Citrate synthase of *Escherichia coli*

Characterisation of the enzyme from a plasmid-cloned gene and amplification of the intracellular levels

Martin S. Robinson, Richard A. Easom, Michael J. Danson and P.D.J. Weitzman*

Department of Biochemistry, University of Bath, Bath BA2 7AY, England

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Guest [J. Gen. Microbiol. (1981) 124, 17–23] has cloned the citrate synthase gene from *E. coli* into ColE1 plasmids. This plasmid-coded enzyme has now been purified and characterised and shown to be indistinguishable from the chromosome-coded enzyme in molecular, catalytic and regulatory properties. The plasmid copy number has been raised to > 200/cell by treatment with chloramphenicol and the effects of this increase on the intracellular level of citrate synthase have been investigated.

Citrate synthase Escherichia coli *Plasmid*

1. INTRODUCTION

Citrate synthase catalyses the introduction of acetyl-CoA into the citric acid cycle and may be an important site of control of this pathway [1,2]. The enzyme from *Escherichia coli* is allosterically inhibited by NADH [3,4] and by α-oxoglutarate [5,6] which are primary end-products of the cycle under aerobic and anaerobic growth conditions, respectively. It is a hexameric protein of identical subunits [7] and contrasts markedly with the dimeric enzymes from Gram-positive bacteria and eukaryotes, all of which are insensitive to the above allosteric effectors [1,2,8,9].

ColE1–*E. coli* hybrid plasmids containing the citrate synthase gene have been identified [10]. Amplification of such plasmid copy numbers in *E. coli* should lead to greatly increased intracellular levels of citrate synthase, facilitating the isolation of the enzyme for detailed structural studies. However, we have shown [1,11] that only minor genetic alterations to the chromosomal citrate synthase gene of *E. coli* can lead to marked changes in the oligomeric and regulatory nature of the enzyme. It was therefore essential to examine the enzyme from the cloned gene to ascertain its retention of the properties of the normal enzyme. In addition, we have increased the plasmid copy number of one *E. coli* strain by taking advantage of the continued replication of ColE1 plasmids when chromosomal replication is stopped by protein synthesis inhibitors. The effects of this increase on intracellular levels of citrate synthase are reported.

* To whom correspondence should be addressed

2. EXPERIMENTAL

2.1. *Materials*

All chemicals used were analytical grade. The strain of *E. coli* used was JA200 (F^+ *thr leu lacY1 trpΔE5 recA1*), kindly provided by Professor J.R. Guest (University of Sheffield). It contained several copies of a synthetic ColE1–*E. coli* hybrid plasmid designated pLC 26-17 incorporating the *E. coli* citrate synthase gene [10].

2.2. *Methods*

The plasmid copy number of *E. coli* JA200 was increased by treatment of the cells with chloram-

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phenicol according to [12]. Cells were grown aerobically at 37°C in L-broth [13] and chloramphenicol was added to the culture in log phase (A_{650} = 0.4) to 25 μg/ml final conc. The culture was shaken for 12 h after which the cells were twice washed free of chloramphenicol and resuspended in fresh medium to the same cell density as that before the addition of the antibiotic. The cells were then grown aerobically at 37°C and harvested after one generation. The total DNA content of *E. coli* JA200, before and after treatment with chloramphenicol, was prepared as in [14]. Separation of the ColE1 DNA from the chromosomal DNA was achieved by vertical gel electrophoresis on 1% agarose gel [15]. DNA was stained with ethidium bromide and the gel was photographed under UV light.

Citrate synthase was assayed spectrophotometrically at 412 nm [16]. The enzyme was purified from *E. coli* JA200 before plasmid amplification. Nucleic acid was removed from a French-press extract with protamine sulphate [17] and the enzyme further purified by ion-exchange chromatography on DEAE–Sephacel using a linear (0.1–0.5 M) KCl gradient. Purification was completed by chromatography on a column of Matrex Gel Red A (Amicon) with a 0–0.5 M NaCl gradient.

SDS–polyacrylamide gel electrophoresis and gel filtration in 6 M guanidine–HCl on Sephacryl S-300 were done as in [9]. Sedimentation velocity analysis was carried out in a Beckman L5-50B analytical ultracentrifuge and determination of Stokes' radii by zonal gel filtration on Sephadex G-200 was performed as in [9]. Cross-linking of the enzyme with glutaraldehyde was according to [9].

3. RESULTS

3.1. *Purification of citrate synthase*

The purification procedure is summarized in table 1. The specific activity of the enzyme in the French-press extract was ~7-fold greater than that found in the *E. coli* strain lacking the plasmid, as in [10]. This increase in the initial specific activity permitted the purification of the enzyme in high yield after only 3 stages (table 1).

3.2. *Molecular and kinetic properties of citrate synthase*

In molecular and kinetic properties the citrate synthase purified from the plasmid-containing strain JA200 was indistinguishable from that obtained from a non-plasmid organism.

In agreement with [7], the citrate synthase was shown to be a hexameric enzyme of which the constituent polypeptides are all of the same molecular size. Thus the enzyme sedimented as a single protein species with sedimentation coefficient ($s°_{20,w}$) of 11.5 (± 0.3) S. Similarly on zonal gel filtration one protein peak was observed and by comparison of its elution volume with those of standard proteins of known molecular size (see [9] for the calibration curve) a Stokes' radius of 5.6 (± 0.1) × 10^{-7} cm was calculated. Taking the partial specific volume (0.73 ml/g) from the amino acid composition, M_r 269000 (± 6000) was determined.

Table 1

Purification of *E. coli* citrate synthase

Step	Vol. (ml)	Total enzyme (units)	Total protein (mg)	Spec. act. (units/mg)	Yield (%)
1. French-press extract	58	5544	3793	1.5	–
2. Supernatant from protamine sulphate	58	5288	3686	1.4	95
3. Pooled, selected fractions from DEAE–Sephacel column	50	5000	270	18.5	90
4. Pooled, selected fractions from red gel A column	8	2003	32	62.6	36

A unit of enzymic activity is defined as that producing 1 μmol CoASH/min

One protein band was found on SDS-gel electrophoresis and on gel filtration in guanidine–HCl; polypeptide chain M_r-values of 44000 (± 2500) and 49000 (± 2000) were estimated by these two methods. These values, taken with that of the native citrate synthase, give 5.8 (± 0.4) polypeptides/enzyme molecule. This hexameric structure was confirmed by SDS gel electrophoresis of the enzyme after cross-linking with glutaraldehyde; 6 protein bands were observed. No more than 6 bands were generated even after extensive modification.

The citrate synthase showed a sigmoidal dependence of velocity on both acetyl-CoA and oxaloacetate concentrations as reported for the enzyme from a non-plasmid strain [18]. Similarly, enzymic activity was inhibited by NADH and α-oxoglutarate in a manner indistinguishable from that observed with the chromosome-coded citrate synthase (fig.1) and was insensitive to ATP.

3.3. *Amplification of plasmid copy number*

The continued replication of ColE1 plasmids in the presence of chloramphenicol [12] was used to increase the plasmid copy number of *E. coli* JA200. DNA was isolated and electrophoresed as in section 2. Photographic negatives of the gels stained with ethidium bromide were scanned in a recording densitometer. It was found that the absorbance ratio of plasmid to chromosomal DNA after chloramphenicol treatment was ~30-times greater than that from untreated cells grown under otherwise identical conditions. Assuming that JA200 originally contained 7–10 copies of the plasmid/cell ([10] and section 3.1 above) the chloramphenicol treatment appears to have increased this to 200–300 copies.

3.4. *Intracellular levels of citrate synthase*

Surprisingly, the specific activity of citrate synthase in cells with the plasmid copy number amplified by chloramphenicol treatment was only 4 units/mg protein, a rise of 2.7-fold over cells with just 7–10 copies of plasmid. Incubation of cells in different concentrations of chloramphenicol (25–200 μg/ml) did not improve on this specific activity. Resuspension of treated cells in amino acid-supplemented media also had no effect, although the citrate synthase activity was increased by 30% in both acetate and acetate/aspartate minimal media whereas it was decreased by 25% in glucose minimal medium. When L-broth was supplemented with 1% glucose the specific activity of the enzyme decreased to 0.1 units/mg protein, a value < 10% of the original JA200 strain.

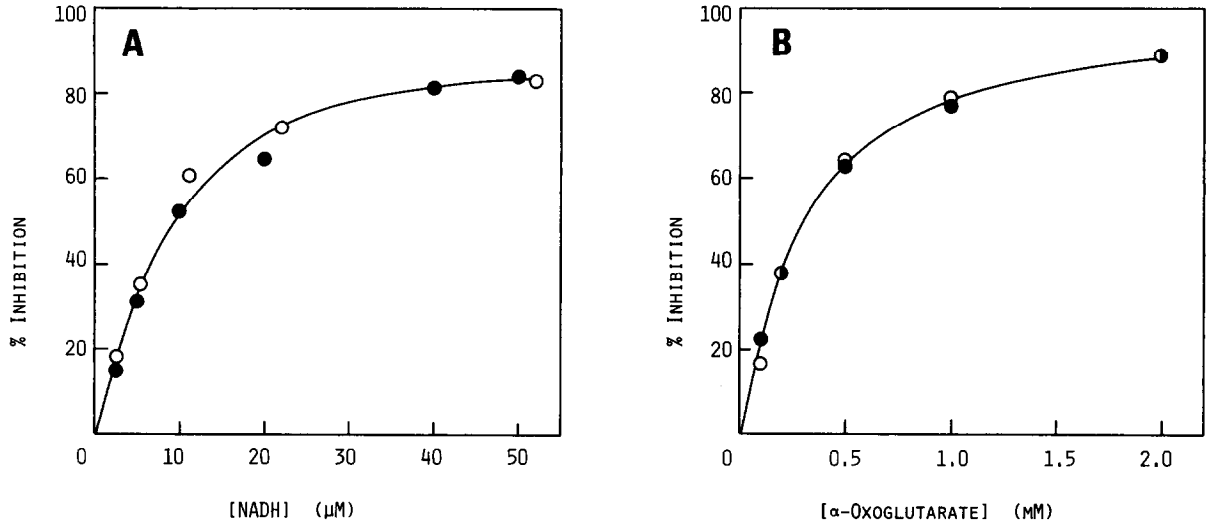

Fig.1. Inhibition of plasmid-coded (○) and chromosome-coded (●) *E. coli* citrate synthase activity by NADH (A) and α-oxoglutarate (B). Citrate synthase was assayed spectrophotometrically at 412 nm [16] using 0.15 mM acetyl-CoA and 0.05 mM oxaloacetate.

4. DISCUSSION

This study aimed to characterise the product of the plasmid-cloned gene *E. coli* citrate synthase [10] and to increase the plasmid copy number to facilitate purification of large amounts of the enzyme.

We have demonstrated that the cloning [10] has been achieved without any apparent modification of the gene, the enzyme produced being indistinguishable from the chromosome-coded enzyme in molecular, kinetic and regulatory properties. Additionally, we have achieved considerable amplification of the cloned gene dosage (up to 200–300 copies/cell) though this was not matched by a corresponding increase in the intracellular levels of enzyme. The maximum specific activity achieved (~5 units/mg protein) is 25-times the level found in non-plasmid strains. Intracellular levels of other enzymes (e.g., NADH dehydrogenase [12]) cloned into plasmids have been amplified to considerably greater extents than that found here for citrate synthase, suggesting that in the present study the capacity for transcription or translation is not a limitation. Rather it is likely that the cell can still regulate its level of the cloned citrate synthase. Support for this is gained from growth on glucose after plasmid amplification when synthesis of citrate synthase was reduced to levels approaching that of the non-plasmid organism.

It is unclear why the amplification of citrate synthase is not as great as the apparent increase in plasmid copy number. It may be that some physiological constraint operates, perhaps to avoid reduction of the concentrations of the substrates, acetyl-CoA and oxaloacetate, to unacceptable levels. Nevertheless, the amplification of citrate synthase currently achieved has permitted a high yield of the pure enzyme to be obtained with relative ease. The demonstration that the plasmid-coded and normal citrate synthases are indistinguishable justifies the use of the cloned gene for structure–function studies on this key regulatory enzyme.

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