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

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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*, 95000 or 97500 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 *k*- *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*, 120000) 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*, of about 79000–83000: 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*, 68500 (Suzuki et al., 1975), and the *Bacillus subtilis* enzyme is considerably larger, *M*, 120000 (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 Feₐ), has been shown to coordinate with the middle carboxyl group, and possibly the hydroxyl group, of the substrate (Kennedy et al., 1987; Wester et al., 1990a, b). Exposure to oxygen during purification results in the loss of the Feₐ 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]⁺⁺ cluster (Emp-tage 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 lac phages were isolated and characterized by their ability to amplify aconitase 5-8-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 lac probe: ATCTGGCTTCACTGCTAATGCA in recC and AACCTTATATCCTGATTCGTC 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 (thr-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 sup E hsdR recB + A recD 099 tonA 21), 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 BamHI site of the pJ 11 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 λEMBL4 derivatives, λJ3F9 and λJ8B6 [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-K39; Wilde et al., 1986), which contains part of the Bacillus subtilis aconitase gene (citB), was the source of the citB probe, a 456 bp Psrl-EcoRI 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 x 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 passages 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-NH42SO4 and loaded at 0·5 ml min-1 on a Phenyl Sepharose CL-4B column (80 x 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 1.0-0·5 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 Sephacyr S-200 HR column (80 x 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 x 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, M, was determined by SDS-PAGE and gel filtration on the Sephacyr S-200 HR column (80 x 2·6 cm) equilibrated and eluted (0·5 ml min-1) with TC buffer. The column was calibrated with standard proteins (M,): β-amylose (200000); alcohol dehydrogenase (150000); carbonic anhydrase (29000); cytochrome c (12300). The standards for SDS-PAGE were (M,): 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 μmol of cis-aconitate formed min-1 ml-1. In reaction 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 tetrathionate tetroxalum; 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

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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.


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 (Meinloth & 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 µg template) and the 456 bp PstI-EcoRI 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 × SSC solution (17.5 g NaCl and 8.2 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 BamHI–EcoRI fragment of AG229 and the 3.2 kb BamHI fragment of J13F9 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 twice with 2 × SSC for 15 min at room temperature, and twice with 0.1 × SSC for 15 min at 65 °C.

Oligonucleotides were labelled with [32P]dATP using the procedure of Arrand (1985) and hybridization was detected by autoradiography. The hybridization temperatures were calculated as 5 °C below the predicted Tm (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 × 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 (Ml): 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 antisera 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 N2, 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 µl serum with 2 units aconitate in a final volume of 1 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 λPE11 E. coli library were plated on E. coli C600 (5 × 108 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 OD600 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 phase 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 µg ml−1) and sampled as described for the phage-infected bacteria. The pelleted bacteria were resuspended in 300 µl TC buffer. Of this, 200 µl was disrupted by ultrasonication (30 s, 10 µm) 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 µl of a suspension with an OD600 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, tetranoitribole tetraxolium, 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, [32P]dCTP and [32P]dATP, were obtained from Amersham. Molecular mass markers for 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 P1ocion Red matrix was prepared as follows: 200 ml Pocion Red H-3B (1%, v/v: ICI) was added to 200 ml Sepharose CL-6B and stirred occasionally for 30 min before adding 100 ml NaCl (20%, v/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, Sephacyr; 6, Procion Red (unbound); 7, Procion Red (bound). (b) Activity-stained non-denaturing PAGE (8% gel) of a sample at the Sephacyr-purified stage. The protein in the active band migrated at $M_r$, 97500 when excised and analysed by SDS-PAGE, as indicated.

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

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Total activity (U)*</th>
<th>Total protein (mg)</th>
<th>Specific activity (U mg$^{-1}$)</th>
<th>Yield (%)</th>
<th>Purification (-fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell extract</td>
<td>2700</td>
<td>8380</td>
<td>0.32</td>
<td>100</td>
<td>1.00</td>
</tr>
<tr>
<td>(NH$_4$)$_2$SO$_4$ ppt.</td>
<td>2110</td>
<td>4130</td>
<td>0.51</td>
<td>78</td>
<td>1.59</td>
</tr>
<tr>
<td>Phenyl Sepharose CL-4B</td>
<td>102</td>
<td>74.3</td>
<td>1.37</td>
<td>3.8</td>
<td>4.28</td>
</tr>
<tr>
<td>Sephacyr S-200 HR</td>
<td>20.4</td>
<td>15.0</td>
<td>1.36</td>
<td>0.76</td>
<td>4.25</td>
</tr>
<tr>
<td>Procion Red (unbound)</td>
<td>0.6</td>
<td>0.46</td>
<td>0.30</td>
<td>0.02</td>
<td>4.06</td>
</tr>
<tr>
<td>Procion Red (bound)</td>
<td>16.2</td>
<td>0.66</td>
<td>24.5†</td>
<td>0.60</td>
<td>76.6</td>
</tr>
</tbody>
</table>

* Units are μmol 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 $M_r$, 97500 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 lac phages and phagemid derivatives. (a) The maps are aligned on the common 400 bp BamHI–EcoRI fragment (BE1). Those for λG229–232 are incomplete because the BglI (G), EcoRV (V) and PstI (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, BamHI; E, EcoRI.

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 (95000) and SDS-PAGE (97500) 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):

\[
\begin{align*}
E.~coli: & \quad -\text{STLREAS KDTLQA- D-T} \\
B.~subtilis: & \quad \text{ANEQKTAADVFQARKTF}
\end{align*}
\]

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): BamHI, 3.3; BglI, 5.8; EcoRI, 5.2; EcoRV, 1.3; HindIII, 0.7; KpnI, none detected; PstI, 10.0 and 1.7; PvuII 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
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 $M_t$ 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 BamHI–EcoRI fragment. Based on their restriction maps, these phages fell into four classes, which are represented by $\lambda$G229-232 in Fig. 2. Five additional classes, which lack the 400 bp BamHI–EcoRI fragment and are represented by $\lambda$G233-237 (not shown), were also detected. This diversity of classes suggests that some considerable rearrangement of the Sau3A partially-digested bacterial DNA fragments had occurred in constructing the $\lambda$PE11 library. The 400 bp BamHI–EcoRI fragment was subcloned from $\lambda$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 $\lambda$ phages containing the entire E. coli genome (Kohara et al., 1987). Plaque hybridizations and Southern blots for $\lambda$4F1, $\lambda$13F9, $\lambda$18B6, $\lambda$E14F6 and $\lambda$1C2 confirmed predictions that $\lambda$13F9 and $\lambda$18B6 should hybridize with the BE1 probe, and restriction analyses confirmed that the $\lambda$13F9 and $\lambda$18B6 inserts overlap the hybridizing regions cloned in the $\lambda$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 $\lambda$-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. 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 (*).
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 BamHI–EcoRI fragment (Fig. 2) possess an active acn gene. Aconitase activity was increased significantly and reproducibly after infection with λG229-231 and λ13F9 (represented by λG229 and λ13F9 in Fig. 4a) but not with λG232-237 or λ18B6. There were corresponding amplifications of immunopositive products co-migrating with aconitase (M, 97500) 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. λG232, M, 112000 and 75000; λG233–234, M, 67500; λG235, M, 79000; λG236, M, 72000; λG237, M, 46000 (Fig. 5).

These results indicate that the 400 bp BamHI–EcoRI 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 λPE11 library. Also, based on the calculated size of the acn coding region and the degree of similarity between the restriction maps of λG230-232, λ13F9 and λ18B6, 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 λG233–237 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 λ13F9 and λ18B6, 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 BamHI–PstI fragment of λ18B6 in pUC119 to generate pGS445, and then inserting the 3.2 kb BamHI
fragment of \( \lambda 13F9 \) into the unique \( BamHI \) 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 \( BamHI \) 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 \textit{in vitro} transcription–translation tests, enzyme analysis and immunochromatographic studies. In an \textit{in vitro} expression system pGS447 directed the synthesis of a polypeptide of the size expected for the \( acn \) gene product (M, 97,500), a minor product of unknown origin (M, 71,000) and \( \beta \)-lactamase (M, 30,000) (data not shown). The aconitase specific activities were reproducibly 20–200-fold greater for \textit{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, 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 \textit{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 \textit{E. coli} linkage map.

The aconitase of \textit{E. coli} resembles those of other organisms in being monomeric and it is immunologically related to the porcine and \textit{Salmonella typhimurium} enzymes. However, its size (M, 97,500) is significantly larger than the mitochondrial enzymes of porcine heart,
bovine heart and S. cerevisiae ($M$, 79000–83000), and smaller than the B. subtilis and Sal. typhimurium enzymes ($M$, 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 & Sonenschein, 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 λacn 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 λacn 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,
oligonucleotide probes encoding N-terminal segments of physical map. First, the relevant HindIII and KpnI 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 (A13F9 and A18B6; Kohara et al., 1987; see Fig. 2).

The biochemical and immunochemical studies indicated that three derivatives of λPE11 and one of λEMBL4, each containing a common 400 bp BamHI–EcoRI fragment, express aconitase and an immunopositive polypeptide of M, 97500. Because most of the λPE11 derivatives seemed to contain non-contiguous fragments from the E. coli chromosome, the acn gene was reconstructed from fragments of the λEMBL4 derivatives, λ13F9 and λ18B6. Convenient restriction fragments from both phages were recombined in pGS447 (Fig. 2) even though it seemed likely that λ13F9 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 focused on deriving the nucleotide sequence of the acn gene and investigating mutants constructed by replacing the chromosomal gene with an in vitro-disrupted derivative.

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