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Production of a Soluble Form of Fumarate Reductase by Multiple Gene Duplication in *Escherichia coli* K12

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1. Ampicillin-hyperresistant mutants of *Escherichia coli* K12 bearing multiple gene duplications in the *ampC* (β-lactamase) gene region of the chromosome overproduced at least six proteins with molecular weights 97000, 80000, 72000, 49000, 33000 and 26500 during anaerobic growth. All but two of the proteins (80000-M_r and 49000-M_r) were also overproduced during aerobic growth. The distribution of the proteins in soluble and particulate cell fractions was investigated.

2. The 33000-M_r and 72000-M_r components were identified as β-lactamase and the *amp*-linked *frdA* gene product, fumarate reductase, respectively. Co-sedimentation of the 26500-M_r component with the fumarate reductase suggested that the smaller protein could be functionally related to the reductase. The lack of correspondence between the amplified proteins and the products of other *amp*-linked genes, *aspA* and *mop*(*groE*), indicated that these genes are not included in the repetitive sequence.

3. Fumarate reductase activities were amplified up to 32-fold by the multiple gene duplications. Two forms of fumarate reductase were produced: particulate (membrane-bound) and soluble (cytoplasmic). Production of the soluble form occurred when the binding capacity of the membrane was saturated. Both forms of fumarate reductase were enzymically active but the soluble form was readily inactivated under assay conditions.

The fumarate reductase of *Escherichia coli* is a membrane-bound flavoprotein which catalyses the interconversion of fumarate and succinate and is distinct from succinate dehydrogenase. A functional fumarate reductase is essential for anaerobic growth on non-fermentable substrates when fumarate is the terminal electron acceptor [1,2]. Synthesis of the enzyme is derepressed under anaerobic conditions but totally repressed by oxygen and nitrate [1,3].

The fumarate reductase structural gene (*frdA*) is located at 92.8 min on the recalibrated *E. coli* linkage map [4], very close to the chromosomally-determined β-lactamase structural gene (*ampC*) and the regulatory gene (*ampA*). A regulatory mutation (*ampA1*) causes a tenfold increase in β-lactamase synthesis and a corresponding increase in bacterial resistance to penicillins [5,6]. Furthermore, studies with highly-resistant mutants by Normark and co-workers [7] have correlated hyperproduction of β-lactamase with multiple repetition of the chromosomal *ampA1 ampC*$^+$ region. The sizes of the repeating units in two strains were estimated as 5000 and 8000 base pairs, each repetition mediating ampicillin resistance to about 10 μg ml^{-1}.

Recently, the fumarate reductase activities of an independent series of ampicillin-hyperresistant mutants isolated by Cole and Guest [8] were found to increase in proportion to the corresponding β-lactamase activities and degree of ampicillin resistance [8]. This indicates that the *frdA* gene is included in the genetic repeating unit. Expression of the *frdA* genes in the repetitive *amp-frdA* gene sequences was still subject to normal repression by oxygen (and nitrate). However, the aerobic repression could be overcome by increasing *frdA* gene dosage, suggesting that the *frdA* gene is controlled by a titratable regulatory component (repressor) expressed from an independent region of the chromosome [8].

This paper reports the production of a soluble (cytoplasmic) form of fumarate reductase by the ampicillin-hyperresistant mutants. The formation of the soluble enzyme appears to be due to saturation of the membrane-binding capacity for fumarate re-

Enzymes. Fumarate reductase (EC 1.3.99.1); nitrate reductase (EC 1.7.99.4); β-lactamase (EC 3.5.2.6); aspartase or L-aspartate ammonia-lyase (EC 4.3.1.1).

ductase. In addition to fumarate reductase and β-lactamase, four other proteins were overproduced by the mutants containing multiple duplications of the *amp-frdA* region.

MATERIALS AND METHODS

Organisms

Spontaneous mutants of *Escherichia coli* K12 resistant to high concentrations of ampicillin were obtained by sequential plating of the parental strain G10 (JRG 997; *ilv metB ampAI*) on L agar [9] containing increasing concentrations of the antibiotic [8]. The strains designated G10, G100, G200, G400 and G800, tolerated, amicillin concentrations of 10, 100, 200, 400 and 800 μg ml^{-1} respectively; they were maintained by routine subculture in L agar containing the corresponding concentration of ampicillin.

Growth of Organisms for Enzymology

Batch cultures (1l) were grown at 37 °C from 50 ml of inocula in a New Brunswick Microferm fermenter. (New Brunswick Scientific Co. Inc, New Brunswick, N.J., U.S.A.). Inocula were grown for 16 h at 37 °C with shaking in conical flasks containing L broth [9]. Ampicillin at the maximum tolerated concentration was added to the inocula in order to maintain selection for the gene duplications. The medium for bulk growth contained (w/v): peptone (0.4%), yeast extract (0.4%) and K_2HPO_4 (0.6%), final pH, 6.8. Aerobic cultures were supplemented with ampicillin at one-tenth the concentration tolerated. Anaerobic cultures were supplemented with glucose (1%) and supplied with oxygen-free N_2 (British Oxygen Corporation) at a flow rate of 500 ml min^{-1}: ampicillin was not added because it retarded anaerobic growth. Cultures were harvested in late exponential phase (after 3 – 4 h) and resuspended (0.25 g wet wt ml^{-1}) in 10 mM potassium phosphate buffer, pH 7.8, without washing. Suspensions were disrupted by passing through a French pressure cell at 68.9 MPa and a few crystals of deoxyribonuclease I were added. The lysates were clarified by centrifuging for 15 min at $10000 \times g$ and the supernatant fluids, designated crude cell-free extracts, were resolved into high-speed supernatant and particulate frations by centrifuging at $100000 \times g$ for 2 h at 4 °C in an MSE Prepspin 50 ultracentrifuge.

Enzymology

Fumarate reductase, nitrate reductase and β-lactamase were assayed spectrophotometrically at 37 °C as described previously [2, 8]. Aspartase (L-aspartate ammonia lyase) was measured by the method of Courtright and Henning [10]. Protein was determined by the method of Lowry et al. [11] using bovine serum albumin as the standard. Enzyme specific activities are quoted in enzyme units/mg protein, where one unit is the amount of enzyme forming 1 μmol product/min.

Sucrose Gradients

Samples (1 ml) of crude extract (from anaerobically grown G800) were carefully layered on linear sucrose gradients (10 – 50%, v/v) containing 10 mM phosphate buffer pH 7.8. Centrifugation was at $150000 \times g$ (35000 rev./min) for 16 h and 4 °C in an MSE Prepspin 50 in a 6×14-ml swing-out rotor. Fractions (600 μl) were collected for enzyme analysis and estimation of sucrose density with an Abbé refractometer. Fractions for sodium dodecylsulphate/polyacrylamide gel electrophoresis were dialysed against 10 mM potassium phosphate buffer pH 7.8 to remove the sucrose before processing by the method of Laemmli [12].

Sodium Dodecylsulphate/Polyacrylamide Gel Electrophoresis

Electrophoresis in 10% (w/v) polyacrylamide gels containing 0.1% sodium dodecylsulphate was performed in the vertical slab gel apparatus described by Studier [13] using the discontinuous sodium dodecylsulphate buffer system of Laemmli [12]. Gels were stained with Coomassie brilliant blue. The molecular weights of protein subunits were estimated by comparison with the following marker proteins: RNA polymerase (165000, 155000 and 39000); β-galactosidase (116000); phosphorylase *b* (94000); conalbumin, type 1 (78000); bovine serum albumin (68000); ovalbumin (43000); lactate dehydrogenase (35000); carbonic anhydrase (29000) and trypsin inhibitor protein (21500).

Chemicals

α-Aminobenzylpenicillin (D-ampicillin, sodium salt) was kindly donated by Beechams Pharmaceuticals (Betchworth, Surrey, U.K.). Benzylpenicillin was purchased from Glaxo Laboratories Ltd (Greenford, U.K.). Benzylviologen and sodium dodecylsulphate were obtained from BDH Chemicals Ltd (Poole, Dorset, U.K.) and the former was reduced by catalytic hydrogenation using palladised asbestos. The sources of commercial enzymes and proteins were: The Boehringer Corporation Ltd (Lewes, East Sussex, U.K.) RNA polymerase, β-galactosidase, phosphorylase *b*, trypsin inhibitor (soybean) and lactate dehydrogenase; The Sigma Chemical Company Ltd (Kingston upon Thames, Surrey, U.K.) ovalbumin, conalbumin, bovine serum albumin and carbonic anhydrase; BDH Chemicals Ltd (Poole, Dorset, U.K.) deoxyribonuclease I.

RESULTS

The fumarate reductase of *Escherichia coli* K12 is a membrane bound enzyme induced during anaerobic growth [14]. Comparisons of the proteins derived from the cytoplasmic membranes of fumarate reductase (*frdA*) amber mutants and the parental strain indicated that the *frdA* gene product is a polypeptide of molecular weight approximately 75000 [14]. The fumarate reductase (*frdA*) and β-lactamase (*amp*) genes are closely linked in the region of the *E. coli* chromosome shown in Fig. 1. Recently, spontaneous mutants possessing multiple gene duplications of the *amp-frdA* region have been isolated [8]. This prompted an investigation of their protein compositions by analytical sodium dodecylsulphate/polyacrylamide gel electrophoresis in order to (a) confirm the size of the *frdA* gene product and (b) detect the products of other genes which may be included within the duplicated region.

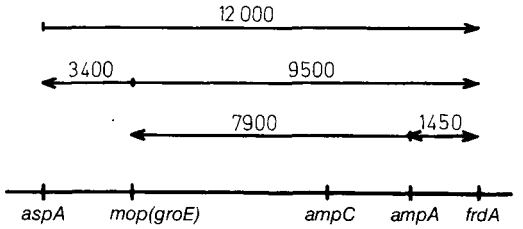

Fig. 1. *Distribution of genes in the* amp-frdA *region of the* Escherichia coli *K12 linkage map.* The approximate positions of the genes in a segment (1.2×10^4 base pairs, 0.25 min) of the 92–93-min region of the linkage map is shown with the number of base pairs, derived from P1 contransduction frequencies [8, 15]. The gene symbols are: *aspA*, aspartase; *mop(groE)*, bacteriophage morphogenesis; *ampC,A*, β-lactamase structural and regulatory genes; *frdA*, fumarate reductase

Sodium Dodecylsulphate/Polyacrylamide Gel Electrophoresis

Analysis of the sodium-dodecylsulphate-soluble proteins of crude extracts of anaerobically grown ampicillin-resistant strains G10, G100, G200, G400 and G800 using sodium dodecylsulphate/polyacrylamide gel electrophoresis showed reproducible increases in at least six components, graded over the range of ampicillin resistance. These components corresponded to polypeptides with molecular weights of 97000, 80000, 72000, 49000, 33000 and 26500. Representative results for three strains, G10, G400 and G800 are shown in Fig. 2 (tracks 1–3). Parallel

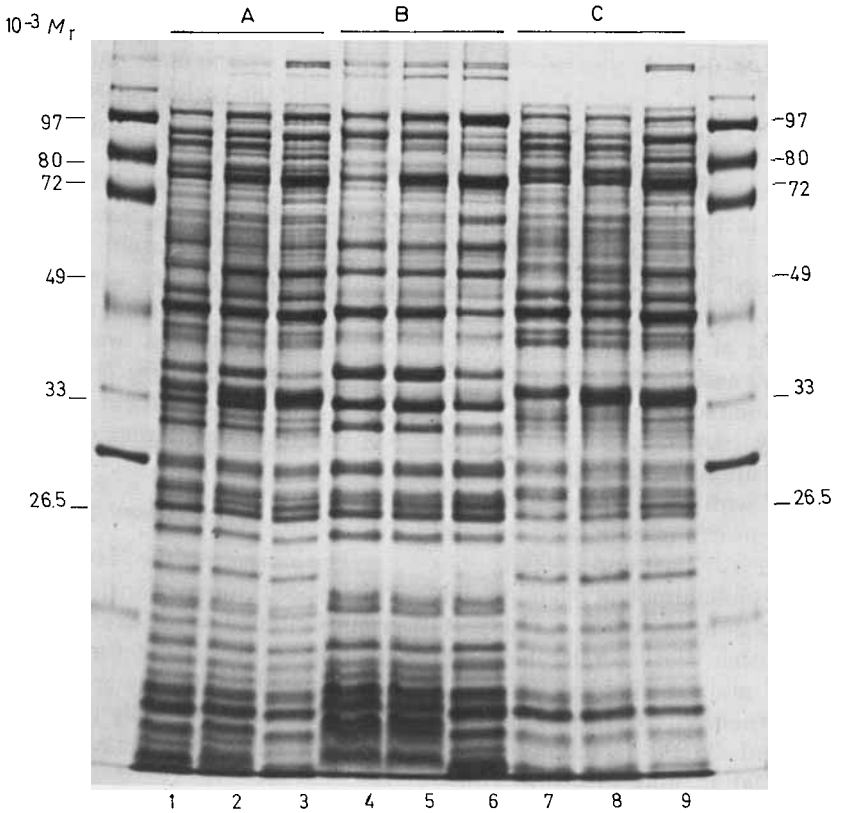

Fig. 2. *Sodium dodecylsulphate/polyacrylamide gel electrophoresis of proteins from anaerobically grown ampicillin-hyperresistant mutants of* Escherichia coli *K12.* Crude cell-free extracts (A) from G10, G400 and G800 were applied to tracks 1–3, particulate fractions (B) of G10, G400 and G800 to tracks 4–6 and high-speed supernatant fractions (C) of G10, G400 and G800 to tracks 7–9, respectively. Protein (50 μg) was added to each track and electrophoresis was for 4 h at a constant current of 26 mA. Molecular weights of the amplified proteins are indicated

assays of fumarate reductase and β-lactamase specific activities and other studies (see below) enabled identification of the 72000-M_r and 33000-M_r components as fumarate reductase (*frdA* gene product) and β-lactamase (*ampC* gene product) respectively. The subunit molecular weight for fumarate reductase is slightly lower than the value reported previously of 75000 [14]. Also, the molecular weight obtained for β-lactamase (33000) is significantly higher than the previous estimates of 29000 [5] and 31400 [7], obtained with the β-lactamase produced by a λ-lysogenic ancestor of strain G10, strain G11a1.

The 97000-M_r, 80000-M_r, 49000-M_r and 26500-M_r proteins could not be identified. The aspartase gene (*aspA*) is sufficiently close to the *amp* region to be included in the repetitive sequence (Fig. 1) [16]. Furthermore, because aspartase contains four identical subunits of molecular weight 48500 [17], the relationship between aspartase and the unidentified 49000-M_r component was investigated. No significant increase in aspartase activity could be detected in the ampicillin-hyperresistant mutants. It was therefore concluded that the 49000-M_r protein is not the aspartase subunit. This conclusion is also supported by the fact that no increase in a protein corresponding to the *mop*(*groE*) gene product, molecular weight 65000 [19,20], could be detected. The *mop*(*groE*) gene is situated between *aspA* and the *amp* region (Fig. 1) [18] and, unless prevented by regulatory factors, its product should have been overproduced if the duplicated region extends as far as *aspA*.

Separation of the crude extracts into particulate or membrane and soluble or cytoplasmic (high-speed supernatant) fractions showed that three proteins (M_r 97000, 72000, 26500) increased in the particulate fraction and five proteins (M_r 80000, 72000, 49000, 33000 and 26500) increased in the soluble fraction (Fig. 2, tracks 4–6 and 7–9 respectively). Also, the results indicated that some of the amplified proteins exist exclusively (M_r 49000 and 33000) or predominantly (M_r 80000) in the soluble or cytoplasmic fraction, others are primarily particulate or membrane proteins (M_r 97000) and, unless some bands contain more than one protein with the same molecular weight, some of the proteins can exist in both soluble and particulate fractions (M_r 72000 and 26500).

The sodium dodecylsulphate/polyacrylamide gel electrophoretic analysis was extended to extracts of aerobically grown organisms. Very similar patterns of protein amplification and distribution to those shown in Fig. 2 were observed for the 97000-M_r, 72000-M_r, 33000-M_r and 26500-M_r components. Exceptions were the 80000-M_r and 49000-M_r components which were not overproduced by the ampicillin-hyperresistant strains during aerobic growth. It appears that expression of the corresponding genes is only permitted during anaerobic growth. By contrast, enzymatic analysis and the appearance of the 72000-M_r component in extracts of aerobically grown organisms (particulate and soluble fractions) confirmed the the earlier report [8] that a high degree of amplification of the *frdA* gene enables it to overcome aerobic repression.

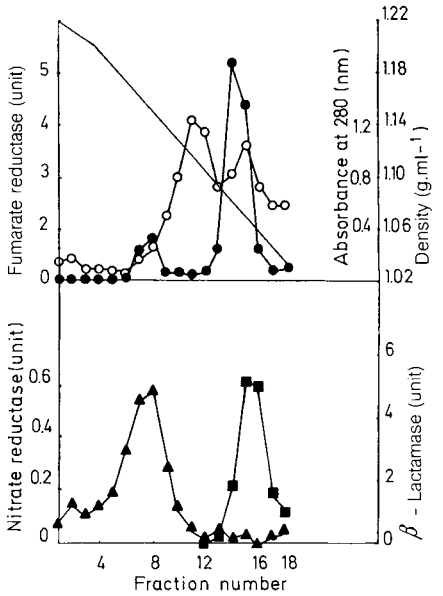

Fig. 3. *Separation of the two forms of fumarate reductase by isopycnic centrifugation.* Crude extract of anaerobically grown G800 was layered on a linear sucrose gradient (10–50%, w/w) and centrifuged at 150000 × *g* for 16 h at 4 °C. Eighteen 600-µl fractions were collected and assayed for fumarate reductase (●), nitrate reductase (▲), and β-lactamase (■) activity. The gradient (——) and absorbance at 280 nm (○) are indicated in the upper section of the figure

A decrease in some proteins, especially components of the particulate fraction, was observed with strain G800 (Fig. 2). This could be a direct consequence of the overproduction of some proteins resulting in an imbalance in total protein synthesis. Minor and less reproducible changes were sometimes detected in other components (Fig. 2) and it was not possible to determine whether these were direct or secondary consequences of the gene duplications.

Sucrose Density Gradient Analysis

The presence of the 72000-*Mr* protein in the soluble (high-speed supernatant) fractions of the amplified strains pointed to the existence of a non-sedimentable (cytoplasmic) form of fumarate reductase. This was investigated by density gradient analysis using crude extracts of anaerobically grown strain G800.

Two peaks of fumarate reductase activity were resolved (Fig. 3). The major peak was located in the low-density region of the gradient, together with the soluble enzyme, β-lactamase. This accounted for 81% of the total fumarate reductase activity. The remaining activity formed a minor peak in the high-density

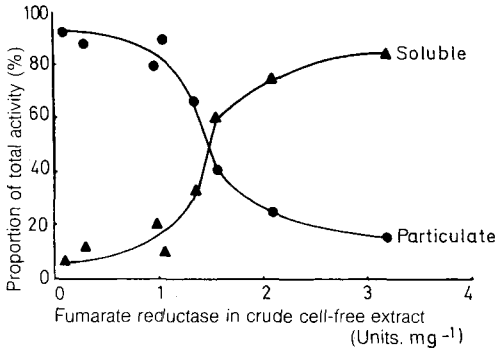

Fig. 4. *The distribution of fumarate reductase activity between particulate and soluble fractions.* The distribution of total fumarate reductase activity in particulate (●) and soluble (▲) fractions of a range of crude cell-free extracts from anaerobically grown mutants of *E. coli* K12 having different ampicillin resistances. The amount of enzyme activity in the two fractions is plotted against the specific activity in the corresponding crude cell-free extract

region of the gradient, which was also occupied by nitrate reductase (Fig. 3). Nitrate reductase is another anaerobically derepressible membrane-bound enzyme and it provides a good internal marker for the particulate fraction. Analysis of fractions from the gradient by sodium dodecylsulphate/polyacrylamide gel electrophoresis showed that both the 72000-M_r and 26500-M_r components co-sedimented in the fractions containing fumarate reductase activity. Major bands corresponding to the β-lactamase and the 80000-M_r and 49000-M_r components were in the soluble fractions, whereas the 97000-M_r components appeared most prominently in the particulate fractions.

Subcellular Distribution of Fumarate Reductase

Crude extracts of anaerobically grown G10, G100, G200, G400 and G800 were fractionated into particulate and soluble (high-speed supernatant) fractions and the distribution of fumarate reductase activity was determined (Fig. 4). The location of the enzyme changed from being predominantly particulate to predominantly soluble, with increasing specific activity in the crude extract. The proportion of soluble enzyme increased steeply when the total specific activity was more than 10 times the basal activity of the parental strain, G10 (0.1 unit · mg protein^{-1}). This suggests that the membranes of anaerobically grown organisms have the capacity to accept 8–10 times more fumarate reductase than is normally formed, before becoming saturated. Similar studies with aerobically grown organisms indicated that the saturation point for the interaction of fumarate reductase with the aerobic membrane was not significantly different. The greatest amplification of fumarate reductase activity was 32-fold relative to the parental strain.

Differences Between the Soluble and Particulate Forms of Fumarate Reductase

The soluble form of fumarate reductase was very unstable, especially at low temperatures. The activity of the soluble fraction of crude extracts fell by 50% after 24 h at −20 °C. No loss of activity of the membrane-bound enzyme was detected after similar treatment.

The progress curves obtained with the two forms of fumarate reductase were markedly different. The initial rate was maintained over relatively long periods with the particulate enzyme, whereas a short but linear initial rate was followed by a rapidly decreasing rate with the soluble enzyme. This was not due to limitation by either of the two substrates. The method of Selwyn [21] was used to test for enzyme inactivation during assay. For this purpose, a set of progress curves obtained with different amounts of enzyme were analyzed by plotting the amount of product formed at different times against the product of the time and the enzyme concentration (Fig. 5). For the particulate enzyme, all the experimental data fell on a single curve, characteristic of a stable enzyme (Fig. 5A). Under the same assay conditions, different curves were obtained with different concentrations of the soluble preparation and this is indicative of extensive inactivation during the course of the reaction (Fig. 5B). The linear type of progress curve could be restored by incubating the soluble enzyme with Mg^{2+} and a membrane fraction prepared from an anaerobically grown *frdA* amber mutant, lacking fumarate reductase activity. This suggests that the enzyme is stabilised by interaction with a component or components of the membrane.

DISCUSSION

Hyperresistance to ampicillin in *Escherichia coli* K12 is mediated by a series of gene duplications around the *ampA1 ampC* region of the chromosome leading to increased synthesis of β-lactamase and fumarate reductase [7, 8]. Analysis by sodium dodecylsulphate/polyacrylamide gel electrophoresis has now shown that another consequence is increased synthesis of at least four other proteins having molecular weights of 97000, 80000, 49000 and 26500. Two of these proteins, with M_r 80000 and 49000 (both soluble), were only amplified during anaerobic growth. This could be explained if expression of the corresponding genes requires a positive regulatory protein, which is inactive or not formed under aerobic conditions.

The membrane-bound fumarate reductase of *Vibrio succinogenes* contains three components, a flavoprotein (M_r 80000–85000), an iron-sulphur protein (M_r 30000) and a cytochrome *b* (M_r 24000) [22]. The co-sedimentation of the 72000-M_r and 26500-M_r proteins

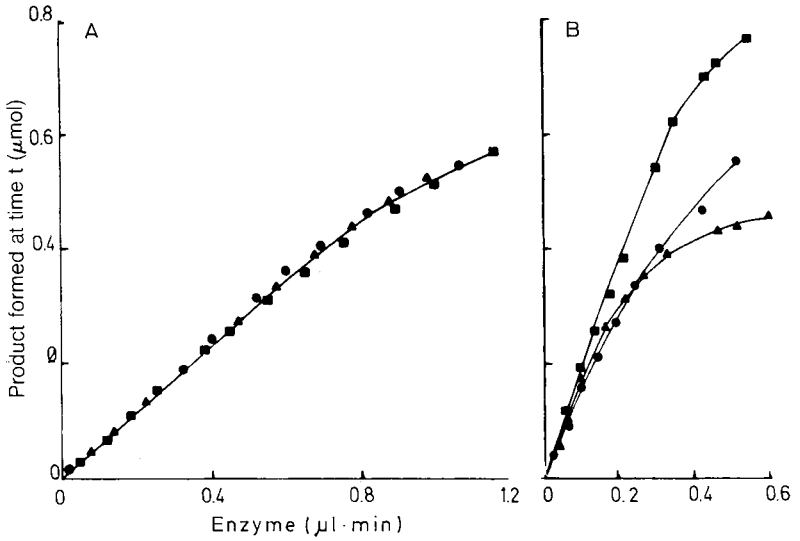

Fig. 5. *Analysis of the progress curves for the particulate and soluble forms of fumarate reductase.* The fumarate-dependent oxidation of reduced benzylviologen was analysed according to Selwyn [18] by plotting the product (succinate) formed at specific times *t* against the product of *t* and the amount of enzyme used (as volume of extract). The reaction was followed spectrophotometrically at 550 nm under He in anaerobic cuvettes (3.2 ml final volume) containing potassium phosphate buffer (300 μmol; pH 7.8), enzyme preparation, reduced benzylviologen (1 μmol) and fumarate (6 μmol) to initiate the reaction. Results were obtained using different volumes of the enzyme preparations. (A) Particulate enzyme: 5 μl (▲), 12.5 μl (●) and 15 μl (■). (B) Soluble enzyme: 2.5 μl μl (▲), 5 μl (●) and 7.5 μl (■)

with the fumarate reductase activity in sucrose density gradients is consistent with a functional relationship. If so, the smaller protein is more likely to be an iron-sulphur protein than a cytochrome because the cytochrome *b* contents of membrane preparations from G10 and G800 were the same by difference spectroscopy.

The six proteins amplified in the ampicillin-resistant mutants could be coded by a single segment of DNA in the *amp-frdA* region of the chrosome containing approximately 10^4 base pairs. This is only sligtly larger than the estimated size (8000 base pairs) of the repeating unit in one of the strains examined by Normark et al. [7]. It is also possible that one or more of the unidentified proteins is amplified indirectly by responding to the overproduction of one of the other proteins rather than as a direct effect of gene dosage.

The discrepancy between the molecular weight estimated for the β-lactamase accumulated by the hyperresistant mutants (33000) and published values (29000 and 31400) for the purified enzmye [5, 7] could be explained if the accumulated enzyme is predominantly in the form of a larger precursor molecule containing an N-terminal polypeptide, normally removed on transfer to the periplasm. This is known to occur for some β-lactamases [23]. No difference between the size of the membrane-bound and cytoplasmic forms of fumarate reductase was detected.

The occurrence of a soluble form of a membrane-bound enzyme is not unique. A non-sedimentable form of nitrate reductase is produced by *hemA* mutants of *E. coli* K12 when the synthesis of the cytochrome *b*, required for membrane incorporation, is impaired [24]. However, it is unusual to find both soluble and particulate forms of an enzyme co-existing in one strain. In recent studies with NADH dehydrogenase, Young et al. [25] observed that despite a 50-fold amplification from plasmid-borne genes, no soluble form was detected. It was concluded that either all of the NADH dehydrogenase produced was present in the membrane fraction or that any unincorporated enzyme was inactive. In the case of fumarate reductase, it appears that once the binding capacity of the membrane has been saturated, further synthesis leads to the accumulation of an active cytoplasmic form.

Comparison of the kinetic properties of the soluble and particulate forms of fumarate reductase indicated that the soluble form is readily inactivated under the conditions of the enzyme assay. This could be due to denaturation of a hydrophobic protein by exposure to an aqueous environment. An explanation proposed by Selwyn [21] involves one of the products as a time-dependent irreversible inhibitor. Preliminary studies have shown that, although both preparations are moderately inhibited by succinate, the soluble form is much more sensitive to succinate at low concentrations.

The amplification of fumarate reductase obtained by multiple gene duplication is several times greater than has been observed using artificially constructed transducing phages carrying the *frdA* gene [26]. The ampicillin hyperresistant strains should thus provide

a valuable source from which the enzyme can be isolated for detailed studies on its structural and kinetic properties.

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