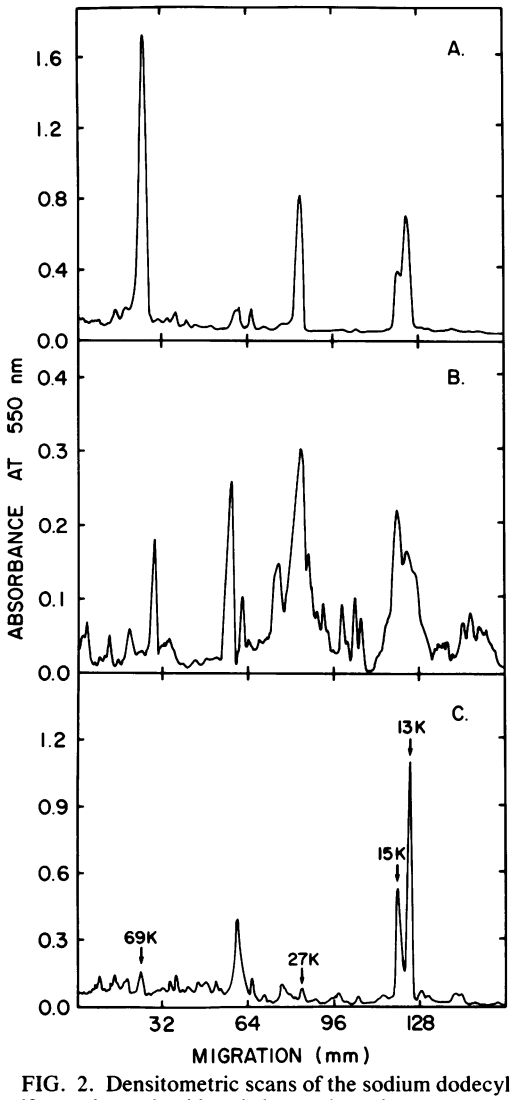

FIG. 2. Densitometric scans of the sodium dodecyl sulfate-polyacrylamide gel electrophoresis pattern. (A) French press membrane vesicles from HB101/pFRD63. (B) Same as (A) but after 30 min of chymotrypsin digestion. (C) Same as (A) but after 6 M urea wash.

Purification of fumarate reductase and assay of activity. The two-subunit form of fumarate reductase was purified by Triton X-100 extraction and hydrophobic exchange chromatography as previously described (3). The activity was assayed by following the oxidation of reduced benzyl viologen (3). One unit of activity corresponds to 1 μmol of benzyl viologen oxidized per min at 24°C. The holoenzyme form was purified by Triton X-100 extraction and sucrose gradient centrifugation as previously described (8).

Chymotrypsin treatment of everted inner membranes. Inner membranes were suspended in 0.2 M NaPi (pH 6.8) to a final protein concentration of 5.3 mg/ml. The membranes were digested for 2 min at 30°C with *N*-α-*p*-tosyl-L-lysine chloromethyl ketone-treated chymotrypsin in 0.2 M NaPi (pH 6.8) at a protein to chymotrypsin ratio of 10/1. The digestion was stopped by the addition of 10 μg of *N*-tosyl-L-phenylalanyl chloromethyl ketone in 3 μl of isopropanol to 200 μl of digestion mixture which contained 0.9 mg of membrane protein. The membranes were harvested by centrifugation at 160,000 × *g* for 10 min in a Beckman Airfuge and washed twice in 50 mM NaPi (pH 6.8).

Urea wash of everted inner membranes. Everted membranes were stripped of the 69- and 27-kd subunits as follows. Typically, 4.2 mg (42 mg/ml) of inner membrane protein from HB101/pFRD63 or HB101/pBR322 was diluted with 9 volumes of 50 mM Tris-hydrochloride (pH 8.0). Urea (9 M) was added to a final concentration of 6 M, and the mixture was incubated at 0°C for 4 h with occasional mixing. The membranes were recovered by centrifugation at 160,000 × *g* for 60 min at 2°C. The membrane pellet was resuspended in 50 mM NaPi (pH 6.8) and sonicated at 4°C for 90 s at a power setting of 40 W in a Branson Probe Type sonicator. The membranes were stored at −70°C.

Reconstitution of stripped inner membranes. For titration experiments, 10 μg of stripped inner membrane protein was incubated with 2 volumes of 1 M NaCl, 2 volumes of 10 mM dithiothreitol, and up to 20 volumes of HB101/pFRD117 cytoplasmic fraction (5.6 mg/ml) or purified two-subunit fumarate reductase (8.9 mg/ml). For alkaline and temperature stability experiments, 40 to 80 μg of membrane protein was incubated with 15 volumes of HB101/pFRD117 cytoplasm. All reconstitution experiments were incubated at 0°C for 60 min and centrifuged at 140,000 × *g* for 15 min at 24°C in a Beckman Airfuge. The membrane pellets were resuspended in 200 mM NaPi (pH 6.8) for titration experiments or in 10 mM NaPi (pH 6.8) for other experiments.

Temperature and pH stability experiments. HB101/pFRD117 cytoplasmic fraction was desalted on a 2-ml column of Sephadex G-25 equilibrated in 10 mM NaPi (pH 6.8). The column was eluted with equilibration buffer, and colored fractions were combined. At the initiation of the experiment, an equal volume of 0.1 M Tris-hydrochloride (pH 8.8) was added to the desalted cytoplasmic fraction and the reconstituted inner membranes (1.4 mg of protein) in 10 mM NaPi (pH 6.8). The samples were incubated at 0°C, and portions were assayed at the indicated times.

For temperature stability experiments, reconstituted inner membrane and HB101/pFRD117 cytoplasmic fractions were prepared as described for pH stability studies. Samples were immersed in a circulating water bath at 45°C.

Electron microscopy. Membranes at a protein concentration of 0.7 mg/ml in 50 mM NaPi (pH 6.8) were absorbed to hydrophilic carbon films and negatively stained with 1% sodium phosphotungstate (pH 7.0). Photographs were obtained in a Phillips model EM300 electron microscope operated at 80 kV. Digestions with proteinase K (E. Merck Biochemicals, Darmstadt) were performed for 18 h at 25°C with a protease concentration of 50 μg/ml. Anti-fumarate reductase antibody was prepared in rabbits, and the immunoglobulin G (IgG) fraction was isolated by the proce-

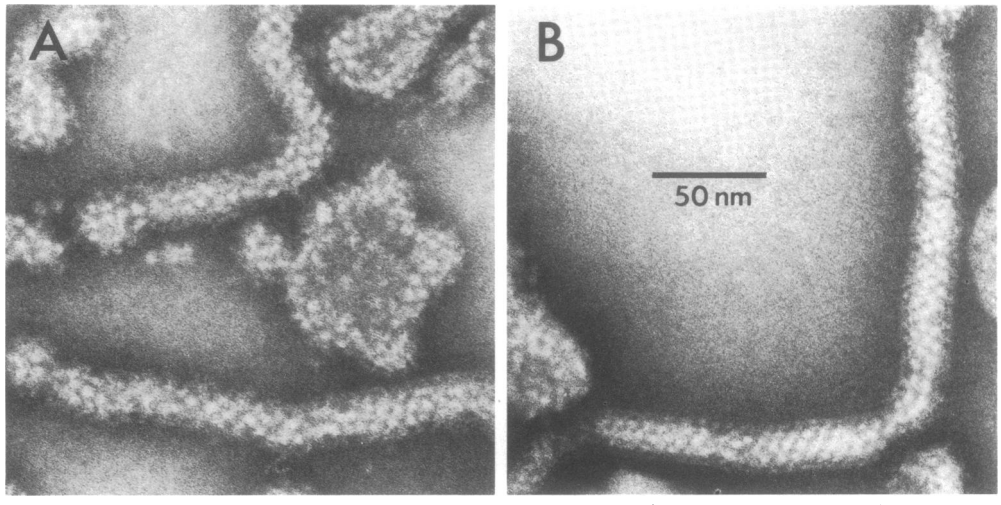

FIG. 3. Electron micrographs. (A) Inner membranes from HB101/pFRD63. (B) Same as in (A) but after chymotrypsin digestion.

dure of Broome and Gilbert (1). Membranes were incubated with 0.1 mg of IgG protein per ml at 25°C for 18 h before electron microscopy.

RESULTS

Visualization of fumarate reductase. We have previously shown that when fumarate reductase is induced by anaerobic growth in the presence of fumarate, the cytoplasmic membrane from HB101/pFRD63 contains dramatically amplified levels of the enzyme (8). Negatively stained preparations of these everted membrane vesicles, prepared by French pressure lysis, were examined in an electron microscope. The cytoplasmic surface appeared to be covered with a high density of knob-like structures. In many of the micrographs, the knobs appeared attached to the membrane by short stalks (Fig. 1B). These knobs were about 4.3 ± 0.3 nm in diameter, and the stalks were about 1 nm in length. Evidence that the knob and stalk structure may in fact be fumarate reductase came from the following studies. Membranes isolated from HB101/pBR322 containing the unamplified (normal) lev-

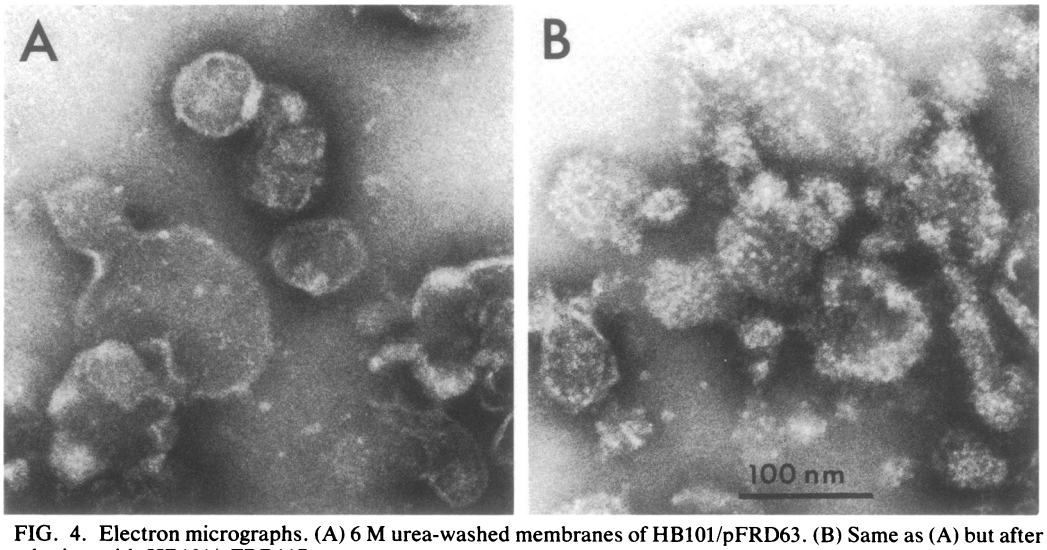

FIG. 4. Electron micrographs. (A) 6 M urea-washed membranes of HB101/pFRD63. (B) Same as (A) but after incubation with HB101/pFRD117 supernatant.

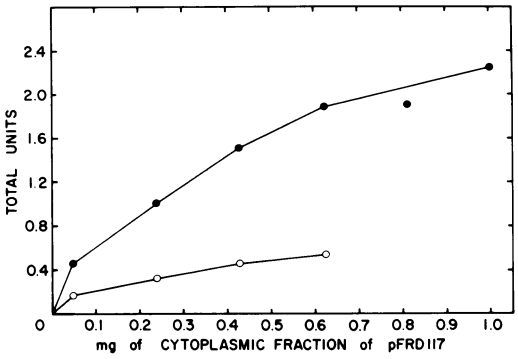

FIG. 5. Reconstitution of the soluble fumarate reductase of HB101/pFRD117 with urea-stripped membranes. Reconstitution was carried out as described in the text. ●, Urea-stripped HB101/pFRD63 membranes; ○, urea-stripped HB101/pBR322 membranes.

el of fumarate reductase had many fewer knobs on the surface (Fig. 1A); treatment of the membranes with proteinase K destroyed all surface structure (Fig. 1C), and sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the resulting vesicles indicated that the 69- and 27-kd subunits were absent (data not shown); antibody prepared against the 69- and 27-kd subunits of fumarate reductase formed a "fuzzy" coating on the vesicle surface and tended to clump the vesicles (Fig. 1D).

In addition to the knob-covered vesicles, we observed an unusual structure in the electron micrographs. This structure appeared to be long tubules covered with a helical array of the knobs (Fig. 1B). The length of these tubes varied greatly, from a few to over 1,000 nm in length. Attempts to separate the tubules from the vesicles by gel filtration in Bio-Gel A5M or by sucrose gradient sedimentation have been unsuccessful, apparently due to fragmentation of the tubules and to the collapse of the vesicles into tube-like structures.

Catalytic portion of fumarate reductase. From the proteinase K digestion experiments, it appeared that the presence of the knobs and stalks correlated with the presence of the 69- and 27-kd subunits. Previous studies (3, 8) have shown that these two subunits from the catalytic portion of the enzyme. To confirm that this structure was indeed the "catalytic head" of the enzyme, two types of experiments were carried out. Treatment of the vesicles with chymotrypsin as described above resulted in removal of the majority of the 69-kd subunit, whereas the 27-, 15-, and 13-kd subunits remained membrane associated, as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Fig. 2B). In the electron microscope, the chymotrypsin-treated vesicles and tubules presented a more uniform appearance. Knob-like structures could still be seen, but these were more distinct and about 20% smaller than untreated knobs (3.4 versus 4 nm; compare Fig. 3A and B). It may be that the 69-kd subunit appears as fuzzy knobs because it is not a tightly packed globular polypeptide. The remnants of the structure seen after chymotrypsin digestion may be the stalks, which appear much more uniform and distinct.

Treatment of the vesicles with 6 M urea resulted in 90% removal of the 69- and 27-kd subunits and small amounts of the 15-kd subunit (Fig. 2C). These vesicles appeared structureless in the electron microscope (Fig. 4A). The urea wash supernatant was inactive, and attempts to regain fumarate reductase activity by dialysis were unsuccessful.

Reconstitution of catalytic portion of fumarate reductase with urea-stripped membranes. Reconstitution studies were carried out to show that the urea-stripped membranes contained intact anchor polypeptides. Two sources of the catalytic portion of fumarate reductase were used for reconstitution. The first was the crude, soluble, detergent-free form of the enzyme synthesized in strains harboring plasmid pFRD117. This plasmid codes only for the 69- and 27-kd subunits and under anaerobic growth conditions produces 10 to 15% of its protein as fumarate reductase. The second source of enzyme was the two-subunit activity purified from the Triton X-100 extract of membranes (of strains harboring plasmid pFRD63) by hydrophobic exchange

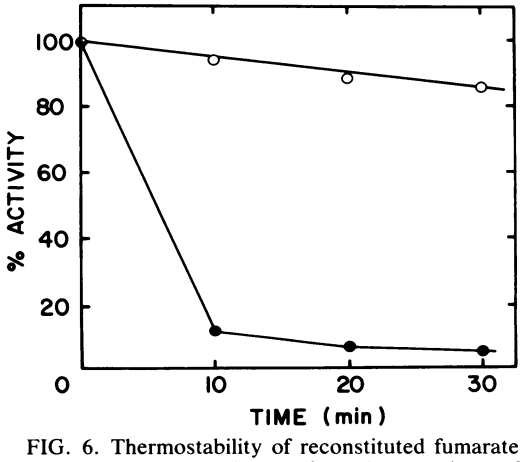

FIG. 6. Thermostability of reconstituted fumarate reductase. Fumarate reductase from the cytoplasm of HB101/pFRD117 (1.8 mg) was reconstituted with urea-stripped French press vesicles (40 μg) of HB101/pFRD63 for 60 min at 0°C, and heat lability at 45°C was measured as described in the text. ●, Cytoplasmic fraction; ○, reconstituted membranes.

TABLE 1. Stimulation of fumarate reductase activity by potassium phosphate[a]

Enzyme	Assay A spec act	Assay B spec act	Ratio B/A
pFRD117 cytoplasmic fraction	14	76	5.4
Purified dimeric fumarate reductase	154	682	4.4
Reconstituted inner membranes	17	21	1.3

[a] Enzyme was assayed in either (A) 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) (pH 6.8) or (B) 0.2 M potassium phosphate (pH 6.8).

chromatography (9). This enzyme is homogeneous but contains trace amounts of deoxycholate. The results obtained with both preparations were essentially identical.

Incubation of urea-stripped vesicles from HB101/pFRD63 with the catalytic portion resulted in membrane-binding activity and visible reconstitution of the knob-like structure (Fig. 4B). This reconstitution was saturable, as shown in Fig. 5, and the binding was specific for vesicles with amplified amounts of anchor polypeptides. Urea-stripped vesicles from HB101/pBR322, which contain only the wild-type level of anchor subunits, did not saturably bind the catalytic heads. The binding was rapid and essentially complete in 10 min. It was independent of temperature between 0 and 37°C, of ionic strength between 0.03 M and 0.2 M NaCl, and of Mg^{2+} ion. The presence of dithiothreitol was necessary for optimal reconstitution.

We took advantage of several properties of the holoenzyme to distinguish between true reconstitution and surface adsorption. As we have shown previously, the anchor polypeptides modulate several catalytic properties of fumarate reductase (8). Thus, the catalytic dimer is very alkaline and thermolabile, whereas the holoenzyme is alkaline and thermostable. When the catalytic portion was reconstituted with anchor polypeptides in the membrane, it regained both alkaline stability (data not shown) and thermostability (Fig. 6). The activity of reconstituted membranes incubated at 45°C for 10 min decreased by approximately 5%, whereas the activity of the HB101/pFRD117 cytoplasmic fraction decreased by over 90%. The small amount of enzyme bound to HB101/pBR322 membranes did not regain these stability properties (data not shown).

The two-subunit form of fumarate reductase requires anions for optimal activity, whereas the membrane-bound form does not (10). As shown in Table 1, the reconstituted enzyme is anion independent, as predicted for true reconstitution.

DISCUSSION

The results in this paper show that fumarate reductase consists of a membrane-extrinsic domain composed of a catalytic dimer (the 69- and 27-kd subunits) and a membrane-intrinsic or anchor portion composed of the 15- and 13-kd subunits. The extrinsic portion likely exists as 4-nm-diameter knobs attached to the cytoplasmic surface of the membrane by short stalks. It appears that the knobs can be removed by

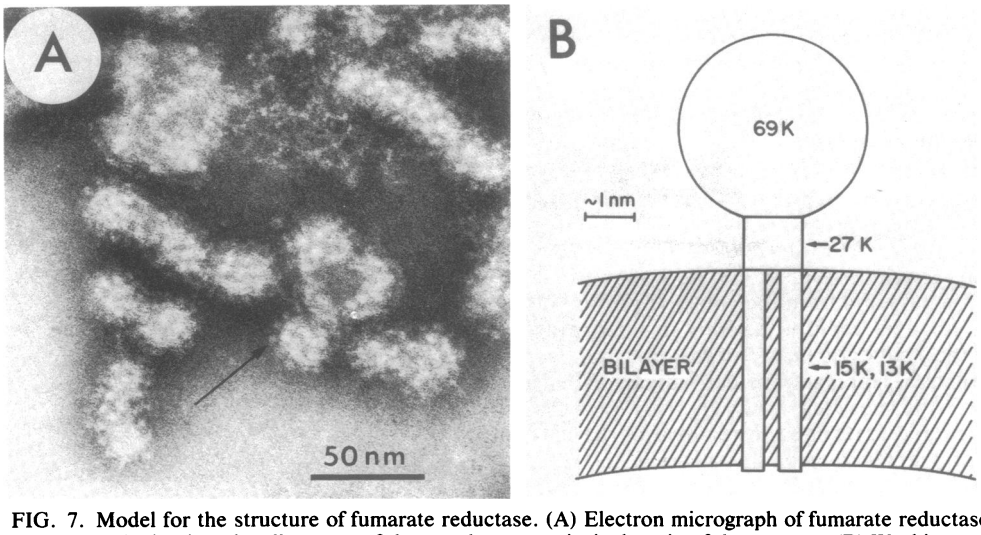

FIG. 7. Model for the structure of fumarate reductase. (A) Electron micrograph of fumarate reductase. The arrows show the knob and stalk aspect of the membrane-extrinsic domain of the enzyme. (B) Working model of the structure based on the electron micrograph.

chymotryptic digestion, leaving remnants of the structure, presumably the stalks, bound to the membrane. Urea washing of the membranes removes both the 69- and 27-kd subunits, and these vesicles appear structureless in the electron microscope. The anchor subunits are apparently unaffected by the urea wash and can specifically bind fresh catalytic dimer. The anchor subunits not only bind the catalytic dimer to the membrane but also modulate the activity and stability of the catalytic subunits. Our working model for the structure of the enzyme is shown diagrammatically in Fig. 7. In many ways this structure is analogous to the F_0/F_1 ATPase of bacterial and mitochondrial membranes, which is composed of an extrinsic F_1 portion and an intrinsic F_0 portion (4). Presumably, the large knob is the 69-kd subunit, and the stalk is the 27-kd subunit. The two anchor polypeptides are shown as transmembranal tubes. The actual subunit contacts, however, remain to be determined. The transmembranal structure of the hydrophobic anchor polypeptides is based on structure analysis of the sequence (5); both proteins consist of three regions composed of very hydrophobic amino acids, each about 23 to 28 amino acids in length, joined by short segments of charged amino acids (5; W. Anderson, B. D. Lemire, and J. H. Weiner, unpublished data). These hydrophobic regions are long enough to cross the membrane as α-helical structures. The subunit interactions and orientations predicted by this model are presently being probed.

Our ability to reconstitute the catalytic dimer with the anchor portion should prove useful for characterizing fumarate reductase mutants and for identifying the amino acids which form the contact regions. The tubular structures covered with a helical array of the fumarate reductase knobs remain a mystery. They are most numerous in stationary phase cells and may result from vesicle collapse as an artifact of the French pressure lysis procedure or other manipulations. Attempts to separate vesicles from tubules to examine lipid to protein ratios and subunit composition have so far been unsuccessful.

ACKNOWLEDGMENTS

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