Identification of a multienzyme complex of the tricarboxylic acid cycle enzymes containing citrate synthase isoenzymes from *Pseudomonas aeruginosa*

Colin G. MITCHELL

Biomedicine and Biotechnology Research Group, Department of Biological Sciences, Napier University, 10 Colinton Road, Edinburgh EH10 5DT, Scotland, U.K.

A multienzyme complex of tricarboxylic acid cycle enzymes, catalysing the consecutive reactions from fumarate to 2-oxoglutarate, has been identified in extracts of *Pseudomonas aeruginosa* prepared by gentle osmotic lysis of the cells. The individual enzyme activities of fumarase, malate dehydrogenase, citrate synthase, aconitase and isocitrate dehydrogenase can be used to reconstitute the complex. The citrate synthase isoenzymes, CSI and CSII, from this organism can be used either together or as the individual activities to reconstitute the complex. No

complex can be reformed in the absence of CSI or CSII. Which CS isoenzyme predominates in the complex depends on the phase of growth at which the cells were harvested and the extract prepared. More CSI was found in the complex during exponential growth, whereas CSII predominated during the stationary phase. The results support the idea of a 'metabolon' in this organism, with the composition of the CS component varying during the growth cycle.

INTRODUCTION

The organization and regulation of metabolic pathways has been a major focus of research over the past few years, with many studies providing evidence that individual enzymes are not randomly distributed in the cell but exist in the form of multienzyme complexes [1–6]. Two distinct lines of investigation have been taken: first, studying the control of enzyme expression by monitoring changes in the flux and the distribution of control through a particular pathway [7] and secondly, investigations of central metabolic pathways such as the tricarboxylic acid cycle, which have revealed the presence of 'metabolons' or organized complexes of sequential enzymes. These strategies have been recently reviewed [8].

One problem that has not been given due attention is the role and the organization of isoenzymes in multienzyme complexes. It has been suggested that different isoenzymic complexes might carry out functional compartmentalization of the pathways of carbohydrate metabolism [9,10], and that individual isoenzymes are distributed between these complexes, with no two isoenzymes existing in the same complex. Although this theory was constructed on the basis of observations in eukaryotic systems, it must be considered a possibility that isoenzymic complexes exist in prokaryotes, which have been shown to possess isoenzymes.

This current paper specifically examines the involvement of multiple forms of the tricarboxylic acid cycle enzyme citrate synthase (CS) (EC 4.1.3.7) in a multienzyme complex. An intriguing diversity of this enzyme exists in living organisms, relating to the nature and molecular interactions of its subunits [11]. Gram-negative bacteria contain a hexameric 'large' enzyme (CSI) (M_r 240 000), controlled allosterically by NADH, whereas eukaryotes and Gram-positive bacteria contain a dimeric 'small' enzyme (CSII) (M_r 100 000), which is subject to isosteric control

by ATP. Studies of *Pseudomonas* species showed that they possessed all CSI, all CSII or varying proportions of both CS types [12]. In particular, a strain of *Pseudomonas aeruginosa* designated PAC514 possessed both forms of CS [13], which have recently been purified and characterized [14]. Whether *Pseudomonas* that possess both CSI and CSII isoenzymes have distinct CS structural genes is not known, but two CS genes exist in *Saccharomyces cerevisiae* [15], *Escherichia coli* [16] and *Bacillus subtilis* [17]. Considering the growing body of evidence for the existence of CS isoenzymes, the question of their organization in a tricarboxylic acid cycle 'metabolon' needs to be addressed.

Many methods have been employed to establish the presence of multienzyme complexes [18], including direct complex isolation [6], specific interactions between purified enzymes [19] and affinity binding of pure enzymes to immobilized enzymes [20]. In this paper, gentle cell disruption by osmotic lysis has been used followed by size-exclusion chromatography [21], to identify a complex of the tricarboxylic acid cycle enzymes from *Ps. aeruginosa* PAC514. In particular, the occurrence of CSI and CSII was studied to try to understand the role that these isoenzymes play in the organization of the multienzyme complex.

EXPERIMENTAL

Materials

The organism used was mutant PAC514 of *Ps. aeruginosa* 8602, and was provided by the Department of Biochemistry, University of Bath, U.K. The organism was previously designated mutant At 14 [22]. Polyclonal antisera were raised against purified pig heart CS, CSI and CSII and all chemicals used were as described previously [14], with the exception of goat anti-rabbit IgG, lactate dehydrogenase (LDH) (rabbit muscle), CS (pig heart),

lysozyme (hen egg), and the sodium salts of NAD^+ , NADP^+ , 2-oxoglutarate, pyruvate, isocitrate, citrate and L-malate (all from Sigma Chemical Co., Poole, Dorset, U.K.). Sephacryl S-200HR and S-300HR were from Pharmacia.

Growth and spheroplast preparation

The organism was grown aerobically in nutrient broth (Oxoid) at 37 °C for 3 h (early exponential phase, $D_{680} = 0.1$), for 8 h (late exponential phase, $D_{680} = 0.8$) or for 25 h (stationary phase, $D_{680} > 1.5$). Spheroplasts were prepared as previously described [21]. Briefly, cells were harvested by centrifugation for 15 min at 4 °C and 18000 *g*. The pellet was washed with 10 mM Tris/HCl, pH 7.2, containing 30 mM NaCl and resuspended at 25 °C to 1 g wet wt per 80 ml in 33 mM Tris/HCl, pH 7.2, containing 1 mM PMSF and 20 % (w/v) sucrose. EDTA and lysozyme were added to final concentrations of 10 mM and 0.5 mg/ml respectively and incubated for 30 min at 25 °C with gentle shaking. The spheroplasts were harvested by centrifugation at 9000 *g* for 15 min, resuspended in 5 ml of cold lysis buffer containing 0.1 M potassium phosphate, pH 7.2, 2.5 mM KCl, 5 mM MgCl_2 , 2 mM cysteine, 1 mM PMSF and 20 % (v/v) glycerol (PG buffer) and, after gentle dispersion, stored for 2 h at 4 °C. This was then centrifuged at 1000 *g* and the supernatant retained. The protein concentration of the supernatant was between 10 and 15 mg/ml. Some experiments involved altering the conditions of the spheroplast lysis, in which case the PG buffer was supplemented with 10 % (w/v) BSA, 14 % (w/v) polyethylene glycol (PEG; M_r 6000–7500), or 1M KCl, or the pH of the standard PG buffer was adjusted to values between 5 and 9.

Size-exclusion chromatography

To identify the presence of a multienzyme complex in the lysed spheroplast supernatant, 1 ml of the spheroplast extract was loaded onto a column (2 cm $\times$ 60 cm) of Sephacryl S-300HR equilibrated at 4 °C in either the standard or one of the modified PG buffers. Fractions (1 ml) were eluted with the same buffer at a flow rate of 15 ml/h and assayed immediately. To resolve the CSI and CSII isoenzymes for reconstitution of the multienzyme complex, chromatography was performed as described above, but with Sephacryl S-200HR.

Concentration of samples

Concentration (5-fold) of the spheroplast lysate or individual enzyme peaks, was done with Macrosep or Microsep centrifugal concentrators (Flowgen, Sittingbourne, Kent, U.K.), with an M_r cut-off of 20000. Centrifugation was performed according to the manufacturer's instructions, but was usually at 3000 *g* for 1–3 h, depending on the volume to be concentrated.

Reconstitution of the multienzyme complex

After size-exclusion chromatography of the spheroplast supernatant, the fractions containing tricarboxylic acid cycle activities not associated with the multienzyme complex were pooled, incubated at 4 °C for 1 h, concentrated and subjected again to size-exclusion chromatography as described above. Several experiments were performed, where the CS used was CSI, CSII, CSI + CSII or pig CS, which are described in the legend to Table 1. Lactate dehydrogenase was included in some cases to demonstrate the absence of non-specific interactions in the formation of the tricarboxylic acid cycle complex.

Sonication of the multienzyme complex

In some experiments, disruption of the multienzyme complex before size-exclusion chromatography was by sonication for a total of 1 min at 4 °C.

Identification and quantitation of CSI and CSII by ELISA

Both CSI and CSII were identified in the multienzyme complex by using an ELISA method described previously [14], except that purified rabbit anti-CSI or anti-CSII IgG was used as the primary antibody. Pure CSI and CSII preparations [14] were used to make quantitative determinations of CSI and CSII during growth-dependent studies. A standard curve was constructed for the ELISA that was linear between 0.2 and 1.8 $\mu\text{g/ml}$ CSI or CSII.

Purification of anti-CSI, anti-CSII and anti-pig heart CS IgG

Preparation of CSI and CSII affinity columns

These were prepared by coupling 0.1 mg of purified CSI, CSII or pig heart CS [14] to 0.2 g of cyanogen bromide-activated Sepharose 4B (Pharmacia), according to the manufacturer's instructions, packed into columns (0.7 cm $\times$ 10 cm, final bed volume 2 ml) and equilibrated in 0.1 M sodium phosphate buffer, pH 7.4, containing 0.5 M NaCl (loading buffer).

Fractionation of polyclonal anti-CSI, anti-CSII and anti-pig heart CS antiserum

Rabbit antiserum raised against CSI, CSII and pig CS [14] was dialysed exhaustively against loading buffer at 4 °C and 1 ml portions were loaded onto the columns prepared above. The columns were washed with loading buffer until the A_{280} of the eluate was zero. Bound antibodies were eluted with 10 mM HCl, and 0.5 ml fractions collected directly into 0.5 ml of 0.1 M Tris/HCl, pH 8.0. Fractions were assayed for the presence of anti-CSI and anti-CSII antibodies by an ELISA method [14].

Preparation of a goat anti-rabbit IgG affinity column

An affinity column was prepared by coupling 1 mg of goat anti-rabbit IgG (Sigma) to 0.5 g of cyanogen bromide-activated Sepharose 4B, according to the manufacturer's instructions, packed into columns (0.7 cm $\times$ 10 cm, final bed volume 5 ml) and equilibrated in loading buffer. Anti-CSI, anti-CSII or anti-pig heart CS antibodies prepared above were dialysed exhaustively against loading buffer, applied to the columns and washed with loading buffer until the A_{280} of the eluate was zero. Bound anti-CSI, anti-CSII and anti-pig CS IgG were eluted with 10 mM HCl and 0.5 ml fractions collected directly into 0.5 ml of 0.1 M Tris/HCl, pH 8.0. Fractions were assayed for the presence of anti-CSI, anti-CSII and anti-pig heart CS IgG by an ELISA method [14]. Fractions containing antibodies were adjusted to 100 μg protein per ml and stored at -70 °C.

Assays

Fumarase was measured by fumarate production with malate as substrate [23]. Citrate synthase was measured by the production of CoA by using Ellman's reagent [24]. Specific activities of CS are given as μmol of CoA produced per min per mg protein. Malate dehydrogenase [25], pyruvate dehydrogenase [26] and 2-

oxoglutarate dehydrogenase [27] were assayed by reduction of NAD⁺. Isocitrate dehydrogenase was assayed by reduction of NADP⁺ and aconitase was assayed by the production of *cis*-aconitate from citrate [28]. Succinate thiokinase was measured as described previously [29]. Protein was assayed with the BioRad reagent.

RESULTS

Resolution of a multienzyme complex

The elution profile shown in Figure 1 indicates that the tricarboxylic acid cycle enzymes succinyl thiokinase, fumarase, malate dehydrogenase, citrate synthase, aconitase and isocitrate dehydrogenase, catalysing the sequential reactions from fumarate to 2-oxoglutarate, can be isolated in a complexed form, possibly associated with the multifunctional enzymes pyruvate dehydrogenase and 2-oxoglutarate dehydrogenase. After a second filtration of the fractions containing the complexed enzymes, the complex was again observed but with about 30 % dissociation to the free, non-complexed enzymes. Altering the complex isolation conditions either increased (adding PEG or BSA) or decreased (1 M KCl or pH extremes) the yield of the complex. The maximum yield of the complex (15 %) was when PEG was added to the standard PG buffer. No complex formation was found at pH 5 or 9. A significant proportion (more than 85 %) of all the enzyme activities did not appear in the complex, but were found as individual low- M_r activities. In the case of CS, where isoenzymes CSI and CSII exist [13], two poorly resolved peaks are seen. For subsequent reconstitution experiments, the fractions containing non-complexed CSI and CSII were pooled, concentrated and re-chromatographed on Sephacryl S-200HR (Figure 2). CSI and CSII are clearly resolved. When the fractions containing the complexed enzymes were sonicated to disrupt enzyme-enzyme interactions, and then re-chromatographed on Sephacryl S-200HR, CSI and CSII were still clearly resolved (Figure 2), showing that they are both present and that there is no differential association of either CSI or CSII with the complex.

Figure 2 Resolution of CSI and CSII from the multienzyme complex and from the non-complex activities by size-exclusion chromatography

To resolve CSI and CSII from the multienzyme complex, fractions 37–43 from Figure 1 were pooled, concentrated 5–10-fold and sonicated as described in the Experimental section. This material was then subjected to size-exclusion chromatography as described in the legend to Figure 1 except that Sephacryl S-200HR was used. The broken line shows the elution profile. To resolve CSI and CSII activities fully for reconstitution experiments, fractions 65–75 from Figure 1, were pooled, concentrated 5–10-fold, and subjected to size-exclusion chromatography as described in the legend to Figure 1 except for the use of Sephacryl S-200HR. The solid line shows the elution profile. The positions of the resolved CSI and CSII isoenzymes are shown. The specific activities (in $\mu\text{mol per min per mg}$ of protein) and yields of pooled fractions containing CSI and CSII to be used in reconstitution experiments were 0.24 and 78 % for CSI and 2.45 and 85 % for CSII respectively.

ELISA of CSI and CSII in the complex

Polyclonal antisera have been raised against the purified CSI and CSII [14]. Antiserum raised against CSI does not cross-react with CSII and that from CSII does not cross-react with CSI [14]. After purification of CSI- and CSII-specific IgG, these were used to determine the presence of CSI and CSII in the complex. With the

Figure 1 Identification of a complex of tricarboxylic acid cycle enzymes by size-exclusion chromatography

Cells were grown to stationary phase and spheroplasts prepared as in the Experimental section. After spheroplast lysis, 1 ml of supernatant was subjected to size-exclusion chromatography (2 cm $\times$ 60 cm Sephacryl S-300HR column, 1 ml fractions, 4 $^{\circ}\text{C}$) in PG buffer supplemented with 14 % (w/v) PEG and enzyme assays were performed as given in the Experimental section. The specific activities of CSI and CSII in the extract prior to chromatography were, respectively, 0.02 and 0.16 $\mu\text{mol per min per mg}$ of protein. Abbreviations used: PDH, pyruvate dehydrogenase; OXO, 2-oxoglutarate dehydrogenase; STK, succinyl thiokinase; IDH, isocitrate dehydrogenase; ACO, aconitase; MDH, malate dehydrogenase; FUM, fumarase; MEC, multienzyme complex.

Figure 3 Detection of CSI and CSII in the multienzyme complex by ELISA

To demonstrate that both CSI and CSII were present in the complex, fractions 38–43 of Figure 1 were assayed by ELISA as described in the Experimental section, using purified anti-CSI (●) or anti-CSII (○) IgG. Pure CSI (▲) and CSII (△) were used as positive controls. Also, after resolution of CSI and CSII from the complex (Figure 2), fractions 14–18 containing CSI and fractions 22–28 containing CSII were tested with anti-CSI (◆) or anti-CSII (◇) IgG. Microtitre plates were coated with up to 5 μg/ml of protein.

Table 1 Reconstitution of a tricarboxylic acid cycle multienzyme complex from the individual enzyme activities

To reconstitute the multienzyme complex, fractions 88–112 containing the individual enzyme activities (IDH, ACO, MDH, FUM) after size-exclusion chromatography (Figure 1) were pooled. Fractions 78–82 containing STK activity after size-exclusion chromatography (Figure 1) were first assayed by ELISA to ensure that no CS activity was present, and those free of CS were added to fractions 88–112. To these combined fractions were added fractions 13–18 (Figure 2) containing CSI (specific activity 0.02 μmol per min per mg of protein) and/or fractions 22–30 (Figure 2) containing CSII (specific activity 0.16 μmol per min per mg of protein). These were then concentrated 5-fold and subjected to size-exclusion chromatography on Sephacryl S-300HR as described in the Experimental section and in the legend to Figure 1. Where a complex was formed, the elution profile was the same as shown in Figure 1, with the exception that STK was not in the complex. Where no complex was formed, only the individual activities of CSI, CSII, IDH, ACO, MDH and FUM eluted at positions similar to those given in Figure 1. Pig-heart CS (specific activity 48.3 μmol per min per mg of protein) replaced CSI and CSII in some experiments and was added to a final concentration of 2 mg/ml. Lactate dehydrogenase at a final concentration of 2 mg/ml was used in some experiments simultaneously with CSI, CSII or pig CS.

Source of CS	Complex detected	CS activity in complex	CS detected by ELISA
No CS added	No	No	No
CSI + CSII	Yes	Yes	Yes
CSI	Yes	Yes	Yes
CSII	Yes	Yes	Yes
Pig CS	No	No	No

purified isoenzymes as positive controls, Figure 3 shows that both CSI and CSII are present, again demonstrating that there is no differential association of CSI or CSII with the complex. Both isoenzymes were inactivated (more than 90%) by their specific IgG (results not shown). Unfractionated antiserum against pig heart CS did not inactivate or react with the CS in the complex.

Reconstitution of the multienzyme complex

When the low- M_r non-complexed enzymes (fractions 64–115, Figure 1) were pooled, concentrated and re-chromatographed,

an elution profile similar to that seen in Figure 1 was observed (results not shown), with the exception that succinyl thiokinase was not present in the complex. It has previously been shown that succinyl thiokinase can associate with 2-oxoglutarate dehydrogenase [27], so the absence of 2-oxoglutarate dehydrogenase from the pooled fractions may account for the absence of succinyl thiokinase from the complex. Pyruvate dehydrogenase and 2-oxoglutarate dehydrogenase were also absent from the pooled fractions, indicating that they are not a requirement for the formation of the complex. This suggests that the enzymes involved in the multienzyme complex can freely undergo association–dissociation reactions and supports the idea of a high- M_r complex or ‘metabolon’ of tricarboxylic acid cycle enzymes [30]. The reconstitution of the high- M_r complex was also performed under conditions where the CS component involved was altered, as shown in Table 1. The low- M_r fractions containing CS activity after purification on Sephacryl S-300HR (Figure 1) were first concentrated and then re-chromatographed on Sephacryl S-200HR, to separate CSI from CSII (Figure 2). CSI- and CSII-containing fractions were assayed by ELISA to ensure that they did not contain the other CS isoenzyme. The resolved CSI and CSII isoenzymes were then used to reconstitute the high- M_r complex. It can be seen that when CSI, CSII or both isoenzymes were used in the reconstitution, a high- M_r complex could be isolated. No complex was formed if CSI and CSII were omitted from the incubation. When pig-heart CS was used instead of CSI or CSII, no detectable high- M_r complex was formed. Lactate dehydrogenase, which was added in some experiments, was not detected in the complex, indicating that the association of CSI and CSII to form a complex is specific. The presence or the absence of CSI, CSII or pig-heart CS from the reconstituted complexes was confirmed by ELISA with specific IgGs. These observations suggest that the formation of the high- M_r complex is dependent on the presence of CSI or CSII from *Ps. aeruginosa* PAC514. Although pig-heart CS catalyses the same reaction as CSI and CSII, its failure to substitute for CSI or CSII suggests that the structural requirements for the formation of an intact high- M_r complex are not present. Indeed, pig-heart CS does not cross-react with antisera against CSI or CSII [14].

Table 2 Growth-dependent variation in the CSI and CSII composition of the tricarboxylic acid cycle multienzyme complex

Cells were harvested at the phase of growth given in the table and a cell-free extract prepared from spheroplasts as described in the Experimental section. The extract was subjected to size-exclusion chromatography on Sephacryl S-300HR to resolve a high- M_r complex (see Figure 1 elution profile), with quantitation of CSI and CSII in the complex being carried out by the ELISA as described in the Experimental section. The data are the means $\pm$ S.E.M for three experiments.

Phase of growth	CSI or CSII:total complex protein (ng/ μ g)	
	CSI	CSII
Early exponential	17.5 $\pm$ 1.4	4.2 $\pm$ 0.7
Late exponential	12.4 $\pm$ 1.0	18.3 $\pm$ 1.2
Stationary	5.6 $\pm$ 0.8	24.6 $\pm$ 1.9

Growth variation in the CS composition of the complex

Pseudomonas aeruginosa PAC514 has been previously shown to display growth-phase-dependent variation in the proportions of CSI and CSII. The 'large' form, CSI, predominates in the exponential phase, whereas the 'small' form, CSII, is the major component in the stationary phase [13]. The data presented in Table 2 show that there is growth-dependent variation in the proportions of CSI and CSII that are associated with the multienzyme complex. As the organism goes from exponential to stationary phase there is a decrease in the amount of CSI and an increase in the amount of CSII associated with the complex.

DISCUSSION

The results presented here support the idea of a tricarboxylic acid cycle 'metabolon' of consecutive enzymes in *Ps. aeruginosa* PAC514 and that the complex can undergo association-dissociation reactions. One aspect of metabolic organization that deserves further investigation is the role that isoenzymes play in these complexes, in organisms where multiple forms of enzymes are known to exist. Multienzyme complexes offer distinct metabolic advantages to the cell, and the nature of the cell *in vivo* seems to favour specific enzyme-enzyme interaction [31–33]. The importance of CS as a structural component in the maintenance of a functional 'metabolon', has been shown in a recent study on *S. cerevisiae* [34], where two CS forms exist, a mitochondrial CS (CS1) and a peroxisomal CS (CS2). Generation of gene-disruption mutants deficient in mitochondrial CS (CS1[−]), peroxisomal CS (CS2[−]) and both CS forms (CS1[−]CS2[−]), showed that the CS2[−] mutant grew on acetate but the CS1[−] mutant did not, suggesting that citrate synthesized by the peroxisomal CS2 could not replace the missing citrate normally synthesized in the mitochondrion. Further investigation [35], using a mutant with a catalytically inactive CS1 constructed by site-directed mutagenesis, showed that when this gene was introduced into the CS1[−] mutant, growth on acetate could be restored, suggesting that the CS1 protein itself, although inactive, plays a structural as well as a catalytic role in the organization of the tricarboxylic acid cycle. The results presented in this paper suggest an important structural role for CSI and CSII in preserving the integrity of the high- M_r complex. However, because impure CSI and CSII preparations were used in the reconstitution experiments, it is possible that another component is involved in complex formation. Srere [8] has suggested that to establish the

validity of the 'metabolon' concept, details of the specific protein-protein interactions in a multienzyme complex must be known, and that one way to achieve this is by the use of chimaeric proteins of isoenzyme pairs. Considering the range of proteins that have been shown to interact with CS [8], it seems logical to use CS in such chimaeric experiments. There are, however, few examples of CS isoenzymes. The yeast system is well documented [15], isoenzymes have been identified and isolated from *Ps. aeruginosa* [14] and recent evidence suggests two CS structural genes in *E. coli* [16] and *B. subtilis* [17].

Several questions about the organization of the complex arise from this study. The organism used has been previously shown to demonstrate a growth-dependent and nutrient-dependent variation in the proportions of CSI and CSII in cell-free extracts. In the exponential phase CSI predominates, whereas in the stationary phase CSII is in excess. Growth on acetate as the sole carbon source promotes CSII formation, whereas growth on glucose promotes CSI formation [13]. In the current study, because both CSI and CSII are capable of associating into the high- M_r complex (Figure 2, Table 1), it was considered possible that the proportions of the two isoenzymes associating with the complex *in vivo* may also vary depending on the prevailing growth conditions. Table 2 shows that this is the case where the amount of CSII associated with the complex is greater in the stationary phase, whereas the amount of CSI is greater in the exponential phase. The term 'ambiquitous' has been used [36] to describe enzymes whose distribution between soluble and particulate forms may vary depending on the prevailing status of the cell in terms of the levels of metabolites capable of influencing that distribution, as well as situations where the isoenzyme composition of the cellular enzyme complement may influence the soluble-particulate distribution. Such an association-dissociation may be of importance in the regulation of CS activity, although in the present study the kinetic constants for CSI in the free and complexed forms were the same, and were similar to those previously reported for the purified enzyme [14]; the kinetics of CSII were also the same in the free and complexed forms (results not shown). This growth-dependent variation is currently being further investigated in this laboratory in chemostat culture.

It has been suggested that where isoenzymes exist, they are associated with distinct multienzyme complexes to form isoenzymic complexes [9]. It is possible that CSI and CSII are associated with different multienzyme tricarboxylic acid cycle complexes and that isoenzymic complexes do exist in *Ps. aeruginosa* PAC514. There is no evidence in this paper for their existence; indeed the data show that either CSI or CSII, or both isoenzymes, seem to associate with a single complex.

The role of multiple forms of CS in *Ps. aeruginosa* PAC514 needs to be addressed, particularly with reference to multienzyme complexes. What makes the situation even more interesting is the recent evidence for different roles of isoenzymes of CS in the cell. In *S. cerevisiae*, mitochondrial CS1 seems to be involved in energy production, whereas the cytoplasmic CS2 regulates glutamate biosynthesis [17]. In *B. subtilis*, two CS genes (*citA* and *citZ*) have been identified [37] that could have different roles depending on the prevailing metabolic conditions: a basal level of CS activity is generated by the CS encoded by the *citA* gene when *citZ* is repressed [37]. It is therefore possible that CSI and CSII could have different roles in *Ps. aeruginosa*. If isoenzymic complexes do exist, then other tricarboxylic acid cycle enzymes should be present as isoenzymes in this organism. The identification and isolation of (iso)enzymes that could potentially interact with CS, such as malate dehydrogenase, is currently under way, to study the specific interaction with CSI and CSII.

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