Occurrence of two distinct citrate synthases in a mutant of *Pseudomonas aeruginosa* and their growth-dependent variation

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Two distinct citrate synthases have been found in a mutant of *Pseudomonas aeruginosa* – a 'large' form (M_r ~250000) and a 'small' form (M_r ~100000). In common with the citrate synthase of wild-type *Ps. aeruginosa* and other aerobic Gram-negative bacteria, the 'large' enzyme is regulated by both NADH and AMP, whereas the 'small' enzyme is insensitive to these effectors. The relative proportions of the two citrate synthases vary with the stage of growth of the bacterial culture. The 'large' form predominates in logarithmic phase, whereas the 'small' form is the major component in stationary phase. The two forms do not appear to be interconvertible by a simple dissociation–association mechanism.

Citrate synthase Pseudomonas aeruginosa *Growth dependence* *Multiple enzyme form*

1. INTRODUCTION

Studies on the citric acid cycle enzyme citrate synthase (citrate oxaloacetate-lyase (CoA-acetylating), EC 4.1.3.7) from a wide range of organisms have demonstrated a clear correlation between the molecular and regulatory properties of the enzyme and the taxonomic grouping of the source organism (review [1]). Two types of citrate synthase (CS) have been identified on the basis of molecular size – a 'large' form (M_r ~250000) which occurs exclusively in Gram-negative bacteria and a 'small' form (M_r ~100000) which is found in Gram-positive bacteria and in eukaryotes. Only one type of CS has hitherto been found to occur in any particular organism.

We have isolated the mutant CSs, having altered structural and regulatory properties [2–4], employing a two-step strategy: a first mutation was used to convert 'wild-type' bacteria to CS-deficient strains; a second mutation resulted in the production of revertants which had regained CS activity. It was amongst such revertants that variants of CS were encountered. In an attempt to extend this approach to species of *Pseudomonas* we examined a mutant of *Ps. aeruginosa* reported in [5] to have very low CS activity (~7% of that of the wild-type). It was anticipated that such a mutant might serve as the CS-deficient starting strain for the second mutation to revertants with modified enzyme. However, the low level of CS in the mutant was found to be different from the wild-type pseudomonad CS by being of the 'small' type and not displaying the regulatory sensitivities towards NADH and AMP shown by the wild-type enzyme. Further investigations on this mutant revealed the additional presence of a 'large' form of CS as well as a marked variation in the relative proportions of the two forms depending on the stage of growth of the bacterial culture. These novel findings are the subject of the present communication.

2. EXPERIMENTAL

The organism used was mutant PAC 514 of *Pseudomonas aeruginosa* 8602 and was kindly provided by Professor Patricia H. Clarke (University College, London); it was previously designated as mutant At 14 [5]. Cells were grown in nutrient broth supplemented with 10 mM glutamate at

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37°C in shaking flasks; growth was monitored by measurement of the turbidity at 680 nm of an appropriately diluted sample. The cells were harvested by centrifugation and disrupted by ultrasonication, and the supernatant solutions obtained after centrifugation were used without further treatment.

Unless otherwise stated, gel filtration was performed at 4°C on a column (1.5 × 25 cm) of Sephadex G-200 equilibrated with 20 mM Tris–HCl (pH 7.0) containing 1 mM EDTA and 0.1 M KCl. Extract (1 ml), to which 10 μl (10 μg) of lactate dehydrogenase (rabbit muscle; Boehringer) had been added, was applied to the column; elution was performed with the buffer used for equilibration and 1 ml fractions were collected.

Citrate synthase and lactate dehydrogenase were assayed spectrophotometrically as in [6]; protein was estimated as in [7].

3. RESULTS AND DISCUSSION

Lactate dehydrogenase (M_r 140000) serves as a convenient 'marker' protein for the identification of 'large' and 'small' CSs by gel filtration. 'Large' CS is eluted ahead of lactate dehydrogenase whereas the 'small' enzyme is eluted after the marker [6].

Examination of an overnight culture of this *Ps. aeruginosa* mutant by gel filtration indicated the presence of a 'small' form of CS (section 1). This enzyme showed no sensitivity towards NADH or AMP in contrast to the 'large' CS of wild-type *Ps. aeruginosa* which is inhibited by NADH and re-activated by AMP [1]. In the course of attempting to purify this mutant CS a large-scale preparation (10 l culture) was undertaken and gel filtration was carried out on a larger column of Sephadex G-200 (2.5 × 35 cm). This experiment clearly revealed the presence of a low level of the 'large' form of CS, though the bulk of the total CS activity appeared as the 'small' form. When fractions containing the 'large' enzyme were tested for their response to effectors, inhibition by NADH and re-activation by AMP were both exhibited. In addition, however, AMP was found to stimulate activity (~2-fold at 0.5 mM AMP) in the complete absence of NADH, a feature not encountered with wild-type *Ps. aeruginosa* CS. This unexpected finding of two apparently distinct forms of CS within one organism prompted an examination of the nature of the enzyme in cells harvested at an early stage in the growth of a culture. When an extract from cells harvested in early logarithmic phase was examined by gel filtration a quite different situation was found; unlike the stationary-phase culture, the CS was predominantly of the 'large' form, only a minor portion of the activity appearing as the 'small' form.

Further experiments were undertaken to investigate and compare the molecular forms of CS

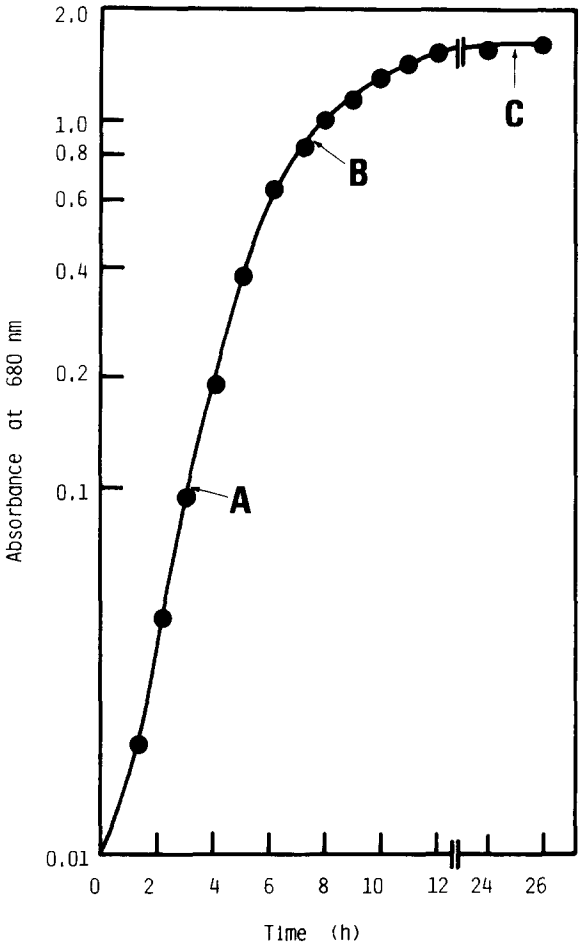

Fig.1. Growth curve of *Ps. aeruginosa* mutant PAC 514. Growth was at 37°C in nutrient broth supplemented with 10 mM glutamate: 5 ml of an overnight (16 h) culture were transferred to 1 l fresh medium and growth was monitored turbidimetrically at 680 nm; (A–C) indicate the 3 points of harvest of the culture at early-logarithmic, late-logarithmic and stationary phases, respectively.

obtained from early logarithmic, late logarithmic and stationary phase cultures of the *Ps. aeruginosa* mutant. Fig.1 shows a growth curve of the mutant and indicates these 3 points of harvest of the culture (at absorbance values of 0.1, 0.92 and 1.7). Gel filtration was carried out on a smaller column (see section 2) to reduce the dilution of enzyme activity which accompanied use of the larger column; this was particularly important when extracts were made from cells harvested at early growth phase and the yield of cells (and hence of enzyme) was extremely low.

Fig.2 shows the pattern of elution of CS activity from cells harvested at late logarithmic phase. Two peaks of activity are apparent, but clearer resolution of the two CSs was achieved by taking advantage of the sensitivity of the 'large' enzyme only to activation by AMP. Thus fig.2 also shows the elution profile when assays were conducted in the presence of 0.5 mM AMP and, by comparison of the two profiles, a third curve representing the 'AMP activation ratio' may be computed. The latter indicates that the 'large' CS is stimulated 2.2-fold (i.e., 120% increase in activity) whereas the 'small' enzyme is totally unaffected. It is thus possible to calculate the proportions of the two forms of CS across the entire profile, and this is illustrated in fig.3B. We have named the enzymes according to the order of their elution – CSI, the first peak of 'large' enzyme and CSII, the second peak of 'small' enzyme. Fig.3A and 3C illustrate the elution profiles, computed by the use of the AMP activation ratio method, of CSI and CSII obtained with cells harvested in early logarithmic and stationary phases of growth. Fig.3 illustrates dramatically the growth-dependent variation in the

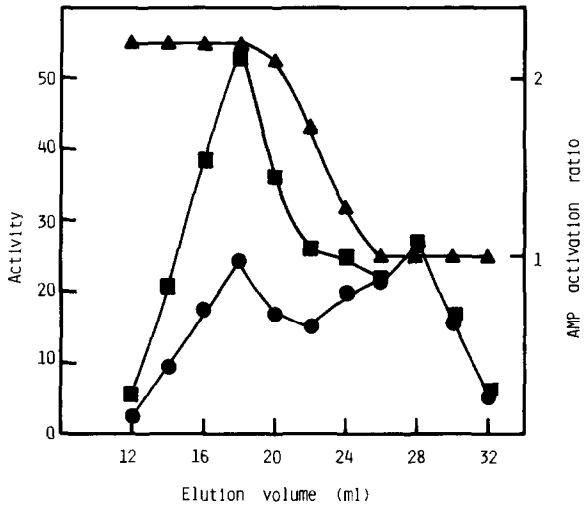

Fig.2. Separation of two forms of citrate synthase by gel filtration. Experimental details were as in the text. Activities are in arbitrary units: (●) enzyme alone; (■) enzyme with 0.5 mM AMP; (▲) AMP activation ratio (activity with AMP : activity without AMP).

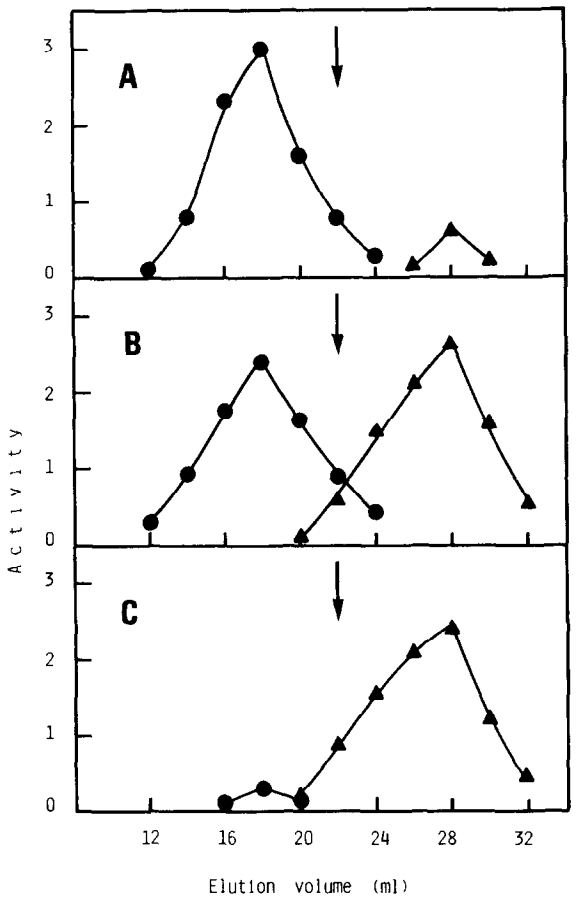

Fig.3. Growth-dependent variation of CSI and CSII. Extracts were prepared from cells harvested at early-logarithmic phase (A), late-logarithmic phase (B) and stationary phase (C) and fractionated on a column of Sephadex G-200 in the presence of lactate dehydrogenase as marker (section 2); (⟶) position of the marker peak. The citrate synthase profile was resolved into distinct peaks of CSI (●) and CSII (▲) by calculation from the AMP activation ratio (see text and fig.2). Activities are in arbitrary units and for convenience of presentation are not the same in A, B and C.

relative proportions of CSI and CSII and represents quite novel findings.

It is conceivable that the two forms of CS represent an association–dissociation system though it is hard to see why this should give rise to different proportions at different points of harvest. Further evidence against a facile interconversion of the two forms was gained from subjecting the separated CSI and CSII to a second gel filtration. Each form behaved as a single stable species and no re-equilibration to a mixture was observed. Furthermore, changes in the procedure for making a cell-free extract (sonication for different times, use of the French press) did not alter the ratios of CSI to CSII.

The occurrence of both 'large' and 'small' CS in this mutant, when hitherto we had encountered only one or the other form in any particular organism, inevitably raised the possibility of contamination of this *Ps. aeruginosa* mutant with a Gram-positive bacterium; the latter might then be the source of the 'small' CSII. However, we guarded against this possibility by working on repeatedly isolated colonies, by checking for the absence of Gram-positive organisms at all stages of growth, and by relying on the assistance of an independent investigator (Dr Dorothy Jones, Leicester University) to confirm the purity of our cultures. In addition it is important to note that when a sample of stationary-phase culture is used to inoculate fresh medium, the new culture passes through the same cycle of events with respect to the relative proportions of CSI and CSII.

In [8,9], two forms of CS were reported in extracts of a marine pseudomonad. The 'large' enzyme was inhibited by NADH and activated by AMP, and the 'small' enzyme insensitive to both these effectors [8,9]. However, dissociation of the 'large' to the 'small' CS was brought about by dialysis against 20 mM phosphate (pH 7) and the reverse association by removal of the phosphate by further dialysis [8,9]. It was not possible to conclude whether these two forms exist in vivo and no growth-dependent variation of the kind reported here was observed.

Here, we have attempted to interconvert CSI and CSII by dissociation and association according to [9] but no such interconversion was observed. These results suggest a measure of independence of the two forms and we suggest that the growth-dependent variation in the two forms is strong evidence for the in vivo existence of two distinct CS enzymes in this organism. If the two CSs exist in vivo it is conceivable that CSII is formed by the breakdown of CSI as the culture passes from logarithmic to stationary phase. However, examination of the specific activities of the two forms shows that this is unlikely to be the case. Thus the specific activity (μmol.min^{-1}.mg protein^{-1}) of CSI was 0.023 in late logarithmic phase and 0.024 in stationary phase; i.e., it remained constant. On the other hand, the specific activity of CSII increased over the same period of growth from 0.024–0.375.

Work is in progress to explore the regulatory mechanisms governing the production of the two citrate synthases in this bacterium, the physiological significance of the existence of the two enzymes and the incidence of this novel system amongst other organisms.

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