The aconitase of *Escherichia coli*: purification of the enzyme and molecular cloning and map location of the gene (*acn*)

CHRISOSTOMOS PRODROMOU, MEGAN J. HAYNES and JOHN R. GUEST*

The Krebs Institute for Biomolecular Research, Department of Molecular Biology and Biotechnology, University of Sheffield, PO Box 594, Firth Court, Western Bank, Sheffield S10 2UH, UK

(Received 24 April 1991; revised 10 July 1991; accepted 24 July 1991)

The aconitase of *Escherichia coli* was purified to homogeneity, albeit in low yield (0.6%). It was shown to be a monomeric protein of M_r 95 000 or 97 500 by gel filtration and SDS-PAGE analysis, respectively. The N-terminal amino acid sequence resembled that of the *Bacillus subtilis* enzyme (*citB* product), but the similarity at the DNA level was insufficient to allow detection of the *E. coli acn* gene using a 456 bp *citB* probe. Phages containing the *acn* gene were isolated from a λ -*E. coli* gene bank by immunoscreening with an antiserum raised against purified bacterial enzyme. The *acn* gene was located at 28 min (1350 kb) in the physical map of the *E. coli* chromosome by probing Southern blots with a fragment of the gene. Attempts to locate the gene using the same procedure with oligonucleotide probes encoding segments of the N-terminal amino acid sequence were complicated by the lack of probe specificity and an inaccuracy in the physical map of Kohara *et al.* (*Cell* 50, 495–508, 1987). Aconitase specific activity was amplified some 20–200-fold in cultures transformed with pGS447, a derivative of pUC119 containing the *acn* gene, and an apparent four-fold activation–deactivation of the phagemid-encoded enzyme was observed in late exponential phase. The aconitase antiserum cross-reacted with both the porcine and *Salmonella typhimurium* (M_r 120 000) enzymes.

Introduction

Aconitase or citrate (isocitrate) hydro-lyase (EC 4.2.1.3) functions in both the citric acid and glyoxylate cycles. It is a dehydratase–hydratase which catalyses the reversible isomerization of citrate and isocitrate via *cis*-aconitate. The mitochondrial aconitases have been purified from a variety of sources and shown to be monomeric proteins with an M_r of about 79 000–83 000: porcine heart (Villafranca & Mildvan, 1971; Zheng *et al.*, 1990); bovine heart (Ryden *et al.*, 1984); and *Saccharomyces cerevisiae* (Scholze, 1983; Gangloff *et al.*, 1990). In contrast, the fluoroacetate-induced enzyme from *Candida lipolytica* seems to be smaller, M_r 68 500 (Suzuki *et al.*, 1975), and the *Bacillus subtilis* enzyme is considerably larger, M_r 120 000 (Dingman & Sonenshein, 1987).

Aconitase is interesting because it contains a cubane-like [4Fe–4S] cluster which is redox inactive, and essential for activity. Indeed, one of the Fe atoms (designated Fe_a), has been shown to coordinate with the middle carboxyl group, and possibly the hydroxyl group, of the substrate (Kennedy *et al.*, 1987; Werst *et al.*,

1990a, b). Exposure to oxygen during purification results in the loss of the Fe_a atom, producing a catalytically inactive enzyme with a [3Fe–4S]⁺ cluster. However, the enzyme can be partially protected by adding citrate during purification, or reactivated by Fe²⁺ under reducing conditions to give a [4Fe–4S]²⁺ cluster (Empage *et al.*, 1983; Kennedy *et al.*, 1983; Kennedy & Bienert, 1988). The structures of the inactive [3Fe–4S] and active [4Fe–4S] forms of the porcine heart enzyme have been solved at 0.21 and 0.25 nm resolution, respectively (Robbins & Stout, 1989a, b).

The gene encoding the mitochondrial aconitase of *S. cerevisiae* has recently been cloned and the primary structure of the enzyme has been deduced from its nucleotide sequence (Gangloff *et al.*, 1990). Similarly, the amino acid sequence of porcine heart aconitase has been deduced from the nucleotide sequence of the corresponding cDNA (Zheng *et al.*, 1990) and the N-terminal region of the *B. subtilis* enzyme has been deduced from a segment of the *citB* gene (Rosenkrantz *et al.*, 1985; Dingman & Sonenshein, 1987).

Very little is known about the *Escherichia coli* aconitase and studies on the aconitase gene (*acn*) have been hindered by the lack of mutants deficient in aconitase. Genes encoding all of the citric acid cycle

Abbreviations: ICDH, isocitrate dehydrogenase; TC, Tris/citrate.

enzymes except aconitase have been cloned, sequenced and located in the *E. coli* chromosome (Miles & Guest, 1987; Bachmann, 1990). Several strategies have now been used to clone the *acn* gene and then to explore the possibility that aconitase is related to the oxygen-labile [4Fe-4S]-containing fumarases (Woods *et al.*, 1988; Bell *et al.*, 1989; Flint *et al.*, 1989). An M13 derivative containing part of the aconitase gene (*citB*) of *B. subtilis* was used as a hybridization probe in previous attempts to clone the aconitase gene (*acn*) of *E. coli* (Wilde *et al.*, 1986). Several putative *lacn* phages were isolated and characterized by their ability to amplify aconitase 5–18-fold upon prophage induction. These phages were subsequently found to contain segments of the *lac* operon and the reason for their ability to amplify aconitase remains obscure (Wilde, 1988). More recently, two sets of weakly hybridizing fragments were detected in digests of *E. coli* DNA, using a purified 456 bp *citB* probe (C. Prodromou, unpublished observations). One corresponded to the *lac* region of the physical map (Kohara *et al.*, 1987), possibly because the probe was contaminated with fragments of *lac* DNA, and the other was traced to the *recC* and *ptrIII* genes at 61 min. These genes each contain a small sequence that is similar to part of the *citB* probe: ATCTGGCTTCACTGCTAATGCA in *recC* and AACCTtATATTCCTGaTGATtTC in *ptrIII* (Finch *et al.*, 1986a, b). Because the *citB* probe failed to detect the *acn* gene of *E. coli*, alternative strategies were adopted based on the purification of aconitase and the use of (i) oligonucleotide probes encoding N-terminal sequences and (ii) immunochemical probes.

This paper reports the purification of the *E. coli* aconitase and the successful isolation, identification and map location of the corresponding gene.

Methods

Bacteria, plasmids and bacteriophages. The strains of *E. coli* K12 were: W3110 (wild-type); DH1 (*thi-1 hsdR17 supE44 recA1 endA1 gyrA96 relA1*), the routine transformation host; C600 (*thr-1 leuB6 thi-1 lacY1 supE44 hsdR tonA21*) as the general host for λ phages; and NM621 (*leu pro thi supE hsdR mcrB⁺ A⁻ recD1009 ton tsx*), kindly provided by Dr M. Masters (University of Edinburgh) for propagating phages from the *E. coli* W3110 gene library (Kohara *et al.*, 1987). The *E. coli* C600 gene library was provided by Dr P. T. Emmerson (University of Newcastle-upon-Tyne): it contained partially *Sau3A*-digested bacterial DNA inserted into the *Bam*HI site of the λ PE11 replacement vector (Arthur *et al.*, 1982). A copy of the *E. coli* W3110 gene library (Kohara *et al.*, 1987) was provided by D. Buck (Schering, Saffron Walden, Essex). The λ EMBL4 derivatives, λ 13F9 and λ 18B6 [254 and 255 in the 'mini-set' of Kohara *et al.* (1987)], were used for subcloning the *acn* gene into the phagemid vector pUC119. A derivative of M13 (M13mp10-KJ9; Wilde *et al.*, 1986), which contains part of the *Bacillus subtilis* aconitase gene (*citB*), was the source of the *citB* probe, a 456 bp *Pst*I–*Eco*RI fragment encoding 152 amino acid residues close to the N-terminus of the enzyme.

DNA from *E. coli*, bacteriophages and phagemids was prepared and manipulated as described by Maniatis *et al.* (1982).

Purification of aconitase. Aconitase was purified from *E. coli* W3110 grown with vigorous aeration at 37 °C for 13 h using 1% (v/v) inocula (Tryptone Soya Broth, Oxoid) in 16 × 500 ml complex malate medium to obtain a high level of aconitase expression (Gray *et al.*, 1966). Subsequent steps were performed at 4 °C and a citrate-containing buffer (TC; Tris/citrate, 20 mM, pH 8.0) was used to protect the enzyme. The bacteria were harvested (14000 g for 15 min), washed and resuspended (1 g wet wt ml⁻¹) in TC buffer and then disrupted by three passes through a French pressure cell (20000 p.s.i.: 138 Pa). The extract was clarified by centrifugation at 20000 g for 30 min and 150000 g for 2 h.

The cell extract was fractionated with (NH₄)₂SO₄ at 35–50% saturation and the pellet fraction (15000 g for 30 min) solubilized in 47 ml TC buffer. A sample (23.5 ml) was mixed with an equal volume of 2 M-(NH₄)₂SO₄ and loaded at 0.5 ml min⁻¹ on a Phenyl Sepharose CL-4B column (80 × 2.6 cm) previously equilibrated with TC buffer containing 1 M-(NH₄)₂SO₄. The column was washed with 200 ml of 1 M-(NH₄)₂SO₄ in TC buffer and bound aconitase was eluted using an 800 ml linear gradient of 1.0–0 M-(NH₄)₂SO₄ in TC buffer at a flow-rate of 3 ml min⁻¹. The active fractions from two columns were concentrated by ultrafiltration in a stirred cell (Filtron, omegacell) with a 3 kDa nominal molecular mass limit. The concentrated sample (4.0 ml approx.) was fractionated by gel-filtration on a Sephacryl S-200 HR column (80 × 2.6 cm) equilibrated in MES/Mg buffer (10 mM-MES/KOH, pH 6.0, with 5 mM-MgCl₂) containing sodium citrate (10 mM, pH 6.0) and eluted at 0.5 ml min⁻¹ with the same buffer. Active fractions were pooled and concentrated approximately fivefold (to 8.5 ml) by ultrafiltration, as above. The sample was then diluted with 10 vols of MES/Mg buffer in order to reduce the concentration of residual citrate before applying to a Procion Red dye affinity column (26 × 1.6 cm). A weakly-active fraction failed to bind, and pure active enzyme was eluted (1–2 ml min⁻¹) with a linear gradient of 0–100 mM-citrate in MES/Mg buffer. Protein was assayed by the procedure of Bradford (1976).

Determination of *M_r*. *M_r* was determined by SDS-PAGE and gel filtration on the Sephacryl S-200 HR column (80 × 2.6 cm) equilibrated and eluted (0.5 ml min⁻¹) with TC buffer. The column was calibrated with standard proteins (*M_r*): β -amylase (200000); alcohol dehydrogenase (150000); carbonic anhydrase (29000); cytochrome *c* (12300). The standards for SDS-PAGE were (*M_r*): β -galactosidase (116000); fructose-6-phosphate kinase (84000); ovotransferrin (76000–78000); bovine serum albumin (66250); pyruvate kinase (64000); ovalbumin (48000 and 45000); lactate dehydrogenase (36400); triose-phosphate isomerase (29700); carbonic anhydrase (29000); myoglobin (17200); cytochrome *c* (12300).

Enzyme assays. Aconitase was assayed spectrophotometrically at 240 nm by following the formation of *cis*-aconitate from isocitrate, as described by Kennedy *et al.* (1983). An absorption coefficient of 3.6 mM⁻¹ was used, and 1 unit (U) of activity was equal to 1 μ mol of *cis*-aconitate formed min⁻¹ ml⁻¹. In reactivation studies, extracts, prepared in Tris/HCl (20 mM, pH 8) or TC buffers, were incubated at 4 °C with 5 mM-dithiothreitol and 0.5 mM-Fe(NH₄)₂(SO₄)₂, and assayed at 10 min intervals. An activity stain for aconitase in non-denaturing polyacrylamide gels was performed by coupling the conversion of *cis*-aconitate to isocitrate to a colorimetric test for isocitrate dehydrogenase (ICDH). Samples containing aconitase were fractionated in 8% (w/v) non-denaturing polyacrylamide gels. The test gel was immersed in 32 ml of the linked assay reagent containing: 10 U ICDH; 12 mg tetranitroblue tetrazolium; 15 mg NADP; 1 mg phenazine methosulphate; 2.5 mM-MgCl₂; 125 mM-Tris/HCl, pH 8.0; 12.5 mM-*cis*-aconitate; and 10% (w/v) gelatine. A control gel was

incubated without *cis*-aconitate, and another was incubated with isocitrate instead of *cis*-aconitate and with no ICDH, in order to stain for ICDH activity.

N-terminal amino acid sequence analysis and oligonucleotide probes. The N-terminus of aconitase was sequenced by automated Edman degradation (Laursen, 1971) at the SERC Amino Acid Sequencing Facility (Department of Biochemistry, University of Leeds). Two pairs of mixed oligonucleotide probes were designed to encode overlapping segments of the N-terminal sequence: S135 (AA[AG] GA[TC] AC[GTAC] TT[AG] CA[AG] GC) and S136 (AA[AG] GA[TC] AC[GTAC] CT[GTAC] CA[AG] GC) encode K-D-T-L-Q-A but use different sets of Leu codons; whereas S139 (GA[AG] GC[GTAC] TC[GTAC] AA[AG] GA[TC] AC) and S140 (GA[AG] GC[GTAC] AG[TC] AA[AG] GA[TC] AC) encode E-A-S-K-D-T using different Ser codons.

Southern blotting and DNA hybridization. Southern blotting was carried out according to Southern (1975) and λ plaques and colonies containing pGS447 and pGS448 were transferred and fixed to nitrocellulose as described by Maniatis *et al.* (1982). A transfer procedure which avoids depurination (Meinkoth & Wahl, 1984) was used with the oligonucleotide probes. The *citB* probe was labelled by incorporating digoxigenin-11-dUTP into a complementary strand of M13mp10-KJ9 by primer extension (1.2 pmol universal primer per μ g template) and the 456 bp *Pst*I-*Eco*RI fragment was released and then purified by electrophoresis in 0.5% agarose. Hybridizations were for 16 h at 55, 60 and 65 °C, the filters were washed twice at the hybridization temperature for 15 min with $2 \times$ SSC solution (17.53 g NaCl and 8.82 g sodium citrate in 1 litre, pH 7.0) and immunodetection was according to the instructions supplied with the Non-Radioactive Labelling Kit (Boehringer Mannheim). The 400 bp *Bam*HI-*Eco*RI fragment of λ G229 and the 3.2 kb *Bam*HI fragment of λ I3F9 were subcloned into M13mp18 (CP1 and CP2, respectively) and then labelled and excised to give probes BE1 and BB1 (respectively) as for the *citB* probe. Hybridization with these probes was for 4 h at 65 °C and the filters were washed with $2 \times$ SSC for 15 min at room temperature, and twice with $0.1 \times$ SSC for 15 min at 65 °C.

Oligonucleotides were labelled with $[\gamma\text{-}^{32}\text{P}]\text{dATP}$ using the procedure of Arrand (1985) and hybridization was detected by autoradiography. The hybridization temperatures were calculated as 5 °C below the predicted T_{diss} (Mason & Williams, 1985): 39 °C for S135; 41 °C for S136; and 41 °C for the combination of S139 plus S140. The filters were washed with $6 \times$ SSC for 15 min at 14 °C and then for 1 min at 44 (S135) or 46 °C (S136, and S139 plus S140).

Gel electrophoresis and immunological techniques. PAGE was performed as described by Laemmli (1970), using denaturing conditions (0.1% SDS in all buffers and 10 or 6% acrylamide) or non-denaturing conditions (no SDS and 8% acrylamide) and biotinylated size markers (M_r : phosphorylase b (97400); catalase (58100); alcohol dehydrogenase (39800); carbonic anhydrase (29000). For Western blotting, samples in SDS-polyacrylamide gels were transferred to nitrocellulose with a Bio-Rad Transblot Electrophoretic Transfer Cell according to the manufacturer's instructions.

The aconitase used for raising antiserum was excised from a polyacrylamide gel (SDS-PAGE, 6%) which had been lightly stained with 0.2% Coomassie blue R-250 in the absence of methanol and acetic acid. The gel slice was frozen in liquid N_2 , crushed to a fine powder, and mixed with an equal volume of Freund's complete adjuvant for injecting a rabbit. Assays for enzyme inactivation were performed by mixing 1–100 μ l serum with 2 units aconitase in a final volume of 1.0 ml 20 mM-Tris/HCl, pH 7.0. The mixtures were incubated for 30 min at 4 °C, centrifuged for 10 min at 15000 g, and the supernatant fluids assayed for aconitase activity.

For plaque hybridization, samples of the λ PE11 *E. coli* library were

plated on *E. coli* C600 (5×10^2 p.f.u. per plate). The plates were incubated at 37 °C for 5–6 h, overlaid with a nitrocellulose filter and incubated for a further 4–6 h, before fixing (Maniatis *et al.*, 1982). The procedure for immunological detection with nitrocellulose filters from Western blots and plaque lifts was performed at room temperature as described by Andrews *et al.* (1989). The dilutions of the primary antiserum and the biotinylated anti-rabbit antibody were 1:1000 and 1:500, respectively, while that of the streptavidin-biotinylated horseradish peroxidase complex was 1:3000.

Analysis of aconitase activity in λ -infected bacteria and phagemid transformants. Cultures of *E. coli* C600 were grown to an OD_{650} of 0.2 in 20 ml LB broth (Maniatis *et al.*, 1982) containing 0.2% maltose and 10 mM-MgSO₄. The cultures were concentrated 20-fold in 10 mM-MgSO₄ for phage adsorption (15 min at room temperature with an m.o.i. of 2) and diluted into 20 ml prewarmed LB broth containing 10 mM-MgSO₄. The infected bacteria were incubated at 37 °C with shaking, and samples (1.5 ml) were pelleted (15000 g for 2 min) and stored at 20 °C prior to analysis.

Phagemid transformants of *E. coli* DH1 were cultured by shaking at 37 °C in 50 ml LB broth containing ampicillin (200 μ g ml⁻¹) and sampled as described for the phage-infected bacteria. The pelleted bacteria were resuspended in 300 μ l TC buffer. Of this, 200 μ l was disrupted by ultrasonication (30 s, 10 μ m) and the clarified supernatant assayed for aconitase activity and protein. The remainder was stored at -20 °C for SDS-PAGE and Western blotting in which each lane contained the equivalent of 10 μ l of a suspension with an OD_{650} of 1.0. The results were reproducible within narrow limits for each type of experiment and typical sets are presented.

DNA-directed transcription-translation. Purified double-stranded pUC119 and pGS447 DNA was used for *in vitro* transcription-translation according to the instructions supplied with the Prokaryotic DNA-directed Translation Kit (Amersham).

Materials. Isocitric acid, *cis*-aconitic acid, tetranitroblue tetrazolium, phenazine methosulphate, isocitrate dehydrogenase, β -galactosidase and alcohol dehydrogenase were obtained from Sigma. Alkaline phosphatase, NADP, T4 DNA ligase, DNA polymerase (Klenow enzyme) and the Non-Radioactive Digoxigenin Labelling Kit were purchased from Boehringer Mannheim. Biotinylated anti-rabbit antibody, streptavidin-biotinylated horseradish peroxidase complex, the Prokaryotic DNA-Directed Translation Kit, L-[³⁵S]methionine and $[\gamma\text{-}^{32}\text{P}]\text{dATP}$, were obtained from Amersham. Molecular mass markers for SDS-PAGE were obtained from BDH, and the prestained SDS-PAGE-, gel-filtration and biotinylated-markers were from Sigma. Restriction enzymes were purchased from Northumbria Biologicals. Oligonucleotides were synthesized with an Applied Biosystems 381A DNA synthesizer. The Procion Red matrix was prepared as follows: 200 ml Procion Red H-3B (1%, w/v; ICI) was added to 200 ml Sepharose CL-6B and stirred occasionally for 30 min before adding 100 ml NaCl (20%, w/v) and 5 ml 5 M-NaOH after a further 30 min. The slurry was left for 3 d at room temperature and then washed sequentially with 1 M-NaCl and 6 M-urea to remove unreacted dye.

Results

Purification and characterization of aconitase

Studies with crude extracts prepared in the presence and absence of citrate showed that the former were 2.5-fold more active, and that the aconitase activities of the latter could be reactivated to the citrate-protected level by incubating with ferrous ions under reducing conditions.

Fig. 1. Purification and activity-staining of the *E. coli* aconitase. (a) Coomassie-stained SDS-PAGE (10% gel) showing samples of active fractions at different stages of purification. Lanes: 1, molecular mass standards; 2, cell-free extract; 3, ammonium sulphate precipitate; 4, Phenyl Sepharose; 5, Sephacryl; 6, Procion Red (unbound); 7, Procion Red (bound). (b) Activity-stained non-denaturing PAGE (8% gel) of a sample at the Sephacryl-purified stage. The protein in the active band migrated at M_r 97 500 when excised and analysed by SDS-PAGE, as indicated.

Table 1. Purification of aconitase from *E. coli* W3110

Purification step	Total activity (U)*	Total protein (mg)	Specific activity (U mg ⁻¹)	Yield (%)	Purification (-fold)
Cell extract	2700	8380	0.32	100	1.00
(NH ₄) ₂ SO ₄ ppt.	2110	4130	0.51	78	1.59
Phenyl Sepharose CL-4B	102	74.3	1.37	3.8	4.28
Sephacryl S-200 HR	20.4	15.0	1.36	0.76	4.25
Procion Red (unbound)	0.6	0.46	1.30	0.02	4.06
Procion Red (bound)	16.2	0.66	24.5†	0.60	76.6

* Units are $\mu\text{mol cis-aconitate formed min}^{-1}$.

† Represents a minimum value due to enzyme instability.

This implied that the *E. coli* enzyme is an iron-sulphur protein resembling the aconitases from other sources. A procedure was devised for purifying *E. coli* aconitase to homogeneity under citrate-protected conditions (see Methods, Table 1 and Fig. 1). The purification was aided by the use of an activity stain which correlated the

activity in non-denaturing gel slices with a polypeptide(s) of M_r 97 500 in SDS-PAGE. Major activity losses occurred during Phenyl Sepharose chromatography and gel filtration, and the overall yield was only 0.6% despite the use of substrate protection. The final product had a minimum specific activity of 24.5 U (mg protein)⁻¹,

Fig. 2. Restriction maps for the segments of bacterial DNA cloned in putative *lacn* phages and phagemid derivatives. (a) The maps are aligned on the common 400 bp *Bam*HI–*Eco*RI fragment (BE1). Those for λG229–232 are incomplete because the *Bgl*I (G), *Eco*RV (V) and *Pst*I (P) sites were only identified in Southern blots with the BE1 probe. The maps for relevant parts of λ13F9 and λ18B6 (total inserts 17.8 and 17.7 kb, respectively; Kohara *et al.*, 1987) are revised in the region of the BE1 probe. The arrows point towards the right arm of the λ vector. The regions common to the λPE11 and λEMBL4 derivatives are indicated by thick lines and the vertical dashed lines. The predicted size and polarity of the aconitase coding region are indicated by hatching within the deduced limits for its location (dashed box). (b) Derivatives of pUC119 containing fragments of λ13F9 and λ18B6 are shown with hatched regions denoting the approximate positions of the aconitase coding region and arrows showing the polarity of the *lac* promoter. B, *Bam*HI; E, *Eco*RI.

which corresponds to a purification of at least 76-fold. Interestingly, most of the active enzyme was bound by the dye affinity matrix whereas an apparently inactive fraction was not bound (Fig. 1). After continued use, the Phenyl Sepharose column gave a non-symmetrical activity-profile, and the enzyme at the leading edge (up to 30% of total activity) had to be discarded, otherwise the final fractions were contaminated with components of M_r 21 500 (unbound) and 41 500 (bound).

M_r estimates obtained by gel filtration (95 000) and SDS-PAGE (97 500) indicated that aconitase is a monomeric protein. As shown below, the N-terminal amino acid sequence of the purified enzyme exhibited some degree of similarity with *B. subtilis* aconitase (Dingman & Sonenshein, 1987) (– denotes an unidentified residue):

E. coli: – STLREAS KDTLQA– D– T

: * : * * * *

B. subtilis: ANEQKTA AKDVFQARKTF

Studies with oligonucleotide probes

Knowledge of the N-terminal amino acid sequence of the *E. coli* aconitase allowed the design of two pairs of mixed

oligonucleotides for use in locating the *acn* gene in the physical map of the *E. coli* chromosome (see Methods). One probe (S135) hybridized to the following set of *E. coli* DNA fragments (kb): *Bam*HI, 3.3; *Bgl*I, 5.8; *Eco*RI, 5.2; *Eco*RV, 1.3; *Hind*III, 0.7; *Kpn*I, none detected; *Pst*I, 10.0 and 1.7; *Pvu*II 10.0 and 1.5. Very little hybridization was detected with an iso-encoding probe (S136), possibly because it lacks the specific Leu codon of the *acn* sequence. Unfortunately, the corresponding set of overlapping fragments could not be found in the physical map of the bacterial chromosome (Kohara *et al.*, 1987). Some of the same fragments (italicized above) were amongst those detected by the related probes (S139 and S140) but even this limited set could not be found in the map. If it is assumed that the N-terminal sequence is correct, these results suggest that the probes are insufficiently specific for the *acn* gene or that there are inaccuracies in the *acn* region of the physical map.

Immunoscreening and map location of the *acn* gene

The antiserum used for screening the λPE11-*E. coli* library gave a strong and reasonably specific reaction with aconitase in Western blots of cell extracts

Fig. 3. Physical map in the 28 min region of the *E. coli* chromosome according to Kohara *et al.* (1987). The sizes (kb) of fragments hybridizing with the BE1 probe (A) are compared with possible counterparts (B), hatched in the map. The fragments in double digests detected with the same probe are also shown. Some of the fragments (C) hybridizing with an oligonucleotide probe (S135) are likewise compared with predicted counterparts (D), stippled in the map. Limits for the deduced positions of the probes and the positions of specified genes (Kroger *et al.*, 1990) are indicated above the map. The predicted location of an extra 0.8 kb of DNA in the physical map is indicated by vertical arrows and the asterisk (*). B, *Bam*HI; Bg, *Bgl*I; E, *Eco*RI; V, *Eco*RV; H, *Hind*III; K, *Kpn*I; P, *Pst*I; Pv, *Pvu*II.

fractionated by SDS-PAGE, although it appeared not to inactivate or precipitate the enzyme under the conditions used (data not shown). In comparable blots, the antiserum cross-reacted with porcine aconitase and with a polypeptide of M_r 120000 (approx.) in extracts of *Salmonella typhimurium*. In plaque hybridizations some 40 out of 7000 plaques were immunopositive and restriction analyses with 16 purified lines showed that 10 contain a common 400 bp *Bam*HI-*Eco*RI fragment. Based on their restriction maps, these phages fell into four classes, which are represented by λ G229-232 in Fig. 2. Five additional classes, which lack the 400 bp *Bam*HI-*Eco*RI fragment and are represented by λ G233-237 (not shown), were also detected. This diversity of classes suggests that some considerable rearrangement of the *Sau*3A partially-digested bacterial DNA fragments had occurred in constructing the λ PE11 library. The 400 bp *Bam*HI-*Eco*RI fragment was subcloned from λ G229 to M13mp18 for preparing the hybridization probe, BE1. This was used to confirm that each of the four classes of phages contained the identical fragment and to define its chromosomal location.

Southern blotting with BE1 identified a set of *E. coli* DNA fragments which could best derive from the 28 min

(1350 kb) region of the chromosomal map (Fig. 3). However, there were some significant discrepancies between the observed and predicted sizes, including the size (1.0 kb) predicted for the 0.4 kb probe. Nevertheless, the proposed location was supported by the hybridization patterns obtained with double digests of *E. coli* DNA (Fig. 3) and by tests with specific clones from the 'mini-set' of λ phages containing the entire *E. coli* genome (Kohara *et al.*, 1987). Plaque hybridizations and Southern blots for λ 4F1, λ 13F9, λ 18B6, λ E14F6 and λ 1C2 confirmed predictions that λ 13F9 and λ 18B6 should hybridize with the BE1 probe, and restriction analyses confirmed that the λ 13F9 and λ 18B6 inserts overlap the hybridizing regions cloned in the λ PE11 derivatives (Fig. 2). The discrepancy between the observed sizes of hybridizing fragments and the physical map ranged from 0.6 to 1.4 kb and, excluding experimental inaccuracies, it would seem that there is approximately 0.8 kb of extra DNA in the physical map relative to both the chromosomal and λ -cloned DNA (Fig. 3). When this discrepancy is taken into account, several of the fragments detected with one of the oligonucleotide probes could be placed, as shown in Fig. 3.

Fig. 4. Aconitase activities of λ *acn*-infected and phagemid-containing *E. coli*. (a) The aconitase specific activities, mU (mg protein)⁻¹, for *E. coli* C600 cultures infected with representative λ PE11 and λ EMBL4 derivatives (m.o.i. of 2) are shown below the corresponding growth curves; ▽, uninfected; ●, λ G229; ○, λ G232; ▲, λ 13F9; △, λ 18B6. The results obtained for λ G230–231 were similar to those for λ G229, and those for λ G233–237 to λ G232. (b) The aconitase specific activities, U (mg protein)⁻¹, of transformants of *E. coli* DH1 grown in LB broth plus ampicillin (200 μ g ml⁻¹) are shown below the growth curves; ▽, untransformed; ◆, pUC119 (vector); ◇, pUC445; ■, pGS447; and □ pGS448. A representative set of typical and reproducible results is shown for each type of experiment.

Biochemical characterization of putative *λacn* phages

Evidence from enzymological and immunochemical studies with infected bacteria showed that four of the six types of phages containing the 400 bp *Bam*HI–*Eco*RI fragment (Fig. 2) possess an active *acn* gene. Aconitase activity was increased significantly and reproducibly after infection with λ G229–231 and λ 13F9 (represented by λ G229 and λ 13F9 in Fig. 4a) but not with λ G232–237 or λ 18B6. There were corresponding amplifications of immunopositive products co-migrating with aconitase (M_r 97 500) from the active phages (Fig. 5). In addition, several cross-reacting fusion or deletion products were amplified with some of the inactive phages, e.g. λ G232, M_r 112 000 and 75 000; λ G233–234, M_r 67 500; λ G235, M_r 79 000; λ G236, M_r 72 000; λ G237, M_r 46 000 (Fig. 5).

These results indicate that the 400 bp *Bam*HI–*Eco*RI fragment encodes part of aconitase, and that some of the immunopositive phages contain a functional *acn* gene despite the considerable rearrangement of bacterial DNA in the λ PE11 library. Also, based on the calculated

size of the *acn* coding region and the degree of similarity between the restriction maps of λ G230–232, λ 13F9 and λ 18B6, it is possible to define the approximate position of the *acn* gene in the 28 min region of the *E. coli* linkage map (Fig. 2). Furthermore, the polarity of the gene is defined by the relative positions of the binding sites for the hybridization probes, S135 (N-terminal) and BE1 (internal). It is not known whether λ G233–237 are immunopositive because they express part of the *acn* coding region, or some other product which cross-reacts with one of the antibodies used for immunodetection.

Studies with a pUC119 derivative containing the *acn* gene

Because the least corrupted DNA sources appeared to be cloned in λ 13F9 and λ 18B6, which contain overlapping inserts from the *acn* region, a fragment from each was combined in pUC119 in an attempt to reconstruct a functional *acn* gene. The strategy involved cloning the 1.08 kb *Bam*HI–*Pst*I fragment of λ 18B6 in pUC119 to generate pGS445, and then inserting the 3.2 kb *Bam*HI

Fig. 5. Western blots of *E. coli* infected with putative *lacn* phages. Total cell protein in samples harvested after 40 min infection (see Fig. 4a) was fractionated by SDS-PAGE (10% gel), blotted and immunostained. The presence of an amplified immunopositive product corresponding to aconitase (ACN), M_r 97 500, (+), and the possession of the 400 bp *Bam*HI-*Eco*RI fragment (O), are indicated. Lanes: ACN, purified aconitase; U₀ and U₄₀, uninfected bacteria at 0 and 40 min, respectively; 1–11, bacteria infected with λ G233 (1), λ G231 (2) λ G229 (3), λ G234 (4), λ G232 (5), λ G235 (6), λ G230 (7), λ G236 (8), λ G237 (9), λ 13F9 (10) and λ 18B6 (11).

fragment of λ 13F9 into the unique *Bam*HI site of pGS445 (Fig. 2). Phagemids containing the larger fragment in two orientations were recovered, pGS447 (desired orientation) and pGS448 (reverse orientation). The 3.2 kb *Bam*HI fragment was cloned in M13mp18 to provide a probe (BB1) for screening transformants during the construction of pGS447 and pGS448.

The presence of a functional *acn* gene in pGS447 was confirmed by *in vitro* transcription-translation tests, enzyme analysis and immunochemical studies. In an *in vitro* expression system pGS447 directed the synthesis of a polypeptide of the size expected for the *acn* gene product (M_r 97 500), a minor product of unknown origin (M_r 71 000) and β -lactamase (M_r 30 000) (data not shown). The aconitase specific activities were reproducibly 20–200-fold greater for *E. coli* DH1(pGS447) than for transformants containing pUC119, pGS445 or pGS448, at different stages in the growth cycle (Fig. 4b). Parallel SDS-PAGE analyses and Western blotting confirmed the dramatic enrichment of aconitase protein and cross-reacting material in the pGS447 transformant (Fig. 6). However, quantitative densitometry of Coomassie-stained gels indicated that the amount of aconitase protein in DH1(pGS447) remained relatively constant at 16–18% of total cell protein, throughout the short-lived, but reproducible, fourfold increase in specific activity in late exponential phase (Fig. 4b). With C600(pGS447) the specific activity increased somewhat earlier and more gradually to a similar maximum before declining, again

with little change in aconitase protein (3.7–4.6% of total). It would thus appear that a significant proportion of the aconitase can be in an inactive form and that a post-translational activation-inactivation mechanism might be operating. It is also apparent that pGS448 expresses an enzymologically-inactive cross-reactive polypeptide of M_r 77 000 (Fig. 6) and, in keeping with the predicted location and polarity of the *acn* gene, this could correspond to an aconitase polypeptide which lacks a substantial C-terminal segment. The extra cross-reactive components observed in some of the DH1 transformants (Fig. 6) are presumably degradation products of aconitase which become apparent due to the high degree of aconitase amplification (pGS447), or to the instability of the truncated form (pGS448).

Discussion

The results describe the purification of aconitase from *E. coli*, the first cloning of a functional prokaryotic aconitase gene (*acn*), and the location and polarity of the *acn* gene at 28 min in the *E. coli* linkage map.

The aconitase of *E. coli* resembles those of other organisms in being monomeric and it is immunologically related to the porcine and *Salmonella typhimurium* enzymes. However, its size (M_r 97 500) is significantly larger than the mitochondrial enzymes of porcine heart,

Fig. 6. SDS-PAGE analysis and Western blotting of total cell proteins of strains transformed with pUC119 derivatives. (a) Coomassie-stained SDS-PAGE (10% gel) of transformants of *E. coli* DH1 sampled at 7.5 h (see Fig. 4b). The positions of biotinylated molecular mass markers are indicated, as are the positions of aconitase (ACN) and a truncated derivative (arrows). (b) Immunostained Western blot of samples fractionated as in (a). Lanes: 1, untransformed host; 2–5, specific transformants with phagemids pUC119 (2), pGS445 (3), pGS448 (4) and pGS447 (5).

bovine heart and *S. cerevisiae* (M_r 79000–83000), and smaller than the *B. subtilis* and *Sal. typhimurium* enzymes (M_r 120000 approx.) The N-terminal sequences of the *acn* and *citB* products are sufficiently similar to suggest that these proteins are homologous. If this is substantiated, the *E. coli* enzyme will join the family of mammalian, yeast and *Bacillus* enzymes (Zheng *et al.*, 1990; Gangloff *et al.*, 1990; Dingman & Sonenshein, 1987). In the case of fumarase, *E. coli* contains two classes of enzyme, of which only one is related to the *Bacillus* and mitochondrial enzymes. It will therefore be interesting to discover whether an analogous situation exists for aconitase. Despite the similarities in N-terminal sequence, the corresponding segment of the *citB* structural gene failed to detect the *acn* gene of *E. coli* when used as a heterologous probe. Weak hybridization to the *recC* and *ptrIII* genes was detected, and to the *lac* region (presumably because the probe was contaminated with *lac* DNA). However, the reason why aconitase is amplified up to 18-fold upon induction of several

putative *lacn* prophages, which were later shown to contain inserts from the *lac* region, remains obscure (Wilde *et al.*, 1986; Wilde, 1988).

Citrate was used to protect aconitase during purification, but 99.4% of the activity was still lost. In the final step the active and inactive enzymes were resolved because the latter failed to bind to the Procion Red matrix. Presumably, the poor recoveries of active and inactive enzyme were due to the inherent instability of the enzyme and to the removal of the inactive form at each stage.

The successful cloning strategy involved raising antibodies to purified aconitase for the immunodetection of *lacn* phages, and the *acn* gene was located at 28 min (1350 kb) in the physical map using a fragment of the gene as a hybridization probe. As a result, all of the citric acid cycle genes of *E. coli* have now been cloned and located in the chromosome. Of these, the *acn* gene is closest but not adjacent to the isocitrate dehydrogenase gene (*icd*) at 25 min (Kroger *et al.*, 1990; Bachmann,

1990). There seem to be several reasons why the mixed oligonucleotide probes encoding N-terminal segments of aconitase (e.g. S135) failed to locate the *acn* gene in the physical map. First, the relevant *Hind*III and *Kpn*I fragments were probably too large for efficient transfer to nitrocellulose (Fig. 3). Second, some additional and complicating fragments were detected due to the degeneracy of the probes. Third, the physical map of the *E. coli* chromosome is inaccurate at the critical position, possibly because there is only a small overlap between the phages which cover this region (λ 13F9 and λ 18B6; Kohara *et al.*, 1987; see Fig. 2).

The biochemical and immunochemical studies indicated that three derivatives of λ PE11 and one of λ EMBL4, each containing a common 400 bp *Bam*HI–*Eco*RI fragment, express aconitase and an immunopositive polypeptide of M_r 97 500. Because most of the λ PE11 derivatives seemed to contain non-contiguous fragments from the *E. coli* chromosome, the *acn* gene was reconstructed from fragments of the λ EMBL4 derivatives, λ 13F9 and λ 18B6. Convenient restriction fragments from both phages were recombined in pGS447 (Fig. 2) even though it seemed likely that λ 13F9 possessed an active intact *acn* gene. In transformants expressing the reconstructed *acn* gene from pGS447 the aconitase specific activity was amplified approximately 200-fold to 150 U (mg protein)⁻¹ and aconitase protein increased to 4–6 or 18% of total cell protein, depending on the strain. There appeared to be disproportionate changes in activity relative to protein content during the course of the growth cycle, as if a post-translational activation–inactivation mechanism may be operating. This could reflect the intracellular redox-state and iron-availability at the corresponding stages in the growth cycle. Nevertheless, the potential for enzyme amplification with pGS447 should lead to greatly improved yields of purified enzyme as well as facilitating studies on the incorporation of iron and other enzymological properties. Current work is focussed on deriving the nucleotide sequence of the *acn* gene and investigating mutants constructed by replacing the chromosomal gene with an *in vitro*-disrupted derivative.

We are very grateful to D. Buck, Y. Kohara, P. T. Emmerson and M. Masters for supplying phages and bacterial strains, S. C. Andrews for assistance with immunological methods, W. W. Kay for helpful advice, and J. B. C. Findlay and J. Keen for deriving the N-terminal amino acid sequence of aconitase. The work was supported by the Leverhulme Trust and the Science and Engineering Research Council, and we are also indebted to Dr W. J. E. Gardner and Haarmann & Reimer (Selby) for their participation in a SERC-CASE studentship (M. J. H.).

References

ANDREWS, S. C., HARRISON, P. M. & GUEST, J. R. (1989). Cloning, sequencing, and mapping of the bacterioferritin gene (*bfr*) of *Escherichia coli* K-12. *Journal of Bacteriology* **171**, 3940–3947.

ARRAND, J. E. (1985). Preparation of nucleic acid probes. In *Nucleic Acid Hybridization, a Practical Approach*, pp. 17–45. Edited by B. D. Hames & S. J. Higgins. Oxford: IRL Press.

ARTHUR, H. M., BRAMHILL, D., EASTLAKE, P. B. & EMMERSON, P. T. (1982). Cloning of the *uvrD* gene of *E. coli* and identification of the product. *Gene* **19**, 285–295.

BACHMANN, B. J. (1990). Linkage map of *Escherichia coli* K-12, edition 8. *Microbiological Reviews* **54**, 130–197.

BELL, P. J., ANDREWS, S. C., SIVAK, M. N. & GUEST, J. R. (1989). Nucleotide sequence of the *fnr*-regulated fumarase gene (*fumB*) of *Escherichia coli* K-12. *Journal of Bacteriology* **171**, 3494–3503.

BRADFORD, M. M. (1976). A rapid and sensitive method for the quantitative determination of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* **72**, 248–254.

DINGMAN, D. W. & SONENSHEIN, A. L. (1987). Purification of aconitase from *Bacillus subtilis* and correlation of its N-terminal amino acid sequence with the *citB* gene. *Journal of Bacteriology* **169**, 3062–3067.

EMPTAGE, M. H., DREYER, J.-L., KENNEDY, M. C. & BEINERT, H. (1983). Optical and EPR characterization of different species of active and inactive aconitase. *Journal of Biological Chemistry* **258**, 11106–11111.

FINCH, P. W., WILSON, R. E., BROWN, K., HICKSON, I. D. & EMMERSON, P. T. (1986a). Complete nucleotide sequence of the *Escherichia coli ptr* gene encoding protease III. *Nucleic Acids Research* **19**, 7695–7703.

FINCH, P. W., WILSON, R. E., BROWN, K., HICKSON, I. D., TOMKINSON, A. E. & EMMERSON, P. T. (1986b). Complete nucleotide sequence of the *Escherichia coli recC* gene and the *thyA*–*recC* intergenic region. *Nucleic Acids Research* **14**, 4437–4451.

FLINT, D. H., EMPTAGE, M. H. & GUEST, J. R. (1989). Fumarase A from *E. coli* contains a [4Fe–4S] cluster. *Journal of Inorganic Biochemistry* **36**, 306.

GANGLOFF, S. P., MARGUET, D. & LAUQUIN, G. J.-M. (1990). Molecular cloning of the yeast mitochondrial aconitase gene (*ACO1*) and evidence of a synergistic regulation of expression by glucose plus glutamate. *Molecular and Cellular Biology* **10**, 3551–3561.

GRAY, C. T., WIMPENNY, J. W. T. & MOSSMAN, M. R. (1966). Regulation of metabolism in facultative bacteria. II. Effects of aerobiosis and nutrition on the formation of Krebs cycle enzymes in *Escherichia coli*. *Biochimica et Biophysica Acta* **117**, 33–41.

KENNEDY, M. C. & BEINERT, H. (1983). The state of cluster SH and S²⁻ of aconitase during cluster interconversions and removal. *Journal of Biological Chemistry* **263**, 8194–8198.

KENNEDY, M. C., EMPTAGE, M. H., DREYER, J.-L. & BEINERT, H. (1983). The role of iron in the activation–inactivation of aconitase. *Journal of Biological Chemistry* **258**, 11098–11105.

KENNEDY, M. C., WERST, M., TELSER, J., EMPTAGE, M. H., BEINERT, H. & HOFFMAN, B. M. (1987). Mode of substrate carboxyl binding to the [4Fe–4S]⁺ cluster of reduced aconitase as studied by ¹⁷O and ¹³C electron-nuclear double resonance spectroscopy. *Proceedings of the National Academy of Sciences of the United States of America* **84**, 8854–8858.

KOHARA, Y., AKIYAMA, K. & ISONO, K. (1987). The physical map of the whole *E. coli* chromosome: application of a new strategy for rapid analysis and sorting of a large genomic library. *Cell* **50**, 495–508.

KROGER, M., WAHL, R. & RICE, P. (1990). Compilation of DNA sequences of *Escherichia coli* (update 1990). *Nucleic Acids Research* **18**, 2549–2587.

LAEMMLI, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature, London* **227**, 680–685.

LAURSEN, R. A. (1971). Solid phase Edman degradation: an automated peptide sequencer. *European Journal of Biochemistry* **20**, 89–102.

MANIATIS, T., FRITSCH, E. F. & SAMBROOK, J. (1982). *Molecular Cloning, a Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

MASON, P. J. & WILLIAMS, J. G. (1985). Hybridization analysis of DNA. In *Nucleic Acid Hybridization, a Practical Approach*, pp. 113–137. Edited by B. D. Hames & S. J. Higgins. Oxford: IRL Press.

MEINKOTH, J. & WAHL, G. (1984). Hybridization of nucleic acids immobilized on solid supports. *Analytical Biochemistry* **138**, 267–284.

- MILES, J. M. & GUEST, J. R. (1987). Molecular genetic aspects of the citric acid cycle of *Escherichia coli*. *Biochemical Society Symposia* **54**, 45–65.
- ROBBINS, A. H. & STOUT, C. D. (1989*a*). The structure of aconitase. *Proteins* **5**, 289–312.
- ROBBINS, A. H. & STOUT, C. D. (1989*b*). Structure of activated aconitase: Formation of the [4Fe–4S] cluster in the crystal. *Proceedings of the National Academy of Sciences of the United States of America* **86**, 3639–3643.
- ROSENKRANTZ, M. S., DINGMAN, D. W. & SONENSHEIN, A. L. (1985). *Bacillus subtilis citB* gene is regulated synergistically by glucose and glutamine. *Journal of Bacteriology* **164**, 155–164.
- RYDEN, L., OFVERSTEDT, L., BEINERT, H., EMPTAGE, H. & KENNEDY, M. C. (1984). Molecular weight of beef heart aconitase and stoichiometry of the components of its iron-sulfur cluster. *Journal of Biological Chemistry* **259**, 3141–3144.
- SCHOLZE, H. (1983). Studies on aconitase species from *Saccharomyces cerevisiae*, porcine and bovine heart, obtained by a modified isolation method. *Biochimica et Biophysica Acta* **746**, 133–137.
- SOUTHERN, E. (1975). Detection of specific sequences among DNA fragments separated by gel electrophoresis. *Journal of Molecular Biology* **98**, 503–517.
- SUZUKI, T., YAMAZAKI, O., NARA, K., AKIYAMA, S.-I., NAKAO, Y. & FUKUDA, H. (1975). The aconitase of yeast: II. crystallization and general properties of yeast aconitase. *Journal of Biochemistry* **77**, 367–372.
- VILLAFRANCA, J. J. & MILDVAN, A. S. (1971). The mechanism of aconitase action. *Journal of Biological Chemistry* **246**, 772–779.
- WERST, M. M., KENNEDY, M. C., BIENERT, H. & HOFFMAN, B. M. (1990*a*). ^{17}O , ^1H , and ^2H electron double resonance characterization of solvent, substrate, and inhibitor binding to the [4Fe–4S] $^{+}$ cluster of aconitase. *Biochemistry* **29**, 10526–10532.
- WERST, M. M., KENNEDY, M. C., BIENERT, H., HOUSEMAN, A. L. P. & HOFFMAN, B. M. (1990*b*). Characterization of the [4Fe–4S] cluster at the active site of aconitase by ^{57}Fe , ^{33}S , and ^{14}N electron double resonance spectroscopy. *Biochemistry* **29**, 10533–10540.
- WILDE, R. J. (1988). PhD Thesis, University of Sheffield.
- WILDE, R. J., JEYASEELAN, K. & GUEST, J. R. (1986). Cloning of the aconitase gene (*acn*) of *Escherichia coli*. *Journal of General Microbiology* **132**, 1763–1766.
- WOODS, S. A., SCHWARTZBACH, S. D. & GUEST, J. R. (1988). Two biochemically distinct classes of fumarases in *Escherichia coli*. *Biochimica et Biophysica Acta* **954**, 14–26.
- ZHENG, L., ANDREWS, M. A., HERMODSON, M. A., DIXON, J. E. & ZALKIN, H. (1990). Cloning and structural characterization of porcine heart aconitase. *Journal of Biological Chemistry* **265**, 2814–2821.