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

By DAVID J. LATOUR AND JOEL H. WEINER*

Department of Biochemistry, University of Alberta, Edmonton, Alberta, Canada T6G 2H7

(Received 2 July 1986; revised 4 September 1986)

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 *frd* 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 8 α (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 sulphhydryl group (Robinson & Weiner, 1982) and a covalently bound 8 α (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.

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*, *AvaI*, *AluI* 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. Kröger (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. *λ*b221c1857*rex*::Tn5 (3 × 10⁹ 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 1 l 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 MPa as 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 M-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

Table 1. *Bacterial strains, phage and plasmids*

Bacteria	Genotype/phenotype	Source
<i>E. coli</i> HB101	F ⁻ <i>hsdR hsdM pro</i> <i>leu gal lac thi recA rpsL</i>	G. McFadden, University of Alberta, Canada
<i>E. coli</i> MI1443	Δ (<i>frdABCD ampC</i>) Sm ^r	M. Iwaya, University of Kentucky, USA
Phage and plasmids		
λ b221cI857 <i>rex</i> ::Tn5	Km ^r	P. P. Dennis, University of British Columbia, Canada
pBR322	Ap ^r Tc ^r	Boehringer-Mannheim
pFRD117	Ap ^r <i>frdAB</i>	This laboratory
pFRD79	Ap ^r <i>frdABCD</i>	This laboratory
pFRD79::Tn5-233	Ap ^r Km ^r <i>frdC</i> ::Tn5	This work
pFRD79::Tn5-239	Ap ^r Km ^r <i>frdA</i> ::Tn5	This work
pFRD79::Tn5-260	Ap ^r Km ^r <i>frdB</i> ::Tn5	This work
pFRD79::Tn5-299	Ap ^r Km ^r <i>frdD</i> ::Tn5	This work

200 μ M-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 103000 *g* 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 μ g 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.

Immunoprecipitations. 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. Catalytic dimer was purified by phenyl-Sepharose chromatography (Dickie & Weiner, 1979) and emulsified in Freund's complete adjuvant at 1 mg protein ml⁻¹. 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*-tosylsilylchloromethyl ketone, *p*-tosylphenylalanylchloromethyl ketone and benzamidine hydrochloride, 1 U aprotinin ml⁻¹ and 0.1 vol. cytoplasmic extract from a culture not expressing fumarate reductase. After incubation for 2 h at room temperature the serum was cleared by centrifugation at 20000 *g* for 20 min. Cleared antiserum or control IgG (15 mg rabbit anti-succinyl-CoA synthetase ml⁻¹) was mixed 5:1 with unlabelled or ³⁵S-labelled cytoplasmic fraction (20–25 mg ml⁻¹) and incubated 5 h at 4 °C. Bovine serum albumin (0.2 mg ml⁻¹) was added to the ³⁵S-labelled samples. Unlabelled immunoprecipitates were harvested by centrifugation at 13000 *g* for 5 min, sonicated at 10 mW for 60 s in 5 vols 10 mM-sodium phosphate/potassium phosphate-buffered isotonic saline, pH 7.4 (PBS), 0.5% C₁₂E₈ (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-precipitates were collected as above. The pellets were homogenized in 25 μ l PBS, 25 μ l 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 μ g 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).

Fig. 1. (a) Restriction map of pFRD79. ■, HindIII fragment carrying *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, *AvaI*; E, *EcoRI*; H, *HindIII*; S, *SalI*; U, *AluI*.

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

Expression of fumarate reductase polypeptides by *frd* mutations

Each of the selected *frd* mutations was examined for expression and cellular localization of FRD polypeptides. When Tn5 was inserted within *frdA* (*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 *frdA*. Following insertion of Tn5 within *frdB* (*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

Fig. 2. (a) SDS-PAGE of unlabelled immunoprecipitates, Coomassie Blue staining. Cytoplasmic fractions and Triton X-100 extracted inner membranes were precipitated with anti-FRDAB serum and electrophoresed as described in Methods. Lane 1, HB101(*frdA*::Tn5) cytoplasmic fraction; lane 2, HB101(*frdB*::Tn5) cytoplasmic fraction; lane 3, HB101(*frdC*::Tn5) cytoplasmic fraction; lane 4, HB101(*frdD*::Tn5) Triton X-100 extract. (b) SDS-PAGE of ^{35}S -labelled immunoprecipitates, autoradiograph. Lane 1, M11443(*frdD*::Tn5) cytoplasmic fraction precipitated with anti-FRDAB serum; lane 2, M11443(*frdD*::Tn5) cytoplasmic fraction precipitated with control immunoglobulin. Molecular mass markers are chick ovalbumin (43 kDa), α -chymotrypsinogen (25 kDa), β -lactoglobulin (18 kDa) and cytochrome *c* (12 kDa).

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* (M11443, 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), α -chymotrypsinogen (25 kDa), β -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* M11443 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 150000 *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.

Strain	Specific activity [units (mg protein) ⁻¹]		Percentage of total activity	
	Envelopes	Cytoplasm	Envelopes	Cytoplasm
HB101/pBR322	21	ND	100	ND
HB101/pFRD117	11.5	42.3	0.6	99.4
HB101/pFRD79	76.7	3.2	56.8	43.2*
HB101/ <i>frdC</i> ::Tn5	19	70.8	0.9	99.1
HB101/ <i>frdA</i> ::Tn5	16	ND	100	ND
HB101/ <i>frdB</i> ::Tn5	12	ND	100	ND
HB101/ <i>frdD</i> ::Tn5	45	32.7	21.5	78.5

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 *Hind*III 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 consensus 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 consensus 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.

The authors wish to thank Dr M. Iwaya for supplying MI1443 and Dr G. D. Armstrong for assistance with raising antisera. This work was supported by grant MT-5838 from the Medical Research Council of Canada.

REFERENCES

- BERG, D. E. (1977). Insertion and excision of the transposable resistance determinant Tn5. In *DNA Insertion Elements, Plasmids and Episomes*, pp. 205–212. Edited by A. I. Bukhan, J. A. Shappiro & S. L. Adhya. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory.
- BERG, D. E., JORGENSEN, R. & DAVIES, J. (1978). Transposable kanamycin-neomycin resistance determinants. In *Microbiology-1978*, pp. 13–15. Edited by D. Schlesinger. Washington, DC: American Society for Microbiology.
- BERG, D. E., WEISS, A. & CROSSLAND, L. (1980). Polarity of Tn5 insertion mutations in *Escherichia coli*. *Journal of Bacteriology* **142**, 439–446.
- BERG, D. E., SCHMANDT, M. A. & LOWE, J. B. (1983). Specificity of transposon Tn5 insertion. *Genetics* **105**, 813–828.
- BIRNBOIM, H. C. & DOLY, J. (1979). A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Research* **7**, 1513–1523.
- BOSSI, L. & CIAMPI, M. S. (1981). DNA sequences at the sites of three insertions of the transposable element Tn5 in the histidine operon of *Salmonella*. *Molecular and General Genetics* **183**, 406–408.
- BOWEN, B., STEINBERG, J., LAEMMLI, U. K. & WEINTRAUB, H. (1980). The detection of DNA-binding proteins by protein blotting. *Nucleic Acids Research* **8**, 1–20.
- BRADFORD, M. M. (1976). Sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* **72**, 248–254.
- CAMMACK, R., PATIL, D. S. & WEINER, J. H. (1986). Evidence that centre 2 in *Escherichia coli* fumarate reductase is a [4Fe–4S] cluster. *Biochimica et biophysica acta* **870**, 538–544.
- CACCHINI, G., ACKRELL, B. A. C., DESHLER, J. O. & GUNSALUS, R. P. (1986). Reconstitution of quinone reduction and characterization of *Escherichia coli* fumarate reductase activity. *Journal of Biological Chemistry* **261**, 1808–1814.
- CLEWELL, D. B. & HELINSKI, D. R. (1972). Effect of growth conditions on the formation of the relaxation complex of supercoiled ColE1 deoxyribonucleic acid and protein in *Escherichia coli*. *Journal of Bacteriology* **110**, 1135–1146.
- COLE, S. T. (1982). Nucleotide sequence coding for the flavoprotein subunit of the fumarate reductase of *Escherichia coli*. *European Journal of Biochemistry* **122**, 479–484.
- COLE, S. T., GRUNDSTRÖM, T., JAURIN, B., ROBINSON, J. J. & WEINER, J. H. (1982). Location and nucleotide sequence of *frdB*, the gene coding for the iron-sulphur protein subunit of the fumarate reductase of *Escherichia coli*. *European Journal of Biochemistry* **126**, 211–216.
- COLE, S. T., CONDON, C., LEMIRE, B. D. & WEINER, J. H. (1986). Molecular biology, biochemistry and bioenergetics of fumarate reductase, a complex membrane-bound iron-sulfur flavoenzyme. *Biochimica et biophysica acta* **811**, 381–403.
- DE BRUIJN, F. J. & AUSUBEL, F. M. (1981). The cloning and transposon Tn5 mutagenesis of the *glnA* region of *Klebsiella pneumoniae*: identification of *glnR*, a gene involved in the regulation of the *nil* and *hut* operons. *Molecular and General Genetics* **183**, 289–297.
- DE BRUIJN, F. J., STROKE, I. L., MARVEL, D. J. & AUSUBEL, F. M. (1983). Construction of a correlated physical and genetic map of the *Klebsiella pneumoniae* *hisDGO* region using transposon Tn5 mutagenesis. *EMBO Journal* **2**, 1831–1838.
- DICKIE, P. & WEINER, J. H. (1979). Purification and characterization of membrane-bound fumarate reductase from anaerobically grown *Escherichia coli*. *Canadian Journal of Biochemistry* **57**, 813–821.
- GOLDBERG, A. L. (1972). Degradation of abnormal proteins in *Escherichia coli*. *Proceedings of the National Academy of Sciences of the United States of America* **69**, 422–426.
- GRUNDSTRÖM, T. & JAURIN, B. (1982). Overlap between *ampC* and *frd* operons on the *Escherichia coli* chromosome. *Proceedings of the National Academy of Sciences of the United States of America* **79**, 1111–1115.
- HADDOCK, B. A. & JONES, C. W. (1977). Bacterial respiration. *Bacteriological Reviews* **41**, 47–99.
- HEDERSTEDT, L. (1983). Succinate dehydrogenase mutants of *Bacillus subtilis* lacking covalently bound flavin in the flavoprotein subunit. *European Journal of Biochemistry* **132**, 589–593.
- HEDERSTEDT, L., MAGUIRE, J. J., WARING, A. J. & OHNISHI, T. (1985). Characterization by electron paramagnetic resonance and studies on subunit location and assembly of the iron-sulfur clusters of *Bacillus subtilis* succinate dehydrogenase. *Journal of Biological Chemistry* **260**, 5554–5562.
- INGLEDEW, W. J. & POOLE, R. K. (1984). The respiratory chains of *Escherichia coli*. *Microbiological Reviews* **48**, 222–271.

- JOHNSON, R. C., YIN, J. C. P. & REZNIKOFF, W. S. (1982). Control of Tn5 transposition in *Escherichia coli* is mediated by protein from the right repeat. *Cell* **30**, 873–882.
- JOHNSON, D. A., GAUTSCH, J. W., SPORTSMAN, J. R. & ELDER, J. H. (1984). Improved technique utilizing nonfat dry milk for analysis of proteins and nucleic acids transferred to nitrocellulose. *Gene Analysis Techniques* **1**, 3–8.
- LAEMMLI, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature, London* **227**, 680–685.
- LAIRD, A. J. & YOUNG, I. G. (1980). Tn5 mutagenesis of the enterochelin gene cluster of *Escherichia coli*. *Gene* **11**, 359–366.
- LEMIRE, B. D., ROBINSON, J. J. & WEINER, J. H. (1982). Identification of the membrane anchor polypeptides of *Escherichia coli*. *Journal of Bacteriology* **152**, 1126–1131.
- LEMIRE, B. D., ROBINSON, J. J., BRADLEY, R. D., SCRABA, D. G. & WEINER, J. H. (1983). Structure of fumarate reductase on the cytoplasmic membrane of *Escherichia coli*. *Journal of Bacteriology* **155**, 391–397.
- LOHMEIER, E., HAGEN, D. S., DICKIE, P. & WEINER, J. H. (1981). Cloning and expression of the fumarate reductase gene of *Escherichia coli*. *Canadian Journal of Biochemistry* **59**, 158–164.
- LUPSKI, J. R., SMILLIE, B. L., BLATTNER, F. R. & GODSON, G. N. (1982). Cloning and characterization of the *Escherichia coli* chromosomal region surrounding the *dnaG* gene, with a correlated physical and genetic map of *dnaG* generated via transposon Tn5 mutagenesis. *Molecular and General Genetics* **185**, 120–128.
- MANDEL, M. & HIGA, A. (1970). Calcium dependent bacteriophage DNA infection. *Journal of Molecular Biology* **53**, 159–162.
- MILLER, J. H. (ed) (1972). *Experiments in Molecular Genetics*, pp. 431–435. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory.
- MILLER, J. H., CALOS, M. P., GALAS, D., HOFER, M., BUCHEL, D. E. & MULLER-HILL, B. (1980). Genetic analysis of transpositions in the *lac* region of *Escherichia coli*. *Journal of Molecular Biology* **144**, 1–18.
- OLIVER, D. (1985). Protein secretion in *Escherichia coli*. *Annual Review of Microbiology* **39**, 615–648.
- PANNEKOEK, H., HILLIE, J. & NOORDERMEER, I. (1980). Relief of polarity caused by transposon Tn5: application in mapping a cloned region of the *Escherichia coli* *uvrB* locus essential for UV resistance. *Gene* **12**, 51–61.
- RENART, J., REISER, J. & STARK, G. R. (1979). Transfer of proteins from gels to diazobenzyloxymethyl-paper and detection with antisera: a method for studying antibody specificity and antigen structure. *Proceedings of the National Academy of Sciences of the United States of America* **76**, 3116–3120.
- ROBINSON, J. J. & WEINER, J. H. (1981). The effects of anions on fumarate reductase isolated from the cytoplasmic membrane of *Escherichia coli*. *Biochemical Journal* **199**, 473–477.
- ROBINSON, J. J. & WEINER, J. H. (1982). Molecular properties of fumarate reductase isolated from the cytoplasmic membrane of *Escherichia coli*. *Canadian Journal of Biochemistry* **60**, 811–816.
- SHAW, K. J. & BERG, C. M. (1979). *Escherichia coli* K12 auxotrophs induced by insertion of the transposable element Tn5. *Genetics* **92**, 741–747.
- SINGH, A. P. & BRAGG, P. D. (1975). Reduced nicotinamide adenine dinucleotide dependent reduction of fumarate coupled to membrane energization in a cytochrome deficient mutant of *Escherichia coli* K12. *Biochimica et biophysica acta* **396**, 229–241.
- SPENCER, M. E. & GUEST, J. R. (1974). Proteins of the inner membrane of *Escherichia coli*: changes in composition associated with anaerobic growth and fumarate reductase amber mutation. *Journal of Bacteriology* **117**, 954–959.
- UNDEN, G. & KRÖGER, A. (1981). The function of the subunits of the fumarate reductase complex of *Vibrio succinogenes*. *European Journal of Biochemistry* **120**, 577–584.
- WEINER, J. H. & DICKIE, P. (1979). Fumarate reductase of *Escherichia coli*. Elucidation of the covalent flavin component. *Journal of Biological Chemistry* **254**, 8590–8593.
- WEINER, J. H., LEMIRE, B. D., ELMES, M. L., BRADLEY, R. D. & SCRABA, D. G. (1984). Overproduction of fumarate reductase in *Escherichia coli* induces a novel intracellular lipid-protein organelle. *Journal of Bacteriology* **158**, 590–596.
- WEINER, J. H., CAMMACK, R., COLE, S. T., CONDON, C., HONORÉ, N., LEMIRE, B. D. & SHAW, G. (1986). A novel mutant of *Escherichia coli* fumarate reductase decoupled from electron transport. *Proceedings of the National Academy of Sciences of the United States of America* **83**, 2056–2060.
- YAMATO, I., ANRAKU, Y. & HIROSAWA, K. (1975). Cytoplasmic membrane vesicles of *Escherichia coli*. I. A simple method for preparing the cytoplasmic and outer membranes. *Journal of Biochemistry* **77**, 705–718.