SELECTION FOR CITRATE SYNTHASE–DEFICIENT MUTANTS WITH FLUOROACETATE

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1. Introduction

Studies on citrate synthases from a wide range of bacteria have revealed various inhibition and activation effects which may be correlated with the metabolic capabilities of the different organisms [1,2]. To complement investigations of naturally-occurring citrate synthase variants we have begun to study artificially-generated mutant forms of the enzyme [3,5]. The strategy was first to generate citrate synthase-deficient (CS^-) mutants and then, by reversion, to regain the enzymic activity. If the revertant enzymes had an altered amino acid sequence, compared with that of the original enzyme, then changes in molecular and regulatory properties might result.

In the course of this work a new procedure for the isolation of CS^- mutants was devised. This relies on the lethal effect of fluoroacetate which is believed to be exerted after its conversion, via citrate synthase, to fluorocitrate [6]. Mutants devoid of citrate synthase cannot produce fluorocitrate and may, as a result, be resistant to the toxic effect of fluoroacetate.

2. Experimental

Four strains of *Escherichia coli* K12 (Kl.1, K2.1, AB 259 and AB 1621) and three CS^- mutants derived therefrom (Kl.1.4, K2.1.4 and AB 1623) were kindly provided by Professor Sir Hans Kornberg, University of Cambridge. A CS^- mutant of AB 259 was isolated as below. *Acinetobacter calcoaceticus*, strain 4B, was isolated by P. D. J. W. from water. Methods of growth in liquid or on solid media were as in [5].

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Mutagenesis was carried out with ethylmethane sulphonate as in [7]. In the case of *E. coli* AB 259 penicillin enrichment was performed by the double-cycle procedure [8]. A mutated culture was first grown in minimal medium containing 10 mM succinate and 1 mM glutamate, washed and transferred to medium containing 10 mM succinate only and treated with ampicillin (20 μg/ml) for 1 h at 37°C. The cells were then transferred and allowed to grow in succinate–glutamate medium and the procedure repeated. The resulting culture was serially diluted and plated onto nutrient-agar plates. Plates with ~100 colonies were replicated onto succinate and succinate–glutamate plates and mutants selected which grew on the latter but not on succinate alone. These were grown in liquid culture and cell-free extracts were tested for citrate synthase activity. By this procedure, a CS^- mutant of *E. coli* AB 259, designated AB 259-CS1, was isolated.

Selection for resistance to fluoroacetate was performed as follows. A culture of *Acinetobacter calcoaceticus* was mutated as above, transferred to 100 ml minimal medium containing 10 mM glutamate and 1 mM fluoroacetate and grown to stationary phase. The culture was then diluted and plated onto glutamate–fluoroacetate plates at ~100 cells/plate and incubated at 30°C for 3 days.

Citrate synthase was assayed spectrophotometrically at 412 nm and 25°C as in [9].

3. Results and discussion

The extreme toxicity of fluoroacetate is believed to depend on its metabolic conversion to fluorocitrate by the same reactions as would metabolise acetate itself. Activation to fluoroacetyl-CoA may occur

either by the action of acetate thiokinase or by the joint action of acetate kinase and phosphotransacetylase. The fluoroacetyl-CoA may then act as a substrate for citrate synthase, generating fluorocitrate which, in many organisms, is a powerful inhibitor of aconitase and thus blocks the citric acid cycle [6].

On this basis organisms devoid of citrate synthase, and hence unable to produce fluorocitrate, might be immune to the toxic effect of fluoroacetate. Citrate synthase deficiency would also result in the inability to synthesise α-oxoglutarate and this might be compensated for by providing glutamate in the growth medium. This anticipated resistance to fluoroacetate was first tested using several CS^- mutants of *E. coli* and their wild-type parental strains. When spread on agar plates containing 10 mM succinate, 1 mM glutamate and 1 mM fluoroacetate, the wild-type strains of *E. coli* (Kl.1, K2.1, AB 259 and AB 1621) failed completely to grow. However, the CS^- mutants derived from these strains (Kl.1.4, K2.1.4, AB 259-CS1 and AB 1623) all grew on this medium thereby demonstrating their resistance to fluoroacetate. We therefore tried the direct method of fluoroacetate resistance to obtain CS^- mutants of *Acinetobacter calcoaceticus,* whose insensitivity to penicillin made the mutagenesis/penicillin enrichment procedure ineffective.

The procedure in section 2 was employed and a large number of mutants were obtained which grew on the glutamate–fluoroacetate medium. From these, mutants were selected for their ability to grow on glutamate but their inability to grow on either acetate or succinate, and analysis of cell-free extracts showed that several had virtually zero levels of citrate synthase. Consistent with the absence of this enzyme was a growth requirement for glutamate, α-oxoglutarate or proline. We have used such CS^- mutants to obtain revertant CS^+ strains either spontaneously or after mutagenesis [10] or by transformation [4]. The method of fluoroacetate resistance therefore does succeed in selecting CS^- mutants and should, in principle, be applicable to a range of other organisms (both prokaryotic and eukaryotic) and should prove a useful additional approach complementing methods which have been applied by other investigators.

Bacterial mutants devoid of citrate synthase have been isolated and studied by other workers. CS^- mutants were isolated from *E. coli* W and *Aerobacter aerogenes* after mutation by UV irradiation followed by penicillin enrichment and selection for glutamate auxotrophs [11] and a CS^- mutant of *E. coli* K12 was isolated by mutagenesis with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine followed by selection for glutamate auxotrophs [12]. A mutant of *Pseudomonas aeruginosa* largely deficient in citrate synthase was isolated by mutagenesis and selection for inability to grow on acetate [13]. A range of citric acid cycle mutants of *Bacillus subtilis* was isolated by heating spores, then plating them out on nutrient-agar containing calcium carbonate [14]. Mutants lacking enzymes of the citric acid cycle accumulated organic acids and this led to the formation of halos around mutant colonies, resulting from solubilization of the calcium carbonate. Among the mutants so isolated were some deficient in citrate synthase. In [15], CS^- mutants of *E. coli* K12 were isolated as double mutants also lacking isocitrate dehydrogenase. Mutants of *E. coli* lacking isocitrate dehydrogenase grew very slowly on medium containing glucose plus glutamate but were overgrown by other mutants found, additionally, to lack citrate synthase [15]. It was suggested that isocitrate dehydrogenase-deficient mutants produce a build-up of citrate or isocitrate which is inhibitory to growth; the CS^- mutants cannot produce such an accumulation and thereby avoid growth inhibition [15].

Resistance to fluoroacetate has been used [16,17] to isolate mutants of *E. coli* lacking acetate kinase or phosphotransacetylase. Resistance to fluoroacetate and fluoroacetamide has also been used [18,19] to obtain mutants of *Aspergillus nidulans,* while resistance to fluoroacetamide has been used [20]to isolate mutants of *Pseudomonas aeruginosa* defective in the structural or regulator genes for amidase.

To our knowledge there have been no published reports on the use of fluoroacetate to isolate CS^- mutants. Following our report on mutant citrate synthases from *A. calcoaceticus* [4], which referred to the isolation of CS^- strains by selection for fluoroacetate resistance, we learned that J. T. Beatty and H. Gest (personal communication) have employed a similar procedure to isolate CS^- mutants from *Rhodopseudomonas capsulata.*

In applying this procedure to other organisms it must be borne in mind that an immunity to the effect of fluoroacetate may be conferred by a variety of mechanisms. Mutations other than in citrate synthase may prevent the generation of fluorocitrate, and there may also be an inability to take up fluoroacetate into the cell. In other cases citrate synthase may not utilise fluoroacetyl-CoA as effectively as acetyl-

CoA [21] or the aconitase may be relatively insensitive to fluorocitrate inhibition [22,23]. Another possibility is that the organism may be able to hydrolyse fluoroacetate to glycollate (e.g. Pseudomonads) and hence escape its toxicity. Nevertheless it is likely that a considerable number of organisms will yield CS^- mutants by the fluoroacetate selection procedure.

Finally, although the effect of fluorocitrate has been ascribed to its inhibition of aconitase, the suppression of growth may not be due simply to blocking of the citric acid cycle at this step, since the presence of glutamate in the growth medium (e.g., with wild-type *E. coli*) did not overcome the toxic effect of fluoroacetate. It may be that the inhibition of aconitase leads to an accumulation of citrate and/or fluorocitrate, and that these latter compounds inhibit other cellular functions [24,25].

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