Investigation of *Escherichia coli* Fumarate Reductase Subunit Function Using Transposon Tn5

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Seventy two Tn5 transposon insertions were isolated in the *frd* operon carried on the multicopy plasmid pFRD79. The polar nature of these mutations permitted examination of the expression and localization of the *jid* polypeptides in novel subunit combinations. The minimal catalytic unit is the FRDA plus B dimer. A transposon within *frdB* (*frdB* : : Tn5) produces inactive, soluble FRDA polypeptide which has covalently attached 8a(N3-histidyl)flavin adenine dinucleotide cofactor. A transposon mutation within *frdC* (*frdC* : : Tn5) produces soluble, catalytically active dimer. An insertion in *frdD* (*frdD* : : Tn5) produces both a soluble trimer composed of FRDABC, and a tetramer of FRDABC and truncated FRDD bound to the inner membrane. Eighty percent of the activity is in the soluble form. Using this mutant, the requirement for FRDD both for optimal activity of the catalytic domain and for proper anchorage in the cytoplasmic membrane was demonstrated.

INTRODUCTION

When the facultative anaerobe *Escherichia coli* is grown anaerobically on medium containing glycerol and fumarate, a simple electron transport chain consisting of the anaerobic glycerol-3-phosphate dehydrogenase, menaquinone, a b-type cytochrome and fumarate reductase is synthesized and assembled in the cytoplasmic membrane (Singh & Bragg, 1975; Haddock & Jones, 1977; Ingledew & Poole, 1984). Fumarate reductase, the terminal electron transfer enzyme, is composed for four non-identical subunits of 69, 27, 15, and 13 kDa (Weiner *et al.*, 1984), encoded by the *frdA*, *B*, *C* and *D* genes, respectively. The *frd* operon has been cloned into multicopy plasmids and sequenced (Cole, 1982; Cole *et al.*, 1982; Grundström & Jaurin, 1982). Strains of *E. coli* harbouring such plasmids amplify fumarate reductase activity in their membranes 5-20-fold (Lohmeier *et al.*, 1981; Weiner *et al.*, 1984).

The holoenzyme has been purified and characterized (Weiner *et al.*, 1984). The 69 kDa polypeptide (FRDA) contains a catalytically essential sulphydryl group (Robinson & Weiner, 1982) and a covalently bound 8a(N3-histidyl)flavin adenine dinucleotide cofactor (Weiner & Dickie, 1979). The 27 kDa subunit contains three iron–sulphur centres (Cole *et al.*, 1982, 1986; Ingledew & Poole, 1984; Cammack *et al.*, 1986). Together these two subunits comprise a membrane-extrinsic catalytic domain which can be purified in water-soluble form (Dickie & Weiner, 1979).

The 15 and 13 kDa subunits (FRDC and FRDD, respectively) are basic and extremely hydrophobic in amino acid composition (Weiner *et al.*, 1984). They form the membrane-anchor domain of fumarate reductase (Lemire *et al.*, 1982). The finding that anions or the anchor polypeptides could stabilize the catalytic dimer against denaturation by either alkali (pH > 8.6) or heat (>45 °C) has led to the proposal that the FRDC and D polypeptides have at least two functions: those of anchoring and stabilizing the catalytic dimer (Robinson & Weiner, 1982; Lemire *et al.*, 1982, 1983).

Abbreviations: GF, glycerol–fumarate medium; DMNH₂, reduced 2,3-dimethyl-1,4-naphthoquinone.

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In *E. coli*, transposon Tn5 confers resistance to the aminoglycoside antibiotics kanamycin and neomycin (Berg et al., 1978). It transposes at high frequency (Johnson et al., 1982) and with little or no insertional specificity (Shaw & Berg, 1979; Berg et al., 1980; Miller et al., 1980). Transposition of Tn5 into a structural gene causes insertional inactivation, which is normally polar on cistrons located promoter-distal to the site of the insertion (Berg, 1977; Berg et al., 1980). Tn5 mutagenesis has been used to generate correlated physical and genetic maps of DNA segments cloned into multicopy plasmids (Laird & Young, 1980; Pannekoek et al., 1980; De Bruijn & Ausubel, 1981; Lupski et al., 1982; De Bruijn et al., 1983).

In this paper Tn5 is used to introduce polar mutations into the plasmid-borne *frd* genes. The altered gene products are examined in terms of the known properties of fumarate reductase and the specific functions of individual polypeptides are discussed.

**METHODS**

**Enzymes and chemicals.** Restriction endonucleases *EcoRI, AatI, AulI* and *SalI* were obtained from Boehringer. *BamHI* and *HindIII* were from Pharmacia. Rabbit anti-succinyl-CoA synthetase IgG was a gift from W. A. Bridger (this department). 2,3-Dimethyl-1,4-naphthoquinone was a gift from A. Kroger (J. W. Goethe University, Frankfurt, FRG). All other chemicals were of the highest grade commercially available.

**Strains and plasmids.** These are described in Table 1. pFRD79 is similar to pFRD63 (Lemire et al., 1982) except that the 4.5 kbp *HindIII* fragment carrying the *frd* operon is reversed in orientation relative to the *EcoRI* site of pBR322. Expression of the *frd* operon is not altered by this change (data not shown).

**Cell growth.** Cells were grown anaerobically for 21 h on glycerol–fumarate medium (GF) supplemented with appropriate antibiotics, as described by Spencer & Guest (1974).

**Tn5 mutagenesis and plasmid isolation.** HB101 (pFRD79) was grown overnight at 37 °C on L-broth (Miller, 1972) supplemented with 20 µg thiamin ml⁻¹, 10 mm-MgSO₄, and 100 µg ampicillin ml⁻¹. *Jb221cl857reX: Tn5 (3 × 10⁶ p.f.u.) was added to 6 × 10⁸ cells of bacterial culture. The culture was incubated for 30 min at 24 °C, then centrifuged for 5 min at 13000 g. The pellet was resuspended in 10 vols L-broth containing thiamin and ampicillin, and the cells were incubated at 30 °C without shaking for 2 h. The cells were harvested as before, then resuspended in 200 µl of medium. Samples (100 µl) of the suspension were spread on L-broth agar plates supplemented with thiamin, ampicillin and 40 µg kanamycin ml⁻¹ and incubated overnight at 32 °C. Resistant colonies were suspended in 2 ml of medium and 250 µl was inoculated into 25 ml L-broth containing thiamin and ampicillin and grown to stationary phase. Samples (5 ml) of stationary phase culture were inoculated into 11 M9 medium (Miller, 1972) with suitable supplements and plasmid DNA was amplified by the method of Clewell & Helinski (1972). Cells were harvested and plasmid DNA was prepared as described by Lohmeier et al. (1981). HB101 cells treated with CaCl₂ (Mandel & Higa, 1970) were transformed with plasmid DNA and Tn5-containing plasmids were selected on L-broth plates supplemented with thiamin, ampicillin and kanamycin.

**Screening Tn5 insertions.** Individual colonies were picked and grown overnight at 37 °C in 2 ml L-broth supplemented with thiamin, ampicillin and kanamycin. Plasmid DNA was isolated from 0.5 ml of the above cultures by the rapid alkaline extraction method of Birnboim & Doly (1979) and digested with 1–3 units of restriction endonuclease, as described by Lohmeier et al. (1981).

**Membrane preparations and detergent extractions.** Crude cell envelopes were prepared by French pressure cell lysis at 110 MPas described by Dickie & Weiner (1979). Inner membranes were prepared by a modification of the method of Yamato et al. (1975). DNAase (10 µg ml⁻¹) and MgCl₂ (25 mm) were added before lysis in 50 mm-Tris/HCl, pH 7.5, 50 mm-NaCl at 34 MPa. At the final step, isolated inner membranes were resuspended in a minimum volume of 200 mm-sodium phosphate buffer, pH 6.8. Detergent extracts were prepared by suspension of inner membranes (10 mg protein ml⁻¹) in 200 mm-sodium phosphate buffer, pH 6.8, 1% (v/v) Triton X-100. The extract was stirred on ice for 1 h. Insoluble debris was removed by centrifugation for 5 min at 15000 g. All solutions contained 2 mM-phenylmethylsulphonyl fluoride and, unless otherwise stated, all procedures were done at between 0 and 4 °C.

**Assays.** Fumarate reductase was assayed by the method of Dickie & Weiner (1979) except that the assay buffer was 200 mm-sodium phosphate, pH 6.8, 0.5 mm-DTT. For anion titrations this buffer was changed to 25 mm-HEPES/NaOH, pH 6.8, 0.5 mm-DTT. One unit of activity equals 1 µmol reduced benzyl viologen oxidized min⁻¹. Proteins were estimated by the method of Bradford (1976) using crystalline bovine serum albumin (BioRad) as standard.

Quinol oxidase activity was measured in 4 ml quartz cuvettes sealed with rubber septa. All buffers and solutions were degassed and saturated with O₂-free Ar, and injected through the septa using Hamilton syringes. 2,3-Dimethyl-1,4-naphthoquinone (5 mM in ethanol) was reduced by the addition of 2 mol NaBH₄ (28 mm in 0.025 mm-NaOH) mol⁻¹ and kept dark and anoxic (DMNH₂). The quinol could be kept stably reduced for at least 3 h. Final substrate concentrations in 3-7 ml 100 mm-sodium phosphate, pH 6.8, assay buffer were 50 µM-DMNH₂ and...
200 µM-sodium fumarate. Oxidation of DMNH₂ was followed at 270 nm, using an absorption coefficient of 16000 l mol⁻¹ cm⁻¹.

SDS-PAGE. Gels (22 × 15 × 0.15 cm) were linear gradients of 12–17% (w/v) acrylamide, 0.32–0.45% (w/v) bisacrylamide and 0–6% (w/v) sucrose. All solutions were according to Laemmli (1970). Gels were stained and destained as described by Lemire et al. (1982).

Thermal stability studies. Fumarate reductase catalytic dimer or mutant enzymes were stored in 200 mM-sodium phosphate buffer, pH 6-8. Immediately before assay the buffer was exchanged by chromatography through 1 ml Sephadex G-25 Fine columns equilibrated with 25 mM HEPES/NaOH, pH 6-8, and coloured fractions were pooled for assay. Membrane-bound forms were pelleted for 20 min at 103 000g in a Beckman Airfuge and resuspended by homogenization in 25 mM-HEPES/NaOH, pH 6-8. Incubations were done at 47°C for up to 60 min at a protein concentration of 1–3 mg ml⁻¹. Samples were removed at intervals and assayed for fumarate reductase activity.

Anion titrations. Samples were prepared in 25 mM-HEPES/NaOH, pH 6-8 as described for thermal stability studies. Enzymes were assayed as described above, in the presence of increasing concentrations of sodium phosphate buffer, pH 6-8.

³⁵S-Labeling. Stationary phase L-broth cultures (2 ml) were washed with two 5 ml volumes of 50 mM-Tris/HCl, pH 7-2, by centrifugation at 5000 g for 10 min followed by resuspension, and inoculated into 60 ml M9 medium supplemented with thiamin, kanamycin, ampicillin and 40 µg ml⁻¹ each of 19 amino acids (lacking methionine). [³⁵S]Methionine (1-0 MBq: 46.6 TBq mol⁻¹) was added and the culture grown anaerobically for 21 h at 37°C.

Immuno precipitation. FRDA and FRDB polypeptides were isolated by excision from Coomassie Blue stained SDS-PAGE gels. The polyacrylamide slices were finely minced and emulsified with Freund's complete adjuvant (Dickie & Weiner, 1979) and stored at -20°C. Procedures were similar to those of Johnson et al. (1979). Rabbit antisera to FRDA, FRDB and purified catalytic dimer were raised by standard methods. Serum (75 mg protein ml⁻¹) was pretreated with 2 mM p-tosyllysylchloromethyl ketone, p-tosylphenylalanlylchloromethyl ketone and benzamidine hydrochloride, 1 U aprotinin ml⁻¹ and 0-1 vol. of 0.15 M-sodium phosphate buffer, pH 7-4. Immuno precipitates were harvested by centrifugation at 13000 g for 5 min, sonicated at 10 mW for 30 s and resuspended in 10 mM-sodium phosphate/potassium phosphate-buffered isotonic saline, pH 7-4 (PBS), 0-5% C₁₅E₆ (octaethyleneglycol monododecyl ether; Nikko Chemicals, Tokyo, Japan) and repelleted as before. For labelled samples, Staphylococcus aureus protein A was added to 0-1 mg ml⁻¹ and incubated a further 2 h at 4°C. The co-precipitats were collected as above. The pellets were homogenized in 25 µl PBS, 25 µl solubilization buffer (Laemmli, 1970) and boiled for 2 min.

Immunoblotting analysis. Gels were prepared as described above in slabs of 11 × 15 × 0.15 cm; 10 µg protein was loaded per well. Protein was transferred to BA85 nitrocellulose paper by passive diffusion (Renart et al., 1979; Bowen et al., 1980) for 24 h. The nitrocellulose-bound protein was decorated by the method of Johnson et al. (1984) with rabbit antisera raised against FRDA or FRDB. Bands were visualized with horseradish peroxidase-coupled goat-antirabbit antibody (BioRad).
RESULTS

Isolation of Tn5 insertion mutations

A total of 302 colonies exhibiting both ampicillin and kanamycin resistance were screened. The EcoRI restriction digestion patterns were compared to that of the parent plasmid pFRD79 (Fig. 1a). Loss of the 3.4 kbp fragment and appearance of a 9 kbp fragment indicated insertion of Tn5 into the \textit{frd} operon distal to the EcoRI site in \textit{frdA}: 72 colonies (23.8\%) showed this pattern. An additional 112 (37.1\%) colonies showed a pattern consistent with Tn5 insertions in the pBR322 vector or the promoter-proximal region of \textit{frdA}, while the remainder (39.1\%) showed a pattern which indicated the presence of both a wild-type pFRD79 and a transposon-carrying plasmid.

The location of the Tn5 insertion in the \textit{frd} operon was mapped for each of the 72 \textit{frd}::Tn5 plasmids by digestion with SalI, BamHI and HindIII. Insertions in the \textit{frdCD} region were further mapped with \textit{AvuI} and \textit{AluI} digestions (Fig. 1c). Of the insertions 37 (51.4\%) mapped in \textit{frdA}, 28 (38.9\%) in \textit{frdB}, 3 (4.2\%) in \textit{frdC} and one (1.4\%) in \textit{frdD}. An additional 2 (2.8\%) were in the \textit{ampC} region (Fig. 1b). Mutant pFRD79::Tn5-239 was chosen as an insertion in \textit{frdA} (\textit{frdA}::Tn5), pFRD79::Tn5-260 as an insertion in \textit{frdB} (\textit{frdB}::Tn5), pFRD79 ::Tn5-233 as an insertion in \textit{frdC} (\textit{frdC}::Tn5), and pFRD79::Tn5-299 as the sole example of an insertion in \textit{frdD} (\textit{frdD}::Tn5).

Expression of fumarate reductase polypeptides by \textit{frd} mutations

Each of the selected \textit{frd} mutations was examined for expression and cellular localization of FRD polypeptides. When Tn5 was inserted within \textit{frdA} (\textit{frdA}::Tn5) no fumarate reductase subunits were detected in the cytoplasm by immunoprecipitation (Fig. 2a, lane 1), or immunoblotting (Fig. 3a, lanes 1 and 2). Similar results were seen with other Tn5 insertions in \textit{frdA}. Following insertion of Tn5 within \textit{frdB} (\textit{frdB}::Tn5), intact FRDA was detected by immunoblotting (Fig. 3a, lanes 1 and 3) and by immunoprecipitation (Fig. 2a, lane 2), but no fragments of FRDB were seen (Fig. 3b, lanes 1 and 3). The antibodies raised against FRDB exhibited some cross-reactivity with FRDA (Fig. 3b, lanes 1 and 3). The FRDA polypeptide

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Fig. 1. (a) Restriction map of pFRD79. ——, HindIII fragment carrying \textit{frd}; ——, pBR322. (b) Genetic map of HindIII fragment: arrows show direction of transcription; numbers mark locations of Tn5 insertions. (c) Restriction map of HindIII fragment: A, \textit{AvuI}; E, EcoRI; H, HindIII; S, SalI; U, \textit{AluI}. 
accumulated in the cytoplasm and contained the covalent flavin cofactor characteristic of fumarate reductase (Weiner & Dickie, 1979) (data not shown). When Tn5 was inserted near the middle of frdC (frdC::Tn5), both FRDA and FRDB accumulated in the cytoplasm (Fig. 2a, lane 3) but a FRDC fragment could not be detected on the gels. The insertion mutation in frdD (frdD::Tn5) gave rise to a unique phenotype. Immunoprecipitation of Triton X-100 extracts of the inner-membrane fraction indicated that a tetramer consisting of FRDA, B, C and a truncated FRDD migrating at an apparent molecular mass of 12 kDa was amplified in the membrane (Fig. 2a, lane 4). No wild-type FRDD could be visualized in the membrane fraction. Furthermore, examination of Fig. 2b, lane 1 shows that a fumarate reductase trimer composed of FRDA, B and C accumulated in the cytoplasm of a strain which does not carry a chromosomal copy of frd (MI1443, Table 1). This trimer apparently reacted poorly with the antisera available, and could only be visualized in radiolabelled immunoprecipitates.
Fig. 3. Immunoblotting analysis of fumarate reductase polypeptides. Immunoblotting was done as described in Methods. In (a) anti-FRDA gamma-globulin was used; in (b) anti-FRDB gamma-globulin was used. Lane 1 SDS-solubilized membranes from HB101(pFRD79); Lane 2 cytoplasm from HB101(frda::Tn5); Lane 3 cytoplasm from HB101(frdb::Tn5). Molecular weight markers are chick ovalbumin (43 kDa), a-chymotrypsinogen (25 kDa), P-lactoglobulin (18 kDa) and cytochrome c (12 kDa).

Amplification of fumarate reductase activity in frd mutations

The presence of a complete frd operon on a plasmid vector (pFRD79) results in an amplification of membrane-bound fumarate reductase activity over wild-type (Table 2). The presence of a plasmid carrying only frdA and frdB (pFRD117) results in a substantial expression of soluble activity. Insertion of a transposon into frdA or frdB did not give rise to detectable activity in the cytoplasm, but normal levels of activity were observed in the cytoplasmic membrane of E. coli HB101, due to the intact chromosomal copy of frd. HB101 harbouring mutation frdC::Tn5 produces large quantities of soluble, cytoplasmic enzyme activity and normal levels of membrane-associated activity. Phenotypically, this plasmid is indistinguishable from pFRD117 (Lemire et al., 1982).

In transposon mutation frdD::Tn5 both the cytoplasmic and membrane fractions of HB101 had amplified levels of fumarate reductase activity. Nearly 80% of the total activity was soluble, while the remainder was bound to the inner membrane (Table 2).

Properties of fumarate reductase produced in strains harbouring frdC::Tn5 and frdD::Tn5

The catalytic dimer and holoenzyme forms of fumarate reductase can be easily distinguished (Lemire et al., 1982; Weiner et al., 1984). E. coli MI1443 is deleted for frd and ampC and cannot
Fig. 4. Anion titration of the cytoplasmic fumarate reductase activity produced by HB101(frdC::Tn5) (●) and HB101(frdD::Tn5) (▼). Assays were done in 25 mM-HEPES/NaOH, pH 6.8, 0.5 mM-DTT, with increasing concentrations of sodium phosphate buffer, pH 6.8.

Table 2. Expression and localization of fumarate reductase

Cells were grown as described in Methods and lysed by two passages through a French pressure cell. Crude envelopes were separated from cytoplasm by centrifugation at 150 000 g for 60 min and the envelopes resuspended by homogenization in a minimal volume of 200 mM-sodium phosphate buffer, pH 6.8. Data are representative of four independent experiments.

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<th>Strain</th>
<th>Specific activity [units (mg protein)⁻¹]</th>
<th>Percentage of total activity</th>
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<td></td>
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<tr>
<td>HB101/pBR322</td>
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<tr>
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<tr>
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<td>32.7</td>
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ND, Not detected.

* Fumarate reductase in the cytoplasm of this strain is not soluble. It is a holoenzyme bound into tubular lipoprotein structures induced by over-expression of the enzyme (Weiner et al., 1984).

Grow anaerobically on GF. pFRD117, which produces dimer, does not permit growth on GF, while pFRD79, which codes for wild-type tetramer, does (C. Condon & J. H. Weiner, unpublished observation). The activity of the dimer, but not the tetramer, is stimulated fivefold by addition of anions to the assay buffer (Robinson & Weiner, 1981). Additionally, the catalytic dimer is rapidly denatured by incubation in alkaline solution (pH 8.6) or at elevated temperature (>45 °C). Holoenzyme, but not dimer, is capable of accepting reducing equivalents from quinone analogues (Cecchini et al., 1986; Weiner et al., 1986). These observations have led to the proposal that the anchor polypeptides (FRDC and FRDD) induce an optimal conformation of the catalytic subunits (Weiner et al., 1984) and are essential for the physiological functions of fumarate reductase. The properties of the two Tn5 insertions in FRDC and D provide further evidence for this model.

Transposon mutation frdC::Tn5 produced catalytic dimer in the cytoplasm, while frdD::Tn5 produced soluble trimer. These two enzyme forms showed different anion dependence. frdC::Tn5 enzyme was stimulated fivefold by anions (Fig. 4), as expected for catalytic dimer and in agreement with the results reported previously (Lemire et al., 1982). frdD::Tn5 trimeric enzyme was not activated by anions. This result indicates that although the frdD::Tn5 enzyme lacked FRDD, it behaved like tetramer with respect to anion dependence.
Fig. 5. (a) Thermal stability (47 °C, pH 6.8) of the cytoplasmic fumarate reductase activity produced by HB101(frDC::Tn5) (○) and HB101(frDD::Tn5) (△); (b) alkaline stability (pH 8.6, 24 °C) of the cytoplasmic fumarate reductase activity produced by HB101(frDC::Tn5) (○) and HB101(frDD::Tn5) (△). Samples were treated and assays were done as described in Methods.

Fig. 6. (a) Thermal stability (47 °C, pH 6.8) of inner-membrane-bound enzyme produced by HB101(pFRD79) (■) or by HB101(frDD::Tn5) (△); (b) alkaline stability (pH 8.6, 24 °C) of inner-membrane-bound enzyme produced by HB101(pFRD79) (■) or by HB101(frDD::Tn5) (△).

To examine this further the thermal and alkaline labilities of the frDC::Tn5 and frDD::Tn5 activities from the cytoplasm and frDD::Tn5 activity from the membrane were measured. Both cytoplasmic enzymes were denatured by incubation at 47 °C with a half-life of about 7 min (Fig. 5a). The frDC::Tn5 dimer was rapidly denatured by incubation at pH 8.6 (Fig. 5b) while the frDD::Tn5 trimer was stable. The frDD::Tn5 tetramer in the inner membrane has stability properties comparable to those of the soluble trimer. It is thermolabile (Fig. 6a), unlike wild-type tetramer and stable to alkaline conditions (Fig. 6b).

The frDD::Tn5 plasmid complements MI1443 to growth on GF. The trimeric enzyme has no detectable fumarate reductase activity in the DMNH₂ assay, while membrane-bound FRDABCD' has 2-(n-heptyl)-4-hydroxyquinoline-N-oxide-sensitive fumarate-dependent DMNH₂ oxidase activity. The ratio of specific activity of the benzyl viologen to DMNH₂ assays is nine for the two membrane-bound enzymes. Thus, the altered tetramer with the truncated FRDD subunit retains physiological activity, while the soluble FRDABC trimer does not. Together these results discriminate the functions of FRDC and FRDD.

DISCUSSION

Seventy two Tn5 insertions (23-8% of the total) in the frd operon distal to the EcoRI site in frdA were isolated. If Tn5 transposed completely at random, 37% of insertions would be expected to lie in this 3.4 kbp region of pFRD79 (9.1 kbp total size). While the transposition of Tn5 is not site-specific (Shaw & Berg, 1979; Berg et al., 1980; Miller et al., 1980), there is a preference for insertion at GC (Berg et al., 1983). Moreover, it was shown that there is a hotspot
for insertion of Tn5 at nucleotide 31 of pBR322, which is part of the HindIII site into which frd is cloned in pFRD79. This may account for the apparent bias for insertion in the vector compared to frd. Bossi & Ciampi (1981) reported that Tn5 inserts preferentially at a concensus sequence which is homologous to a 12 bp region in the terminal repeats of the transposon. The frd sequence has been computer-searched for this concensus sequence, and while it was found seven times when four mismatches were allowed, the locations did not match any of the insertions mapped (V. Paetkau, personal communication). The selectivity of Tn5 insertion is exemplified in Fig. 1, which shows a striking paucity of insertions within the frdCD region, compared to flanking sequences. The length of DNA coding for the small polypeptides and FRDB is similar, yet 28 insertions were mapped in frdB and only four in the CD region. A possibility which cannot be ruled out at this time is that insertions did occur in this region, but the resulting mutations were lethal.

Expression analysis of a number of insertions within frdA and frdB indicated that truncated fragments were never detected by the methods used. One possibility is that the cell recognized these aberrant polypeptides and rapidly cleared them by proteolysis (Goldberg, 1972). Truncated fragments of succinate dehydrogenase flavoprotein and iron–sulphur protein subunits have been detected in the cytoplasm of Bacillus subtilis, but they are unstable unless special precautions are taken to preserve them (Hederstedt, 1983; Hederstedt et al., 1985). Intact FRDA accumulated in the cytoplasm of strains carrying Tn5 mutations in frdB, although neither fumarate reductase (fumarate-dependent benzyl viologen oxidase) activity (Table 2) nor succinate dehydrogenase [succinate-dependent 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) reductase] activity (data not shown) was observed. This is in agreement with the results of Cecchini et al. (1986), who also showed that the FRDA subunit was inactive as succinate dehydrogenase, but in contrast to the results reported by Unden & Kröger (1981) for the fumarate reductase of Wolinella succinogenes. They observed that the isolated 79 kDa covalent-flavin-containing subunit (equivalent to E. coli FRDA) was catalytically active, but only when associated as a homodimer. The monomer itself was inactive. This leads to the suggestion that the minimal catalytic unit for the E. coli enzyme is the FRDAB dimer.

It was previously shown that FRDC plus FRDD anchor and stabilize the catalytic dimer and permit fumarate-dependent oxidation of quinone analogues (Cecchini et al., 1986; Weiner et al., 1986). However, although these two subunits appear structurally similar (Weiner et al., 1984) no information has been available as to why two different polypeptides are needed. The Tn5 insertion in frdD addresses this question. The FRDABC trimer found in the cytoplasm of strains harbouring the frdD::Tn5 plasmid has properties intermediate between those of dimer and tetramer. The presence of FRDC confers stability at high pH, but not at high temperature. It renders the catalytic domain insensitive to anion stimulation by phosphate. It is not sufficient to allow binding of fumarate reductase to the inner membrane. It does not allow fumarate-dependent oxidation of quinone analogues.

The membrane-bound mutant tetramer expressed by the frdD::Tn5 plasmid elucidates the role of FRDD. The truncated FRDD (FRDD') is capable of allowing membrane insertion of the complex, but at an apparently reduced efficiency (21-5% membrane-bound in frdD::Tn5 vs 100% membrane-bound in pFRD79; Table 2). This effect could also be due to competition between proteolysis of the FRDD' fragment, giving rise to soluble trimer, and insertion of the mutant tetrameric complex into the membrane (Oliver, 1985). It has already been noted that intact FRD subunits tend to accumulate, while truncated polypeptides do not. FRDD' does not confer thermal stability on the catalytic domain, implicating the missing C-terminal fragment in this role. FRDD', together with FRDC, permit the use of quinone analogues as reducing agents and apparently normal physiological activity of the fumarate reductase complex. It cannot be distinguished whether FRDD' alone is responsible, or the interaction between it and the rest of the subunits forms the site of interaction with the quinones of the electron transport chain.

It is interesting to note that in strains bearing the frdD::Tn5 plasmid there is only trimer in the cytoplasm and only tetramer in the cytoplasmic membrane. It is inferred from this that FRDA and B are assembled from a cytoplasmic pool, and that the hydrophobic FRDC polypeptide can associate with this dimer in a soluble form. On addition of the FRDD (or
FRDD') polypeptide the complex becomes membrane bound, but the order of subunit assembly or membrane insertion remains unclear. It is not known why this putative assembly pathway produces only mutant tetramer in a strain that harbours both frdD::Tn5 and a wild-type frd operon (HB101), unless overproduction of mRNA from the multicopy plasmid overwhelms that from the chromosome. Experiments to further elucidate this pathway and the role of FRDC and FRDD in quinone binding are being done.

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Fumarate reductase subunit function


