Enterobactin synthase polypeptides of *Escherichia coli* are present in an osmotic-shock-sensitive cytoplasmic locality

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The terminal reactions in the synthesis of the siderophore enterobactin (Ent) by *Escherichia coli* require the EntD, E, F and B/G polypeptides. The idea that these molecules form a complex (Ent synthase) that is membrane-associated was re-evaluated. *In vitro* results provided no evidence in support of the proposal: (i) Ent synthase activity occurred normally under conditions where membrane was either absent or disrupted by high concentrations of neutral detergents, and (ii) immunoprecipitation experiments conducted on extracts engaged in Ent synthesis failed to detect any association among the Ent polypeptides. However, Western blot analyses showed that EntE, F and B/G were released from cells by osmotic shock and freeze/thaw treatment but not by conversion of cells to spheroplasts. These results demonstrated that EntE, F and B/G belong to the Beacham group D class of proteins. The shockability of a given group D Ent protein was unaffected by the absence of either EntB/G or EntD and, for EntB/G, the N-terminus was sufficient for release by osmotic shock. The behaviour of group D proteins is generally attributed to their association (partial, loose or transient) with cytoplasmic membrane; therefore, the results are indirect evidence that Ent synthase interacts with membrane *in vivo*. At the very least, the data indicate that EntE, F and B/G are compartmentalized in *E. coli* and, because other biosynthetic enzymes for siderophores and surfactants are related to these Ent proteins, suggest that this entire protein class may be sequestered *in vivo*.

Keywords: enterobactin synthase, iron, osmotic shock, siderophore, *Escherichia coli*

INTRODUCTION

Iron is an essential element for *Escherichia coli*, as it is for almost all organisms. Like many bacteria and fungi, *E. coli* responds to iron-deficient environments by synthesizing and secreting a small compound with high affinity for Fe(III). Such iron-scavenging molecules are termed siderophores; uptake and intracellular dissociation of Fe(III)-siderophore complexes provides the necessary iron. The siderophore produced by all *E. coli* strains is enterobactin (Ent), a 670 Da cyclic trimer of dihydroxybenzoylserine (DBS) (Neilands, 1981a). That

the task faced by the Ent system is formidable is evident from estimates that a rapidly growing *E. coli* cell must assimilate 10^6 Fe ions per division cycle (Braun, 1985; Earhart, 1987).

Six proteins are required for Ent biosynthesis. The first three steps, in which chorismate is converted to dihydroxybenzoic acid (DHBA) by the EntC, B/G and A proteins, are well characterized (Walsh *et al.*, 1990). In contrast, the latter steps, in which the EntD, E, F and B/G polypeptides convert DHBA and Ser to Ent, are not clearly understood. DNA sequence homologies (Guilvout *et al.*, 1993; Turgay *et al.*, 1992) and enzymological data (Walsh *et al.*, 1990) indicate that, as originally proposed by Bryce & Brot (1972), the terminal reactions in Ent synthesis are similar to those for peptide antibiotics. That is, the three amide bonds of Ent are made non-ribosomally, presumably by the protein

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Abbreviations: ACP, acyl carrier protein; DBS, dihydroxybenzoylserine; DHBA, dihydroxybenzoic acid; TCA, trichloroacetic acid.

thiotemplate mechanism (Kleinkauf & von Dohren, 1990; Marahiel, 1992). EntE (homodimer of 59 kDa subunits) and EntF (142 kDa) are both activating enzymes; EntE forms the DHBA-acyladenylate (Rusnak *et al.*, 1989) and EntF activates and binds Ser to itself probably via a 4'-phosphopantetheine cofactor (Reichert *et al.*, 1992; Rusnak *et al.*, 1991). According to current models (Ammerlaan, 1994; Walsh *et al.*, 1990), Ent synthesis continues with formation, on EntF, of DBS by a transamidation reaction between activated DHBA and Ser, and is completed when three DBS molecules are linked by transesterification. EntB/G and EntD are essential but their roles have not been determined. Ent B/G (32.5 kDa) seems to be a bifunctional protein (Staab & Earhart, 1990) required both for an early step in Ent biosynthesis (EntB activity; Rusnak *et al.*, 1990) and an unidentified later step (EntG activity) (Greenwood & Luke, 1976, 1980; Woodrow *et al.*, 1975); the early and late synthetic activities of Ent B/G are separable by mutation and appear to reside in the N- and C-termini of the protein, respectively. That EntB and EntG activities are on the same polypeptide is supported by the detection (unpublished results) of homology between the C-terminus of EntB/G and the N-terminus of iron-regulated HMWP2 of *Yersinia enterocolitica* (Guilvout *et al.*, 1993); the homologous regions include a consensus substrate amino acid binding site for enzymes engaged in non-ribosomal peptide synthesis (Schlumbohm *et al.*, 1991). EntD (23 kDa) is reported to be an integral cytoplasmic membrane protein (Armstrong *et al.*, 1989); its gene shows homologies to two genes (*sfp* and *gsp*) of *Bacillus* spp. that are thought to be necessary for peptide antibiotic secretion (Borchert *et al.*, 1994; Grossman *et al.*, 1993).

The EntD, E, F and B/G proteins are believed to form a multi-enzyme complex (Ent synthase) that carries out the reaction $3\text{Ser} + 3\text{DHBA} + 6\text{ATP} \rightarrow \text{Ent} + 6\text{AMP} + \text{PP}_i$ (Crosa, 1989; Silver & Walderhaug, 1992). The idea that this complex exists was first suggested by Greenwood & Luke (1976, 1980), who further postulated it was associated with the cytoplasmic membrane. Direct evidence for association among EntD, F and B/G consists of gel filtration and DEAE Sephadex chromatographic data, which demonstrated that under certain conditions some co-elution occurs (Greenwood & Luke, 1976; Woodrow *et al.*, 1979). Specifically, in decreasing order of stability, the complexes detected were EntDFG, EntDF and EntDG. Membrane-association of the complex, postulated to be through EntD (Greenwood & Luke, 1976; Woodrow *et al.*, 1979), would provide an efficient means to couple Ent synthesis and secretion. This idea gained support from the observation that EntD can be detected on the cytoplasmic face of the cytoplasmic membrane (Armstrong *et al.*, 1989). On the other hand, (i) EntE has never been reported as part of a complex, (ii) EntE, F and B/G have been purified when overexpressed (Reichert *et al.*, 1992; Rusnak *et al.*, 1989, 1990), and partially purified when present in normal derepressed quantities (Bryce & Brot, 1972; Greenwood & Luke, 1976) by standard procedures for soluble

proteins, (iii) the chromatographic conditions under which Ent protein complexes were detected (slow flow rate and low Sephadex exclusion limit) are those which favour non-specific interactions among proteins and (iv) localization artefacts resulting from overexpression of Ent biosynthetic proteins have been reported (Elkins & Earhart, 1988) and may account for the association of EntD with cytoplasmic membrane.

Therefore, two commonly held ideas regarding the latter steps of Ent biosynthesis, i.e. that a complex of enzymes is responsible and that the complex is associated with the cytoplasmic membrane, have not been rigorously established. This work was undertaken to re-examine the localization and association of the Ent synthase proteins. *In vitro* work provided no evidence for the existence of a stable membrane-bound complex. However, studies with intact cells indicate that much of each of the EntE, F and B/G proteins must be present in an unusual cellular domain. These proteins could be released from cells by osmotic shock but not by converting cells to spheroplasts. EntE, F and B/G can therefore be placed among the group D proteins (Beacham, 1979), along with such members as EFTu, thioredoxin (Lunn & Pigiet, 1982) and DnaK (El Yaagoubi *et al.*, 1994).

METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids employed are described in Table 1. pAUT119Af was constructed by standard techniques (Sambrook *et al.*, 1989) from previously described plasmids (Coderre & Earhart, 1984; Studier *et al.*, 1990); it directs the synthesis of a hybrid protein encoded by the first 12 codons of T7gp10 fused in-frame to the C-terminal 186 codons of *entD*. This plasmid complements *entD* mutations *in vivo* and *in vitro* but the predicted fusion protein has not been detected (Ammerlaan, 1994).

Growth of bacteria. M9 and LB-dipyridyl media were described by Miller (1972) and Chenault & Earhart (1991), respectively; Chelex 100 resin (Bio-Rad) was used to prepare iron-depleted M9 medium as before (McIntosh & Earhart, 1977). TMM medium, deficient in iron and phosphorus (Nossal & Heppel, 1968), consists, per l, of Tris (12.11 g), NaCl (4.68 g), KCl (2.24 g), NaH_2PO_4 (60 mg), NH_4Cl (1.07 g), ZnCl_2 (0.27 mg), Na_2SO_4 (40 mg), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (247 mg) and CaCl_2 (22 mg) to pH 7.4 with HCl. Glucose (to 0.2%) was added to TMM, otherwise the concentrations of glucose and supplements to M9 and TMM were as described (Pierce & Earhart, 1986).

Osmotic shock. The osmotic shock procedure was that of LaVallie *et al.* (1993). The plasmolysing solution (SET) consisted of 20% (w/v) sucrose, 2.5 mM EDTA. Na_2 and 20 mM Tris/HCl, pH 8.0. Depending on the experiment, the shocking solution (4 °C) was deionized water, 2.5 mM EDTA. Na_2 and 20 mM Tris/HCl, pH 8.0 (ET), or cation solutions (CaCl_2 or MgCl_2) at 50, 100, 200 or 500 μM . Proteins in the shockate fraction were pelleted by addition of an equal volume of cold 10% trichloroacetic acid (TCA). Total (unfractionated) cells, shockate proteins and shocked cells were resuspended in equal volumes so that fractions from equal numbers of cells could be directly compared. Cells were

Table 1. Bacterial strains and plasmids

Strain/plasmid	Genotype/relevant characteristics	Reference/source
Strains		
W3110	F ⁻ <i>thyA36 deoC2</i> IN (<i>rrnD</i> – <i>rrnE</i>)	J. R. Walker, The University of Texas at Austin
AB1515	F ⁻ <i>thi-1 trpE38 purE42 proC14 leu-6 lacY1 mtl-1 xyl-5 rpsL109 azi-6 fhuA23 tsx-67 supE44</i>	Langman <i>et al.</i> (1972)
AB1515.24	<i>entB</i> ::Tn5 <i>entG</i> derivative of AB1515 (EntB ⁻ G ⁻)	Staab & Earhart (1990)
AB1515.43	<i>entB</i> ::Tn5 <i>entG</i> derivative of AB1515 (EntB ⁺ G ⁻)	Staab & Earhart (1990)
AB1515.5-4	<i>entD</i> ::Tn5 derivative of AB1515	Ammerlaan (1994)
AN93	<i>entE</i> 405 <i>purE</i> ⁺ derivative of AB1515	I. G. Young, Australian National University, Canberra
AN117	<i>entF</i> <i>purE</i> ⁺ derivative of AB1515	I. G. Young
AN102	<i>fepC</i> <i>purE</i> ⁺ transductant of AB1515	Cox <i>et al.</i> (1970); Chenault & Earhart (1991)
1D2	<i>fes</i> derivative of AB1515	Pierce <i>et al.</i> (1983)
Plasmids		
pPC12	pBR322 carrying 4.0 kb <i>EcoRI</i> fragment, <i>entD</i> ⁺	Coderre & Earhart (1984)
pGEM3Z	Ap ^r , T7 promoter, pUC19 multicloning site, <i>lacZ</i> α peptide, SP6 promoter	Promega
pET-11a	Ap ^r , T7 promoter, <i>lac</i> promoter, T7 <i>gp10</i> RBS and first 12 codons, <i>Bam</i> HI cloning site, T7 terminator	Studier <i>et al.</i> (1990)
pAUT100r	<i>entD</i> ⁺ , 3.1 kb <i>Bam</i> HI fragment of pPC12 into <i>Bam</i> HI site of pGEM3Z	This study
pAUT102	<i>entD</i> ⁺ , 1.0 kb <i>Stu</i> I– <i>Bam</i> HI fragment of pAUT100r into <i>Sma</i> I/ <i>Bam</i> HI-digested pGEM3Z	This study
pAUT119Af	<i>entD</i> ⁺ , T7gp10–EntD fusion protein, 0.9 kb <i>Taq</i> I fragment (filled) of pAUT102 into filled <i>Bam</i> HI site of pET-11a	This study

resuspended in water and TCA-precipitable material was resuspended in 0.3 M KOH.

Freeze/thaw. Cells were resuspended in ET to OD₄₂₀ = 5 (Bausch & Lomb Spectronic 20) and frozen immediately in a dry-ice/ethanol bath for 15 min. Cells were allowed to thaw at room temperature and then harvested by centrifugation (4 °C). The supernatant was made 5% TCA. Thawed cells and TCA-precipitable material were resuspended in equal volumes as described above.

Spheroplasting. Cells were converted to spheroplasts by the method of Kaback (1971). Cells were resuspended in 20% (w/v) sucrose, 30 mM Tris/HCl, pH 8.0 solution. Lysozyme and EDTA were added with gentle stirring to final concentrations of 0.7 mg ml⁻¹ and 10 mM, respectively, and the stirring of the suspension was continued for 30 min at room temperature. Spheroplasts were harvested by centrifugation, the supernatant was made 5% TCA, and spheroplasts and supernatant proteins were resuspended as described above.

Electrophoresis of proteins and Western blot analysis. Samples were mixed with an equal volume of loading buffer (10% glycerol, 0.125 M Tris/HCl, 1% β -mercaptoethanol, 4.6% SDS, and 1 mg bromophenol blue ml⁻¹), boiled for 5 min and analysed on 11 or 15% polyacrylamide gels (Lugtenberg *et al.*, 1975). Pre-stained protein molecular mass standards (high-range; Gibco-BRL), were used and where appropriate gels were stained with Coomassie Brilliant Blue G. Western blot analyses were performed as described by Staab & Earhart (1990). Antisera against EntB, E and F were prepared as described by Ammerlaan (1994) and Staab & Earhart (1990), anti-PhoA antiserum was purchased from 5

Prime → 3 Prime Inc.; anti-T7gp10 antiserum was from Novagen, and anti-acyl carrier protein antiserum was provided by C. Rock, St Jude Children's Research Hospital, respectively. Horseradish-peroxidase-conjugated goat anti-rabbit IgG (Bio-Rad) served as secondary antibody.

Ent synthase assays. Extracts were prepared from overnight cultures and from shockate fractions, which were obtained from mid-exponential phase (OD₄₂₀ = 0.5) cultures. Overnight cultures were harvested, resuspended in CS buffer (10 mM Tris/HCl, pH 8, 5 mM DTT) and passed through a French pressure cell at 18000–20000 p.s.i. (124.2–138 MPa) three times. Cell debris was subsequently removed by centrifuging for 20 min at 20000 g (Greenwood & Luke, 1976). Shockate fractions were concentrated in an Amicon unit using membranes with a molecular mass cut-off of 10 kDa. The Ent synthase assay was performed as described by Greenwood & Luke (1976). In initial experiments (Table 2), extracts were prepared from cells grown in LB-dipyridyl medium and each sample was assayed in quadruplicate. The four values of a given sample were always within 10% of the mean. Also, specific activities varied directly with the degree of iron starvation [as monitored by the Arnow (1937) assay], and iron starvation was more consistently achieved when cells were grown in iron-deficient minimal media. Therefore, subsequent experiments were carried out with extracts from cultures grown in de-ferrated M9 or TMM medium and samples were assayed in duplicate. Representative values are shown.

Radioimmune precipitations. AB1515.5-4/pAUT119Af cells were grown at 37 °C in de-ferrated M9 medium containing ampicillin (200 μ g ml⁻¹), kanamycin (30 μ g ml⁻¹), 1 mM

Table 2. Ent synthase activities of wild-type and mutant *E. coli* strains

Source of extract	Relevant genotype	Reaction conditions	Serine incorporated [nmol (mg protein) ⁻¹]
None	NA	No protein	0.5
AN102	<i>ent</i> ⁺ <i>fepC</i>	No ATP	2.1
AN102	<i>ent</i> ⁺ <i>fepC</i>	No DHBA	2.1
AN102	<i>ent</i> ⁺ <i>fepC</i>	Complete	17.1
AN102 high*	<i>ent</i> ⁺ <i>fepC</i>	Complete	20.8
1D2	<i>ent</i> ⁺ <i>fes</i>	Complete	10.8
AB1515	<i>ent</i> ⁺	Complete	12.2
AB1515.5-4	<i>entD</i> ::Tn5	Complete	2.1
AB1515.5-4/ pAUT119Af	<i>entD</i> / <i>entD</i> ⁺	Complete	9.6
AN93	<i>entE</i>	Complete	2.1
AN117	<i>entF</i>	Complete	1.4
AB1515.43	<i>ent(G)</i> ::Tn5	Complete	2.0

* Extract subjected to an additional centrifugation (2 h, 160 000 g) to remove membrane.

IPTG, and either 20 µM FeCl₃ (high iron) or 1 µM FeCl₃ (low iron) to OD₄₂₀ 0.8. Radiolabel (EXPRES³⁵S³⁵S, DuPont/NEN) was added (1.3 mCi l⁻¹; 48.1 MBq l⁻¹) for 10 min, the cultures harvested and extracts prepared as for an Ent synthase assay. Extracts containing Ent synthase substrates were incubated at 37 °C for 5 min, at which point 20 µl antiserum was added. Binding was allowed to proceed for 1 h during which the samples were gently rocked at 4 °C. Antibody-bound proteins were then precipitated by addition of 20 µl Protein A-Sepharose beads (Pharmacia). After two washes with CS buffer containing 2% Tween 20, bound proteins were released and solubilized with 1× sample loading buffer and analysed by SDS-PAGE (15%). Labelled proteins were detected by autoradiography on Kodak XRP-1 film.

Phenolate assay. The method of Arnow (1937) was used to quantify the phenolate content of culture supernatants. Dihydroxybenzoic acid served as the standard.

Protein assay. Protein concentrations were determined by the Bradford (1976) assay using BSA as the standard.

RESULTS

In vitro Ent synthase activity

Evidence that Ent synthase exists as a membrane-associated multienzyme complex was sought from assays of the enzyme under a variety of conditions. Production of Ent synthase is repressed by growth in iron-replete media so all extracts were prepared from iron-starved cells. Strain AN102 (Chenault & Earhart, 1991; Cox *et al.*, 1970) was used as the source of normal enzyme; because it is defective in a cytoplasmic membrane protein (FepC) necessary for Ent-mediated iron uptake it is unusually sensitive to iron starvation, which makes it a good source of Ent (Neilands, 1981b) and Ent synthase. Results presented in Table 2 verify that EntD, E, F and B/G are all required for *in vitro* activity. The same low specific activities for these mutants were found when ATP was omitted from the reaction mixtures. The data also show that the *entD* mutation of AB1515.5-4

can be complemented *in vitro* by a putative hybrid EntD protein (T7gp10-EntD) encoded by plasmid pAUT119Af. The presence of ferriEnt esterase, the *fes* gene product, had as anticipated, no effect on Ent synthase assays, as shown by the specific activities of extracts of AB1515 and its *fes* derivative 1D2. (The assay measures the conversion of [¹⁴C]Ser into forms soluble in ethyl acetate; the degradation products resulting from esterase cleavage of Ent and FeEnt are still soluble in ethyl acetate.) Other experiments (not shown) demonstrated that addition of polyclonal antibodies specific for EntE, EntF or EntB/G all reduced Ent synthase activity in AB1515.5-4/pAUT119Af extracts. Antibodies directed against the T7gp10 epitope of the hybrid EntD did not reduce Ent synthase activity in these extracts, an unexpected result particularly if EntD is part of a multienzyme complex.

Two observations bear on the *in vivo* complexity of Ent synthase. (i) Extracts prepared by sonication rather than by passage through a French pressure cell exhibited highly variable but generally very low activity. The significance of this unusual effect of the manner of lysis is unclear. (ii) Extracts subjected to high-speed centrifugation (2 h, 160 000 g), a procedure which should pellet membranes and their associated proteins, had a higher rather than reduced specific activity compared to normal crude extracts (Table 2, AN102 high). Solubilized membrane pellets were inactive. Examination of the reaction products by TLC and HPLC showed that both 'high-speed' and regular extracts formed the same products (some Ent, primarily DBS) (Ammerlaan, 1994). These data suggest the Ent proteins are cytosolic.

Experiments were performed to test the effects on Ent synthase activity of agents influencing membrane and protein complex stability. As shown in Fig. 1, ionic detergents drastically reduced enzymic function; the *K*_i values for SDS and Sarkosyl are 0.025 and 0.05%,

Fig. 1. Effect of detergents on Ent synthase activity. Extracts were prepared from AN102 grown at 37 °C in iron-depleted M9 medium. ○, SDS; ●, Sarkosyl; □, Triton X-100; ■, Tween 20; △, NP-40

respectively. Likewise, NaCl was also inhibitory ($K_i = 150$ mM; data not shown). Remarkably, however, addition of non-ionic detergents Triton X-100, Tween 20, and NP-40 to the reaction mixtures at concentrations up to 10% had little effect. *In vitro* Ent synthase resistance to inhibitory effects of non-ionic detergents argues strongly against a mandatory need for membrane and also suggests that Ent synthase does not exist as a stable complex.

Radioimmune precipitations of Ent synthase polypeptides

Immune precipitations were performed to test for association among the EntD, E, F and B/G proteins. Preliminary experiments (Ammerlaan, 1994) determined that the antisera had good specificity; in Western blots, anti-EntE serum immunoprecipitated only EntE and a non-iron-regulated 65 kDa polypeptide, anti-EntF serum precipitated EntF and a non-iron-regulated 52 kDa protein, and anti-EntB/G antibodies recognized, in addition to EntB/G, two non-iron-regulated proteins of 68 and 72 kDa. Polyclonal antiserum against the first 12 amino acids of T7gp10, present at the N-terminus of the hybrid EntD protein, failed to detect EntD. The insensitivity of the Ent synthase reaction to detergent permitted the precipitation to be carried out as Ent synthesis was ongoing, in this case in the presence of 2% Tween 20. As shown in Fig. 2, even under the non-stringent conditions used only one iron-regulated protein of Ent synthase, the cognate protein of the antibody added, was immunoprecipitated. Similar results were obtained when Tween 20 was omitted (Ammerlaan, 1994). In lane 3, a protein with the migration properties of EntB (lane 7) was apparently precipitated along with EntE. However, the appearance of this protein was not reproducible and it was not detected under other even

Fig. 2. Radioimmune precipitations against Ent-synthesizing extracts. Immune precipitations were carried out against radiolabelled AB1515.5-4/pAUT119Af extracts from cells grown under low (odd-numbered lanes) and high (even-numbered lanes) -iron conditions. Extracts were set up as Ent synthase reactions with 2% Tween 20. Precipitations were carried out using antibodies directed against T7gp10 (lanes 1 and 2), EntE (lanes 3 and 4), EntF (lanes 5 and 6) and EntB/G (lanes 7 and 8). The expected migration of Ent proteins is indicated along the left edge of the gel. The arrow indicates an uncharacterized iron-regulated protein of 85–90 kDa.

less stringent conditions, such as in the absence of Tween 20. In addition, apparently the same iron-regulated 85–90 kDa polypeptide was present in lanes 3, 5 and 7; the significance of this observation is unknown. Nothing can be concluded regarding EntD; our laboratory has been unable to observe EntD by a variety of expression systems (Coderre & Earhart, 1989), Western blots, or radioimmune precipitation (Ammerlaan, 1994).

Release of EntB/G, E and F by osmotic shock

Periplasmic proteins are released from cells during the formation of spheroplasts and by osmotic shock; only the latter procedure also results in the release of several non-periplasmic enzymes, termed group D proteins (Beacham, 1979). Results shown in Fig. 3 demonstrate that EntE, F and B/G belong to the group D protein class. Each of these proteins is found in the shockate (lane 2); EntE and EntB/G are present almost exclusively in this fraction whereas EntF, although obviously present in the shockate, remains primarily with the shocked cells. Importantly, the Ent proteins are not released by spheroplasting, unlike the control periplasmic protein alkaline phosphatase (PhoA). FepB, the periplasmic binding protein for ferrienterobactin, behaved like PhoA (data not shown). The appearance of Ent synthase proteins in the shockates was not the result of cell breakage, as can be seen from the results with acyl carrier protein (ACP), a small (8.86 kDa) cytoplasmic polypeptide. Other cytoplasmic proteins tested were DnaA (50 kDa) and the τ and γ subunits of DNA polymerase III (75 and 55 kDa, respectively) of *E. coli*

Fig. 3. EntB/G, EntE and EntF are group D proteins. Osmotic shock, freeze/thaw and spheroplasting were performed on AB1515 cells grown to mid-exponential phase (OD_{420} 0.5) in TMM. Fractions from equal numbers of cells were analysed by Western blots after electrophoresis on 11% polyacrylamide gels. Only relevant portions of blots are shown. Lanes: 1, TC (total cells); 2, shockate; 3, shocked cells; 4, supernatant after freeze/thaw; 5, cells after freeze/thaw; 6, supernatant after spheroplasting; 7, spheroplasts. PhoA and ACP served as controls for periplasmic and cytoplasmic proteins, respectively. Molecular masses (kDa) of the polypeptides are: EntF, 142; EntE, 59; PhoA, 43; EntB/G, 32.5; ACP, 8.86 but migrates as 20 (C. Rock, personal communication).

AB1515 and, from *E. coli* W3110 grown in M9/lactose medium, β -galactosidase (116 kDa); all were found in shocked cells and not the shockates (data not shown). The localization characteristics of the Ent synthase proteins were neither strain-specific nor medium-dependent, as similar results were obtained with *E. coli* W3110 as well as with AB1515 and in M9 as well as in TMM medium.

Group D and periplasmic proteins are also released by freeze/thaw treatment, although not as effectively as by osmotic shock (Lunn & Pigiet, 1982). EntE and B/G are released by freeze/thaw but only trace amounts of EntF were detected in the soluble fraction (Fig. 3, lanes 4 and 5).

Divalent cations suppress release of Ent synthase proteins

Release of group D proteins thioredoxin (Lunn & Pigiet, 1982) and DnaK (El Yaagoubi *et al.*, 1994) is blocked by addition of divalent cations to the shocking fluid. Increasing concentrations of $CaCl_2$ steadily reduced the amount of EntE, F and B/G in shockates, such that at 0.5 mM $CaCl_2$ almost all EntF and EntE were retained in

Fig. 4. Calcium suppression of osmotic shock release of Ent synthase components. AB1515 cells grown in TMM were plasmolysed in SET, harvested, and resuspended in $CaCl_2$ solutions of concentrations (μ M) indicated at the top of (a). Proteins in shockates (Sh), shocked cells (SC), and total cells (TC) were resolved by electrophoresis in 11% polyacrylamide gels. (a) Coomassie Brilliant Blue-stained gel. (b) Western blot analyses of Ent synthase polypeptides present in shockates and shocked cells.

the shocked cells, as was most of EntB/G (Fig. 4). Similar results were observed when $MgCl_2$ was used in place of $CaCl_2$. These results differ from previous data of others in that this diminution of proteins released from the shockate fraction was not specific to group D proteins.

Osmotic shock of Ent synthase mutants

If Ent synthase proteins exist in a complex, a defective or absent unit of the complex could alter its intracellular location and thereby influence the osmotic shock retention of other components. To test this possibility, strains with mutations in each of the Ent synthase components were subjected to the shock procedure (Fig. 5). AB1515.43 has a Tn5 insertion in the 3' end of *entB/G*; the truncated protein can be detected and is $EntB^+G^-$. That is, it is still an active isochorismate-pyruvate hydrolase but cannot carry out the EntG reaction. In contrast, AB1515.24 is defective in both reactions; the Tn5 insertion is in the 5' end of *entB/G* and no product can be observed. In both mutants, EntE and EntF were released normally. Similarly, point mutations in *entE* (AN93) and *entF* (AN117) did not

Fig. 5. Osmotic shock release of Ent synthase proteins from cells bearing mutations in *entD*, *entE*, *entF* or *entB/G*. Strains designated at the top of the figure were grown in TMM to OD₄₂₀ 0.5 and subjected to osmotic shock. Fractions from equal numbers of cells were analysed as described in Fig. 3. Abbreviations are as in Fig. 4; the asterisk denotes the truncated EntB⁺G⁻ protein.

eliminate the shockability of their cognate proteins or that of other Ent synthase components. Lastly, AB1515.5-4 (Tn5::*entD*) likewise had no effect on retention of other Ent synthase components, a finding of interest since EntD has been proposed to anchor the putative complex to membrane. In summary, the shockability of other Ent synthase polypeptides was independent of EntB/G and EntD; evidence with respect to EntE and EntF was similar but less rigorous because of the nature of the mutation involved.

Ent synthase activity of shocked cells and shockates

The Ent synthase activity in extracts prepared from shocked AB1515 and W3110 cells was approximately 63% of that of normal extracts. The corresponding shockates did not have significant Ent synthase activity.

DISCUSSION

The primary finding of this work is that several Ent synthase components meet the definition of group D proteins, i.e. they are cytoplasmic proteins found in cell supernatants following osmotic shock but not after spheroplasting (Beacham, 1979). Western blot analyses disclosed that most of the EntE and EntB/G proteins are released by osmotic shock treatment. Shockates contained lesser, variable amounts of EntF, which may be related to the fact that EntF is an unusually large (142 kDa) polypeptide. Cytoplasmic proteins DnaA, β -galactosidase, DNA polymerase III subunits τ and γ , and ACP were not released by shock, showing that the presence of Ent proteins in shockates was not the result of cell breakage. The results with the small (8.86 kDa) ACP molecules confirm previous findings of Van den Bosch *et al.* (1970).

Nine group D proteins, most of which are enzymes concerned with nucleoside catabolism, were initially identified by Beacham (1979). Since that time, thio-redoxin (Lunn & Pigiet, 1982) and DnaK (El Yaagoubi *et al.*, 1994) have been so categorized. The three Ent proteins thus bring the total of *E. coli* group D proteins to 14 but it is likely that many more exist. The present work suggests that biosynthetic enzymes for other siderophores, particularly those that involve non-ribosomal synthesis of peptide bonds, may be group D

proteins. In fact, evidence of Viswanatha *et al.* (1987) indicates that aerobactin synthase has an unusual intracellular location. More generally, because EntE and EntF and now EntB/G share homologies with proteins involved in thio-template-directed peptide synthesis, it is possible that many proteins concerned with the biosynthesis of surfactants and peptide antibiotics may be members of the group D class.

Whether or not any EntA or EntC, two proteins required for early steps in Ent biosynthesis, are present in shockates has not yet been determined. No antibodies are available for either and assays for these enzymes are difficult (Walsh *et al.*, 1990). However, results shown in Fig. 5 with AB1515.43 demonstrate that the EntB activity (isochorismate-pyruvate hydrolase, the catalyst that converts the product of EntC into the substrate for EntA) of EntB/G is shockable in the absence of EntG; the N-terminus of the protein is sufficient for osmotic shock release. Definitive evidence regarding EntD in shockates is lacking. Shockates were devoid of Ent synthase activity and attempts to perform enzymic complementation with extracts from Ent synthase mutants supplemented with shockates from wild-type cells were similarly negative. Possibly components in addition to EntD, E, F and B/G may be necessary for Ent synthase activity (Bryce & Brot, 1972); lack of these auxiliary factors could explain the absence of activity in shockates even if EntD were present. There is no ready explanation for the failure of shockates to provide the necessary activity to mutant extracts, since group D proteins in shockates have often been shown to be active (Jacobson & Rosenbusch, 1976; Munch-Peterson & Mygind, 1976; Page & Burton, 1978).

Whether or not Ent synthase exists as a complex *in vivo* remains unknown. *In vitro* data showed (i) the enzyme was active in the presence of high concentrations of neutral detergents and (ii) immunoprecipitation experiments conducted under conditions where the enzyme would be active failed to provide any evidence for a complex. However, EntB/G, E and F all share the unusual property of being a group D protein, which suggests they might be in close proximity to one another *in vivo*. Additional indirect evidence possibly indicative of an *in vivo* complex is the low activity of extracts prepared by sonication and the absence of Ent synthase

activity in shockates. The complex, if it exists, may be transitory. Furthermore, the apparent ability of EntE, F and B/G to reside in several cellular locations (i.e. the cytoplasm and a shock-sensitive region) raises the possibility that *in vivo* the enzyme may be active in only one of the two regions.

The osmotic shockability of group D proteins is generally attributed to their loose or partial association with the inner surface of the cytoplasmic membrane (Beacham, 1979); indeed, such a peripheral membrane association has been reported for EFTu (Jacobson & Rosenbusch, 1976), DnaK (El Yaagoubi *et al.*, 1994) and thioredoxin (Lunn & Pigiet, 1982). Lunn & Pigiet (1982) suggested, more specifically, that group D proteins are located at zones of adhesion between inner and outer membrane and, for thioredoxin, have adduced evidence in support of this proposal (Bayer *et al.*, 1987; Lunn & Pigiet, 1986). Therefore, that EntE, F and B/G are group D proteins is indirect evidence that some fraction of them is membrane-associated. In fact, in a preliminary report EntB/G was detected in cytoplasmic membrane fractions (Hantash & Earhart, 1995) and more recently some EntE and EntF have been similarly localized (unpublished observations). This association was not required for *in vitro* Ent synthase activity, however, as the enzyme was active in high-speed supernatants and in detergent, conditions where membrane would be absent or disrupted, respectively. The relationship of the *in vitro* cytoplasmic activity to *in vivo* activity remains the major question.

One other type of polypeptide recently identified as being osmotically shockable may provide additional insight into the *in vivo* positioning of group D proteins. Periplasmic proteins synthesized with no signal sequences are group D proteins (Bowden *et al.*, 1992; Derman *et al.*, 1993). Evidence suggests that these altered proteins are present in the cytoplasmic membrane export machinery at the time of their release by shock. Possibly, then, identification of a protein as being in the group D class may indicate that not only is at least some portion of the protein loosely associated with membrane but that the membrane site is one at which transmembrane passage occurs. This is consistent with properties of several group D proteins in addition to signal sequence mutants and thioredoxin; Beacham (1979) has discussed the possible role of many group D proteins in nucleoside uptake as well as catabolism and DnaK has a role in protein transmembrane targeting and secretion (Wild *et al.*, 1996). Applied to Ent synthase, this hypothesis would suggest that the latter stages of Ent synthesis occur in association with an unidentified inner membrane export apparatus. Unfortunately, nothing is known regarding how Ent leaves the cytoplasm, and the results shown in Fig. 5 eliminate EntD as providing the putative localizing function necessary for shockability.

Lastly, the data further emphasize the ability of certain cytoplasmic proteins to be compartmentalized or to exist in several cellular locations in prokaryotic cells. In addition to group D proteins, prominent examples of such odd and interesting proteins include FtsZ

(Lutkenhaus, 1993), SecA (Economou & Wickner, 1994), and CheA and CheW (Maddock & Shapiro, 1993).

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