Purification and characterization of citrate synthase isoenzymes from *Pseudomonas aeruginosa*

Colin G. MITCHELL,*†‡ Sean C. K. ANDERSON* and E. M. T. EL-MANSI*

* Biomedicine and Biotechnology Research Group, Department of Biological Sciences, Napier University, 10 Colinton Road, Edinburgh EH10 5DT, U.K., and † Department of Biochemistry, University of Bath, Claverton Down, Bath BA2 7AY, U.K.

Two types of citrate synthase (CS) have been purified from *Pseudomonas aeruginosa*, a 'large' form (CSI) and a 'small' form (CSII). The M_rs of the CSI and CSII isoenzymes were determined to be 240000±16000 (mean±S.E.M.) and 80300±3800 respectively. Chemical cross-linking of the native enzymes with either dimethyl suberimidate or glutaraldehyde followed by electrophoretic analysis by SDS/PAGE showed that CSI is a hexamer and CSII is a dimer. SDS/PAGE showed that CSI and CSII each consist of a single subunit type, of M_r 42000±2000 and M_r 36500±2000 respectively. CSI and CSII were also shown to be distinct kinetically, immunologically and in terms of their regulatory properties. It is suggested that the CS isoenzymes are products of different structural genes.

INTRODUCTION

The Krebs cycle enzyme citrate synthase (CS) (EC 4.1.3.7) catalyses the reaction:

$$\text{Acetyl-CoA} + \text{oxaloacetate} + H_2O \rightarrow \text{citrate} + \text{CoA} + H^+$$

A complex diversity relating to this enzyme has been shown to exist in living organisms regarding the nature and molecular interactions of its subunits [1–3]. Gram-negative bacteria contain a hexameric 'large' enzyme (M_r 240000) which is subject to allosteric control by NADH. Eukaryotes and Gram-positive bacteria contain a dimeric 'small' enzyme (M_r 100000) which is subject to isosteric control by ATP. Recently, a strain of *Pseudomonas aeruginosa* has been shown to possess both large (CSI) and small (CSII) forms of CS. This organism also showed a growth-dependent variation in the proportions of CSI and CSII, where CSI was predominant during the exponential phase and CSII in excess during the stationary phase of growth [4,5]. This was an unusual finding, and further studies of the *Pseudomonas* group itself revealed a more complex diversity, where species possessed all CSI, all CSII, or varying proportions of both CS types [6]. In those *Pseudomonads* which possess both CSI and CSII isoenzymes, the question arises as to whether they also possess distinct CS structural genes. Although the existence of two CS genes in *Saccharomyces cerevisiae* is well documented [7], there has, until recently, been no direct evidence for the existence of distinct CS genes in bacteria. Evidence has now been provided suggesting the presence of two CS genes in *Escherichia coli* [8] and *Bacillus subtilis* [9], organisms considered for many years to contain a single molecular form of CS.

CS has been characterized from a variety of sources [10–18]. However, this has been carried out in organisms which had been thought to contain one CS type. In this paper, we have for the first time purified to homogeneity multiple forms of CS from a single source, in this case *Pseudomonas aeruginosa* PAC514. We see this as a significant step in answering some of the intriguing questions regarding the structure, regulation and the role *in vivo* that this CS diversity poses.

EXPERIMENTAL

Materials

Chemicals used were of analytical quality or the highest commercially available grade. CoA, oxaloacetate, BSA, cytochrome *c* (horse heart), myoglobin (whale skeletal muscle), carbonic anhydrase (bovine erythrocyte), β-galactosidase (*E. coli*), IgG (rabbit), goat anti-rabbit IgG conjugated to alkaline phosphatase, *p*-nitrophenyl phosphate and 5′,5′-dithiobis-(2-nitrobenzoic acid) were from Sigma Chemical Co. (Poole, Dorset, U.K.). Glutaraldehyde and dimethyl suberimidate (DMS) were from Pierce Chemical Co. (Rockford, IL, U.S.A.). Fructose bisphosphate aldolase (rabbit muscle), fumarase (pig heart), catalase (bovine liver), CS (pig heart), glyceraldehyde 3-phosphate dehydrogenase (rabbit muscle), lactate dehydrogenase (rabbit muscle), malate dehydrogenase (pig heart) and phosphorylase *a* (rabbit muscle) were from Boehringer (Mannheim, Germany). Matrex Gel Red-A was from Amicon Ltd. (Woking, U.K.). The organism used was mutant PAC514 of *Pseudomonas aeruginosa* 8602 and was kindly provided by the Department of Biochemistry, University of Bath. This organism was previously designated as mutant At14 [19].

Assays

CS was assayed spectrophotometrically at 412 nm and 25 °C as previously described [20]. Protein was measured in impure fractions by the method of Lowry et al. [20a] or in purified fractions using the Bio-Rad protein reagent. Specific activity is given as μmol of CoA produced·min^{-1}·mg of protein^{-1}.

Preparation of cell extracts

Cells were grown in nutrient broth (Oxoid) to stationary phase and harvested by centrifugation at 18000 *g* for 20 min at 4 °C. Cells were resuspended in 20 mM Tris/HCl, pH 7.0, containing 1 mM EDTA and 0.1 M KCl and a cell extract was prepared by sonication as previously described [6].

Abbreviations used: CS, citrate synthase; DMS, dimethyl suberimidate.

‡ To whom correspondence should be addressed, at Napier University.

Purification of CS

Ion-exchange chromatography

A column (2.5 cm × 50 cm) containing DEAE-Sephacel (Sigma) was equilibrated at 4 °C in 20 mM Tris/HCl, pH 7.0, containing 1 mM EDTA and 50 mM KCl (Tris/EDTA buffer). The cell extract (210 ml) was equilibrated in this buffer before loading on to the column. The column was washed in the same buffer until the A_{280} was less than 0.05. Elution was achieved by a linear gradient (0.05–0.5 M) of KCl. Selected pooled fractions were dialysed exhaustively against Tris/EDTA buffer.

Dye-ligand chromatography

Pooled DEAE-Sephacel fractions were loaded on to a column (1.5 cm × 30 cm) of Matrex Gel Red-A, previously equilibrated in Tris/EDTA buffer. The column was washed in the same buffer until the A_{280} was less than 0.05 and subsequently washed by a two-step process as described elsewhere [21]. Briefly, the column was first washed with Tris/EDTA buffer containing 0.2 mM oxaloacetate and 0.2 mM CoA. This procedure eluted one of the CS isoenzymes, CSII. The other isoenzyme (CSI) was eluted using a linear gradient (0–3 M) of KCl. After both elution steps, pooled fractions were dialysed exhaustively against Tris/EDTA buffer.

Gel filtration

A column (1.5 cm × 50 cm) of Sephacryl S-200 HR (Sigma) was equilibrated in Tris/EDTA buffer, and the pooled fractions from the KCl elution of Gel Red-A were subjected to gel filtration. Selected fractions were pooled and analysed by SDS/PAGE.

Polyacrylamide-gel electrophoresis

This was performed in the presence of 0.1% (w/v) SDS as described previously [22]. Protein bands were observed using a silver staining procedure [23].

M_r determination by gel filtration

(a) Dissociated enzyme

Gel filtration was performed using Sephacryl S-300 HR in 6 M guanidinium chloride [13], and the distribution coefficients (K_D) for each protein were calculated as described previously [24].

(b) Undissociated enzyme

Gel filtration was performed as described elsewhere [13], with the exception that Sephacryl S-300 HR was used. Standard proteins were *E. coli* β-galactosidase (M_r 465000), bovine liver catalase (M_r 244000), pig heart lactate dehydrogenase (M_r 142000), pig heart CS (M_r 98000) and pig heart malate dehydrogenase (M_r 67000), detected as described previously [25–27].

Chemical cross-linking

Protein was incubated at 25 °C for up to 1 h at a concentration of 100 μg·ml^{-1}. Cross-linking using glutaraldehyde was as previously described [28] and was carried out in 50 mM sodium phosphate buffer, pH 7.5, containing 1 mM mercaptoethanol, 20% (v/v) glycerol and up to 0.25% (v/v) glutaraldehyde. Cross-linking using DMS was as described previously [29] and was carried out in 20 mM triethanolamine, pH 8.0. DMS was at a final concentration of 10 mg·ml^{-1}. The cross-linking reaction was terminated by the addition of an equal volume of 1 M Tris/HCl, pH 8.0. Samples were dialysed exhaustively against deionized water and freeze-dried in preparation for SDS/PAGE.

Amino acid analysis

Hydrolysis was carried out *in vacuo* for 24, 48 and 72 h in 6 M HCl. Samples were subjected to amino acid analysis by HPLC using a Spherisorb-5 RP-18 (220 mm × 2.1 mm) column after derivatization with phenyl isothiocyanate [30]. Cysteine was measured at 256 nm after conversion to *S*-β-(pyridylethyl)-L-cysteine [31]. Amino acids were quantified using a Shimadzu C-R5A Chromatopac integrator.

Preparation of antisera

Antisera against CSI and CSII were raised in rabbits (New Zealand White; female; 3–6 months). A preimmune bleed was taken prior to primary immunization with 400 μg of purified CS in 50% (v/v) Freunds complete adjuvant. Booster injections of 200 μg of protein in Freunds incomplete adjuvant were given on days 12 and 30. Bleedings were performed on days 35 and 45.

Determination of CS reactivity to antiserum by ELISA

Purified CS (1 μg·ml^{-1} in PBS) was incubated on 96-well microtitre plates for 1 h. Plates were washed in PBS containing 0.05% (v/v) Tween-20 (PBS/T-20) and, after blocking in PBS/T-20 containing 10% (w/v) BSA for 1 h, they were incubated for 1 h with dilutions of anti-CS antiserum in PBS/T-20. The second antibody was goat anti-rabbit IgG conjugated to alkaline phosphatase. Immunoreactivity was detected by incubating with *p*-nitrophenyl phosphate in 50 mM carbonate buffer (4.24 g·l^{-1} Na_2CO_3, 5.04 g·l^{-1} $NaHCO_3$), pH 10.14, and measuring the absorbance at 405 nm after 30 min. All incubations were performed at 25 °C.

Kinetic analysis

The Michaelis constants (K_m values) were obtained from purified preparations of CSI and CSII using the standard CS assay conditions. When altering the concentration of any one of the substances, the concentrations of the other components were maintained at saturation level. The data from a plot of the dependence of initial rate on the concentration of each substrate were analysed by the direct linear method [32]. The effects of NADH, ATP and AMP were evaluated at oxaloacetate and acetyl-CoA concentrations at, or near, the K_m values.

RESULTS

Purification of CS isoenzymes

The CS isoenzymes (CSI and CSII) were purified 104-fold and 273-fold respectively, as shown in Table 1. The initial DEAE-Sephacel procedure did not resolve CSI and CSII as previously reported [6]. Resolution was achieved by dye-ligand chromatography using Matrex Gel Red-A, a medium that has been widely used in the purification of CS from a variety of sources [13,21,33,34]. Specific elution of CSII was achieved by using 0.2 mM oxaloacetate/CoA. CSI was not eluted by this procedure, but only with KCl. The apparent low yield of CSI of 2.5% is due to the growth-dependent variation in the proportions of CSI and CSII [4,5]. In the stationary phase, CSI would normally account for about 10% of the total CS activity present in the cell extract, with CSII accounting for the rest. The activities recovered are in good agreement with this variation. Both enzymes were shown to be homogeneous by the criteria of a single band being observed

Table 1 Purification of CSI and CSII from *Pseudomonas aeruginosa* PAC514

Details of the purification are given in the Experimental section. OAA, oxaloacetate.

Fraction	Activity (units)	Recovery (%)	Protein (mg)	Specific activity (units/mg)
Crude extract	161	100	3577	0.045
DEAE-Sephacel (0.05–0.5 M KCl) (CSI + CSII)	86	53	65	1.32
Matrex Gel Red-A (0.2 mM OAA/CoA) (CSII)	39	24	3.4	11.50
Matrex Gel Red-A (0–3 M KCl) (CSI)	6	4	7.9	0.76
Sephadex G-200 (CSI)	4	2.5	0.85	4.71

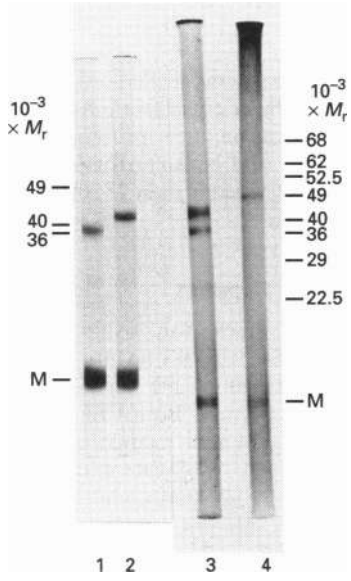

Figure 1 SDS/PAGE of purified CSI and CSII

This was carried out as described in the Experimental section. Lanes 1 and 2 are 6.5% cross-linked gels showing purified CSII and CSI respectively (three standard protein markers to the left); lanes 3 and 4 are 7.5% cross-linked gels showing a mixture of purified CSI/CSII and pig heart CS respectively (eight standard protein markers to the right). Whale muscle myoglobin (M_r 17200; a minor contaminant in this standard is seen at M_r 24000) was used as reference marker in each lane (M).

after silver staining on SDS/PAGE, and a single peak being obtained after gel filtration in 6 M guanidinium chloride. CSII was stable at 4 °C for at least 6 months, but CSI had lost 45% of its activity after this period. For long term storage, and to stabilize CSI in particular, both enzymes were stored in the presence of 20% (v/v) glycerol and 0.2 mM oxaloacetate.

Determination of the subunit M_r

SDS/PAGE of the purified CSI and CSII is shown in Figure 1. Each isoenzyme migrated as a single protein band and, when compared with M_r standards, gave a polypeptide chain M_r for CSI of 42000±2000 (mean±S.E.M.) and for CSII of 36500±2000. When co-electrophoresed, the CSII subunit clearly migrates further than the CSI subunit, and both migrated further than pig CS, which has a subunit M_r of 49000. The data from gel

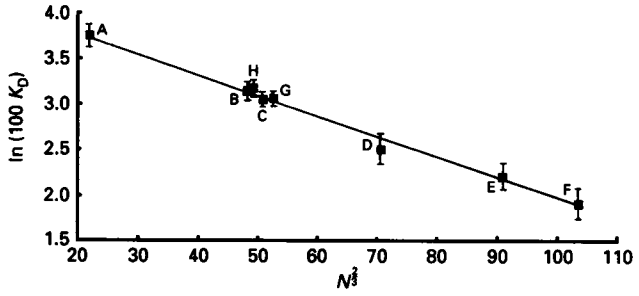

Figure 2 Gel filtration of CSI and CSII on Sephacryl S-300 in guanidinium chloride

Gel filtration was performed at pH 5.0 as described in the text. N is the number of amino acids in the polypeptide chain, and K_D is the distribution coefficient, calculated as described in the Experimental section. Standard proteins were: A, horse heart cytochrome c (N = 104); B, rabbit muscle glyceraldehyde-3-phosphate dehydrogenase (N = 330); C, rabbit muscle fructose bisphosphate aldolase (N = 358); D, BSA (N = 579); E, rabbit muscle phosphorylase a (N = 841); F, *E. coli* β-galactosidase (N = 1021); G and H represent CSI and CSII respectively.

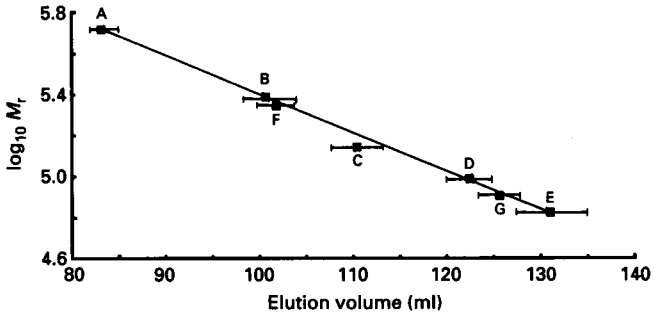

Figure 3 Determination of the M_r of native CSI and CSII by gel filtration on Sephacryl S-300

Gel filtration was performed at 4 °C in 20 mM Tris/HCl buffer, pH 8.0, containing 1 mM EDTA and 100 mM KCl as described in the Experimental section. Standard proteins were: A, *E. coli* β-galactosidase (M_r 465000); B, bovine liver catalase (M_r 244000); C, pig heart lactate dehydrogenase (M_r 142000); D, pig heart CS (M_r 98000); E, pig heart malate dehydrogenase (M_r 67000); F and G represent CSI and CSII respectively.

filtration in Sephacryl S-300 are shown in Figure 2. This is a plot of $\ln(100\ K_D)$ versus $N^{\frac{2}{3}}$, where N is the number of amino acids in the polypeptide chain [35]. The K_D value for CSI was 0.21±0.01 (mean±S.E.M.) and for CSII it was 0.25±0.01. These values represent 369 and 341 amino acids per polypeptide chain for CSI and CSII respectively, corresponding to an M_r of 40600±2000 for CSI and an M_r of 37500±2000 for CSII, which is in excellent agreement with the results from SDS/PAGE.

M_r determination of native CSI and CSII

Figure 3 shows the M_r determination of native CSI and CSII by gel filtration. CSI has an M_r of 240000±16000 (mean±S.E.M.) and CSII has an M_r of 80300±1900.

Amino acid analysis

The amino acid compositions of CSI and CSII given in Table 2 suggest a total of 718 residues for dimeric CSII (359 per polypeptide chain) and 2298 residues for hexameric CSI (383 per polypeptide chain).

Table 2 Amino acid composition of CSI and CSII

The values, from four experiments, are the means (±S.E.M.) of the number of amino acid residues/polypeptide chain of CSI and CSII. Samples were hydrolysed in 6 M HCl at 105 °C for 24 h, 48 h and 72 h. Serine and threonine were estimated by extrapolation to zero time. The 72 h values were used for valine and isoleucine.

Amino acid	CSI	CSII
Gly	38.4±0.5	33.2±0.7
Ala	25.4±0.3	31.8±0.4
Val	14.0±0.2	13.6±0.5
Leu	33.7±0.4	29.1±1.0
Ile	19.1±0.2	17.5±0.7
Phe	9.5±0.3	8.0±0.4
Tyr	6.9±0.2	8.1±0.6
Trp	1.0±0.1	1.0±0.2
Ser	37.7±0.6	26.1±1.0
Thr	17.7±0.2	25.2±0.8
Cys	4.0±0.5	4.0±0.7
Met	6.4±0.3	7.7±0.5
Asp	43.8±0.9	38.7±0.9
Glu	34.0±0.6	42.1±1.0
Lys	35.4±0.5	23.7±0.7
Arg	30.4±0.5	24.2±0.6
His	11.4±0.5	12.4±0.5
Pro	14.6±0.6	13.3±0.5

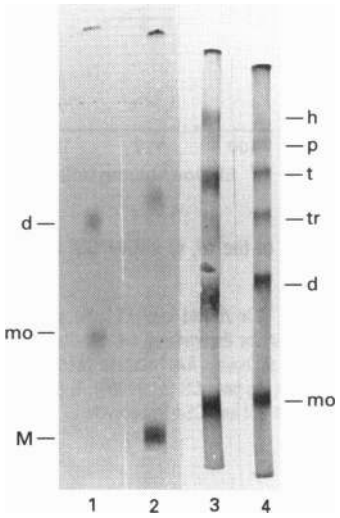

Figure 4 Determination of the number of subunits in CSI and CSII by SDS/PAGE after glutaraldehyde cross-linking

This was carried out as described in the Experimental section using 5% cross-linked gels. Lanes 1 and 2 show purified CSII cross-linked with 0.05% (v/v) and 0.25% (v/v) glutaraldehyde respectively. Lanes 3 and 4 show purified CSI cross-linked with 0.25% (v/v) and 0.05% (v/v) glutaraldehyde respectively. The positions of bands corresponding to monomer (mo), dimer (d), trimer (tr), tetramer (t), pentamer (p) and hexamer (h) are indicated. Lane 2 also included whale muscle myoglobin (M_r 17200) as a reference marker (M).

Chemical cross-linking of CS

Both CSI and CSII in the native form were treated with either glutaraldehyde (Figure 4) or DMS (results not shown) and then subjected to SDS/PAGE. Two protein bands corresponding to CSII were observed, while six bands were seen for CSI. The M_rs for the bands were as follows: CSI, 40300, 82000, 124500, 167000, 210500 and 255000; CSII, 36500 and 78500. This suggests that CSI is hexameric, while CSII is dimeric. Treatment of the enzymes with higher concentrations of either cross-linker resulted in an intensification of the protein bands corresponding to the dimer, tetramer and hexamer for CSI and the dimer for CSII. No species of M_r greater than 255000 for CSI and 78500 for CSII were observed.

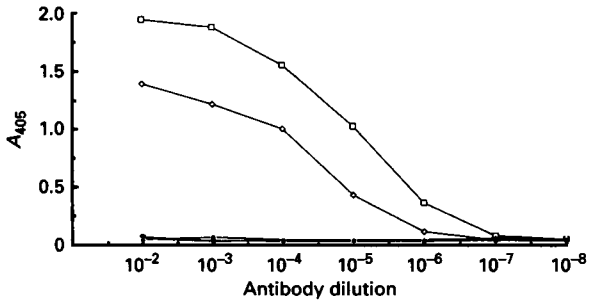

Figure 5 Determination of CSI and CSII immunoreactivity by ELISA

This was performed as described in the Experimental section. □, Anti-CSI antiserum incubated with CSI; ■, anti-CSI antiserum incubated with CSII; ◇, anti-CSII antiserum incubated with CSII; ◆, anti-CSII antiserum incubated with CSI; ▲, buffer control for anti-CSI and anti-CSII antiserum.

Kinetics and regulatory properties

The apparent K_m values for oxaloacetate [960±42 μM (mean±S.E.M.) for CSI; 2.0±0.1 μM for CSII] and acetyl-CoA (860±56 μM for CSI; 50±6 μM for CSII) are significantly different for the two enzymes. Both CSI and CSII exhibited a hyperbolic dependence of rate against concentration of both oxaloacetate and acetyl-CoA. NADH inhibition of CSI (78% at 0.1 mM) was shown to be allosteric, while CSII was shown to be less sensitive to NADH inhibition (15% at 1 mM). CSI was not inhibited by 1 mM ATP, while CSII showed 30% inhibition at the same concentration. The apparently low levels of inhibition of CSII by NADH and ATP may very well be a non-specific effect, as reported for other 'small' CS types [1]. CSI but not CSII was allosterically activated by AMP (360% at 1 mM). AMP also relieved the inhibition by NADH, which has been reported for other 'large' CS forms [36]. The inhibition of CSI by 0.1 mM NADH can be completely overcome by the addition of 1 mM AMP. This has also been previously reported in impure preparations of PAC514 [5].

Immunological characterization

Polyclonal antisera against both CSI and CSII were tested for their reactivity in an ELISA assay. Figure 5 shows that antiserum raised against CSI, although it did react with CSI, did not cross-react with CSII. Similarly, antiserum raised against CSII reacted with CSII but not with CSI. The same pattern of reactivity was seen after Ouchterlony double diffusion and in CS activity assays (results not shown).

DISCUSSION

The molecular diversity of Krebs cycle enzymes, particularly CS, has been the subject of intensive investigation for many years. Gram-positive bacteria were shown to possess a 'small' CS while Gram-negative bacteria contained a 'large' CS. The demonstration that *Pseudomonas* species had multiple forms of CS

suggested that this diversity was more complex than was first appreciated [6].

The presence of multiple forms of CS is not unknown in micro-organisms. The identification of two CS genes in *Saccharomyces cerevisiae* is well documented [7] but, until recently, there was no evidence that CS isoenzymes existed in bacteria other than *Pseudomonas*, or indeed that multiple genes existed to encode bacterial CS enzymes. Evidence has now emerged for the presence of two CS genes in the Gram-negative *E. coli* [8], and in the Gram-positive *B. subtilis* [9].

The results presented in this paper describing the purification of CS isoenzymes from *Pseudomonas aeruginosa* PAC514 strongly suggest that the CS isoenzymes, CSI and CSII, are distinct in terms of structure, kinetic properties and immunological reactivity. Gel filtration gave an M_r of 240000 for CSI and an M_r of 80300 for CSII for the native enzymes. The M_r for CSI is consistent with M_r values for other large CS forms, while the value for CSII, although lower than the M_r for pig heart CS [37], is very similar to the M_r for the small CS from *B. subtilis* [13]. Determination of the subunit M_r by SDS/PAGE and gel filtration in 6 M guanidinium chloride gave values of 42000 for CSI and 36500 for CSII. Amino acid analysis (Table 2) showed significant differences between CSI and CSII in the content of alanine, serine, threonine, glutamic acid, lysine and arginine. Although this in itself does not suggest different structural CS genes, the number of amino acids in each polypeptide chain of CSI and CSII is 383 and 359 respectively, which is in good agreement with the data from Figure 2. It is unclear why the subunits of CSI and CSII have different M_r values, but it is suggestive of distinct structural genes. The subunit M_r estimates show that CSI contains six identical subunits and is hexameric, while CSII is dimeric with two identical subunits. Again, this is consistent with the subunit composition of other large and small CS isoenzymes [10–18,21]. The chemical cross-linking study shows that, after SDS/PAGE, CSI gave six distinct bands while CSII gave two distinct bands, confirming the respective hexameric and dimeric structures. The immunological characterization of CSI and CSII, using an ELISA assay, Ouchterlony diffusion and inhibition of CS activity, showed that there was no cross-reaction of specific antiserum with the other CS form. This was an unexpected finding, given that they both catalyse the same chemical reaction, but since they differ in subunit composition and regulatory properties, significant structural differences must exist. Neither CSI nor CSII cross-reacted with antiserum raised against pig CS (a small CS) (results not shown), again showing that structural variation can exist within a single enzyme type. The immunological differences again suggest that CSI and CSII may be the products of distinct structural genes. However, such immunological differences may also arise due to the more complex assembly of the hexamer compared with the dimer.

It is clear that the role of multiple forms of CS in *P. aeruginosa* PAC514 needs to be addressed. In *S. cerevisiae*, the mitochondrial CS has been proposed to be involved in the generation of energy, while the cytoplasmic CS is used in the route to glutamate synthesis [7]. In *B. subtilis*, where two CS genes have been identified [38], the isoenzymes may have different roles depending on the prevailing metabolic conditions. The CS encoded by the *citA* gene may be used to generate a basal level of CS activity, when the other CS gene, *citZ*, is repressed [38]. It is tempting to speculate that CSI and CSII may also have different metabolic roles in *P. aeruginosa*, and therefore we are currently investigating the growth-dependent variation and expression of CSI and CSII [4,5] in continuous culture.

We thank Napier University Research and Consultancy Committee for a Research Studentship to S.C.K.A. and research support to C.G.M. Part of this work was carried out by C.G.M. while at Bath University and was funded by the Science and Engineering Research Council.

REFERENCES

1 Weitzman, P. D. J. and Danson, M. J. (1976) Curr. Top. Cell. Regul. **10**, 161–204
2 Weitzman, P. D. J. (1981) Adv. Microb. Physiol. **22**, 185–244
3 Danson, M. J. (1988) Adv. Microb. Physiol. **29**, 165–230
4 Solomon, M. (1982) Ph.D. Thesis, University of Bath
5 Solomon, M. and Weitzman, P. D. J. (1983) FEBS Lett. **155**, 157–160
6 Mitchell, C. G. and Weitzman, P. D. J. (1983) J. Gen. Microbiol. **132**, 737–742
7 Rosenkrantz, M. T., Alam, K., Kim, K., Clark, B. J., Srere, P. A. and Guarente, L. P. (1986) Mol. Cell. Biol. **6**, 4509–4515
8 Patton, A. J., Hough, D. W., Towner, P. and Danson, M. J. (1993) Eur. J. Biochem. **214**, 75–81
9 Jin, S. and Sonenshein, A. L. (1994) J. Bacteriol. **176**, 4669–4679
10 Singh, M., Brookes, G. C. and Srere, P. A. (1970) J. Biol. Chem. **245**, 4636–4640
11 Moriyama, T. and Srere, P. A. (1971) J. Biol. Chem. **246**, 3217–3223
12 Tong, E. K. and Duckworth, H. W. (1975) Biochemistry **14**, 235–241
13 Robinson, M. S., Danson, M. J. and Weitzman, P. D. J. (1983) Biochem. J. **213**, 53–59
14 Bhayana, V. and Duckworth, H. W. (1984) Biochemistry **23**, 2900–2905
15 Suissa, M., Suda, K. and Schatz, G. (1984) EMBO J. **3**, 1773–1781
16 Evans, C. T., Owens, D. D., Simegi, B., Kispal, G. and Srere, P. A. (1988) Biochemistry **27**, 4680–4686
17 Donald, L. J., Molgat, G. F. and Duckworth, H. W. (1989) J. Bacteriol. **171**, 5542–5550
18 Sutherland, K. J., Henneke, C. M., Towner, P., Hough, D. W. and Danson, M. J. (1990) Eur. J. Biochem. **194**, 839–844
19 Skinner, A. J. and Clarke, P. H. (1968) J. Gen. Microbiol. **50**, 183–194
20 Srere, P. A., Brazil, H. and Gonen, L. (1963) Acta Chem. Scand. **17**, S129–S134
20a Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. **193**, 265–275
21 Mitchell, C. G. and Weitzman, P. D. J. (1983) FEBS Lett. **151**, 260–264
22 Shapiro, A. L., Vinuela, E. and Maizel, J. V. (1970) Biochem. Biophys. Res. Commun. **28**, 815–820
23 Morrissey, J. H. (1981) Anal. Biochem. **117**, 307–310
24 Belew, M., Fohlman, J. and Janson, J.-C. (1978) FEBS Lett. **91**, 302–304
25 Bergmeyer, H. U. (1974) in Methods of Enzymatic Analysis (Bergmeyer, H. U., ed.), 2nd edn., vol. 1, pp. 452–453, Academic Press, London and New York
26 Bergmeyer, H. U. (1974) in Methods of Enzymatic Analysis (Bergmeyer, H. U., ed.), 2nd edn., vol. 1, pp. 481–482, Academic Press, London and New York
27 Bergmeyer, H. U. (1974) in Methods of Enzymatic Analysis (Bergmeyer, H. U., ed.), 2nd edn., vol. 1, pp. 485–486, Academic Press, London and New York
28 Nucci, R. (1978) J. Mol. Biol. **124**, 133–145
29 Davies, G. E. and Stark, G. R. (1970) Proc. Natl. Acad. Sci. U.S.A. **66**, 651–656
30 Aitken, A., Geisow, M. J., Findlay, J. B. C., Holmes, C. and Yarwood, A. (1989) in Protein Sequencing: A Practical Approach (Findlay, J. B. C. and Geistow, M. J., eds.). pp. 69–84, IRL Press, Oxford
31 Cavins, J. F. and Friedman, M. (1970) Anal. Biochem. **35**, 489–494
32 Eisenthal, R. and Cornish-Bowden, A. (1974) Biochem. J. **139**, 715–720
33 Schendel, F. J., August, P. R., Anderson, P. R., Hanson, R. S. and Flickinger, M. C. (1992) Appl. Environ. Microbiol. **85**, 335–345
34 Weitzman, P. D. J. and Ridley, J. (1983) Biochem. Biophys. Res. Commun. **112**, 1021–1026
35 Ryden, L. (1971) FEBS Lett. **18**, 321–325
36 Weitzman, P. D. J. and Jones, D. (1968) Nature (London) **219**, 270–272
37 Bloxham, D. P., Parmlee, D. C., Kumar, S., Wade, R. D., Ericsson, L. H., Neurath, H., Walsh, K. A. and Titani, K. (1981) Proc. Natl. Acad. Sci. U.S.A. **78**, 5381–5385
38 Jin, S. and Sonenshein, A. L. (1994) J. Bacteriol. **176**, 4680–4690

Received 9 December 1994/14 March 1995; accepted 22 March 1995