Carbamate kinase can replace in vivo carbamoyl phosphate synthetase. Implications for the evolution of carbamoyl phosphate biosynthesis

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Abstract The exclusive involvement of carbamate kinase (CK) in fermentative ATP production and of carbamoyl phosphate synthetase (CPS) in the production of carbamoyl phosphate (CP) for pyrimidines and arginine biosynthesis was challenged by the finding of CK as the only activity synthesising CP in the archaea *Pyrococcus furiosus* and *Pyrococcus abyssi*. We now show that CK can replace CPS in vivo: transformation of *Escherichia coli* devoid of the CPS gene with plasmids encoding the CK from *P. furiosus* or from *Enterococcus faecalis* (which uses CK for making ATP) restores the ability of CPS-deficient *E. coli* to grow in the absence of arginine and uracil if ammonia and bicarbonate are present.

Key words: Carbamate kinase; Carbamoyl phosphate synthesis; Arginine synthesis; Pyrimidine synthesis; Thermophilic archaea; *Pyrococcus furiosus*

1. Introduction

Carbamoyl phosphate (CP), the first committed precursor of pyrimidines and arginine, is generated by carbamoyl phosphate synthetases (CPSs) from ATP, bicarbonate and ammonia (or glutamine). CPSs are large, complex, highly regulated enzymes that catalyse irreversibly the reaction: $2ATP+HCO_3^-+NH_3$ (or glutamine) $\rightarrow 2ADP+P_i$(+glutamate)+$NH_2CO_2PO_3^{2-}$ (CP) [1]. CP can also be produced in the fermentative degradation of arginine by catabolic ornithine transcarbamylase, but this CP is used to make ATP by the enzyme carbamate kinase (CK) [2] according to the reaction: $ADP+NH_2CO_2PO_3^{2-} \rightarrow ATP+NH_2CO_2^-$ (carbamate) [3]. CK and CPS differ widely in size, domain organisation and three-dimensional structure [4,5]. However, a CPS was reported in the hyperthermophilic archaea *Pyrococcus furiosus* [6] and *Pyrococcus abyssi* [7] having all of the characteristics of a CK (amino acid sequence [6], three-dimensional structure [8] and catalytic properties [9]). The reaction catalysed by CK is reversible, and the substrate of the reverse reaction, carbamate, is formed chemically from bicarbonate and ammonia [3], raising the possibility that CK might play in these archaea the role played by CPS in other organisms, the synthesis of CP for anabolic purposes.

To try to clarify if CK can replace CPS in vivo, we have expressed the CK from *P. furiosus* in a strain of *Escherichia coli* with a deletion of the *carAB* gene, which encodes CPS. We show that the expressed CK restores the ability of the CPS-deficient cells to grow in the absence of arginine and pyrimidines if ammonia and bicarbonate are present. Furthermore, the CK of *Enterococcus faecalis* [10], which is used in the latter organism to make ATP fermentatively from arginine [2,3], was also found to support the growth of CPS-deficient *E. coli* in arginine and pyrimidine-free medium. Thus, CK can replace efficiently CPS in vivo, raising the possibility that in some organisms, such as pyrococci, CK may have the role of making CP for biosynthetic purposes.

2. Materials and methods

2.1. Cell strains, plasmids and growth conditions

E. coli strain L814 [11], which lacks both *carA* and *carB* genes (*carA* and *carB* encode the small and large *E. coli* CPS subunits) and plasmid pLLK12 [11], which carries the *carAB* genes inserted in the *Bam*HI site of pUC19 and which confers ampicillin resistance, were generous gifts of Dr. Carol J. Lusty (The Public Health Research Institute, NY, USA). Plasmids pCK41 [10] and pCPS184 [9], two pET-15b (Novagen) derived plasmids carrying the genes for enterococcal and pyrococcal CK, respectively, behind the T7 promoter, and conferring ampicillin resistance, were provided by Dr. A. Marina and Dr. S. Ramón-Maiques. To allow the expression in L814 of the plasmid-encoded CKs, the cells were lysogenised for phage λDE3 using a commercial kit (λDE3 lysogenisation kit, from Novagen) and are called L814T7. For expression of pyrococcal CK, the cells were transformed also with the plasmid pSJS1240 [12,13], encoding tRNAs for rare *E. coli* codons for arginine and isoleucine and conferring spectinomycin resistance (this plasmid was a gift of Dr. S.J. Sandler, Department of Microbiology, University of Massachusetts, MA, USA). Procedures for making competent cells and for transformation with plasmids, and the composition of the minimum medium are as described earlier [9,10,14]. The medium used with transformed cells also contained 0.1 mg/ml ampicillin and, in the case of the pSJS1240 cotransformants, 0.05 mg/ml spectinomycin. When indicated, it contained also 0.6 mM isopropyl β-D-thiogalactopyranoside (IPTG) or the specified amount of ammonium bicarbonate. The initial inoculum consisted of cells that had been grown in Luria–Bertani medium (containing antibiotics as indicated above) to an optical density of 0.6 and that were centrifuged, washed twice and suspended in minimum medium with the indicated additions to an initial absorbance of 0.2. When IPTG was used, the cells, prior to centrifugation, were exposed for 3 h to 0.6 mM IPTG, and then were centrifuged, washed and suspended as above in minimum medium containing 0.6 mM IPTG. The growth of cells at 37°C with orbital shaking (300 cycles/min) was monitored by the optical absorbance of the culture at 600 nm.

2.2. Enzyme activity assays and electrophoretic methods

Cells isolated by centrifugation were sonicated in 20 mM Tris–HCl,

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Abbreviations: CK, carbamate kinase; CP, carbamoyl phosphate; CPS, carbamoyl phosphate synthetase; IPTG, isopropyl β-D-thiogalactopyranoside

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pH 8, containing 1 mM ethylene diamine tetraacetic acid and 0.3 mM phenylmethylsulphonyl fluoride, and the extract was clarified by centrifugation and was desalted by centrifugal gel filtration [15] in the same buffer. CK activity was assayed at either 37 or 60°C, in the direction of CP synthesis, by conversion of the CP to citrulline with ornithine transcarbamylase [9]. One enzyme unit corresponds to the production of 1 μmol CP min^{-1} at the specified assay temperature. Protein in the extracts was assayed according to Bradford [16] using bovine serum albumin as a standard. Extracts were subjected to SDS–PAGE in 10% polyacrylamide gels followed by Western blotting and immunostaining with alkaline phosphatase using as first antibody a monoclonal (mAbCK2 [10]) or a polyclonal mouse antibody recognising enterococcal and pyrococcal CK, respectively. Densitometric quantification of stained bands was carried out by comparison with the purified target enzymes, run in parallel tracks, using a scanner and the Sigmagel program (Sigma).

3. Results and discussion

Since *E. coli* has a single CPS that produces the CP needed for making both arginine and pyrimidines [17], *E. coli* strain L814T7, which lacks this CPS [11], cannot grow in minimum medium (Fig. 1A,B, open circles) except when arginine and uracil are added (Fig. 1B, closed circles). Supplementation with only one of these compounds does not result in growth of these cells. Transformation with plasmid pLLK12, which encodes *E. coli* CPS, restores the ability of the cells to grow in the absence of arginine and uracil (Fig. 1A, inverted triangles). Transformation with pCK41 (Fig. 1, squares) or with pCPS184 (Fig. 1, upright triangles), which encode the CKs from *E. faecalis* and *P. furiosus*, respectively, results in growth in the absence of uracil, if arginine is added. However, growth does not occur when uracil is added and arginine is absent. Thus, it appears that the expressed enterococcal or pyrococcal CKs (revealed in Fig. 2 by SDS–PAGE and Coomassie-staining) supply enough CP to cope with the demands of pyrimidine synthesis but not enough to cope with the larger requirements of arginine synthesis.

In these experiments IPTG was used and growth was delayed in the cells transformed with pCK41 and, particularly,

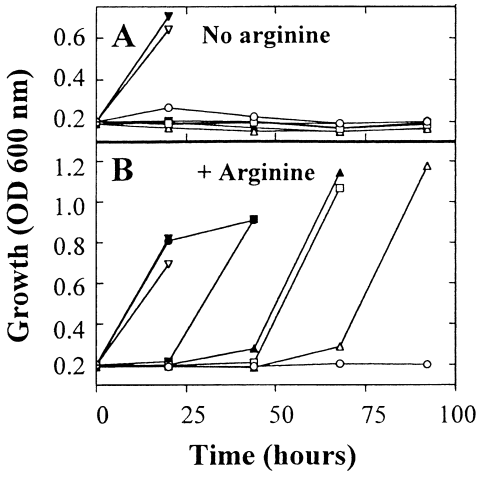

Fig. 1. Influence of transformation with pLLK12 (inverted triangles), pCK41 (squares) or pCPS184 (upright triangles) on growth of L814T7 *E. coli* cells in the presence or absence of arginine and/or uracil. Circles denote non-transformed *E. coli*. Growth was carried out in minimum medium in the presence of 0.6 mM IPTG as indicated in Section 2. The medium was either unsupplemented with arginine (A) or supplemented with 0.06 mg/ml arginine (B). Closed symbols denote supplementation with 0.04 mg/ml uracil.

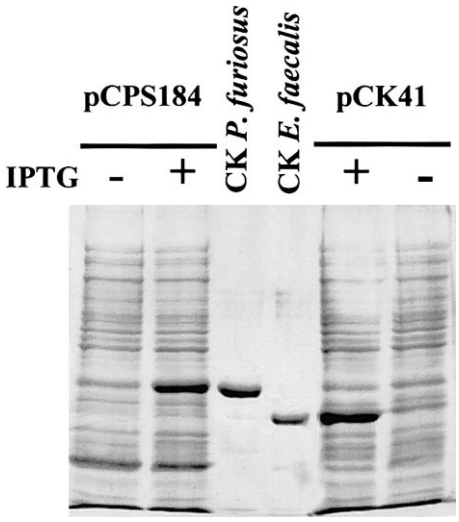

Fig. 2. SDS–PAGE and Coomassie staining of L814T7 *E. coli* cells transformed pCPS184 or pCK41, prior or immediately after incubation for 3 h with 0.6 mM IPTG. The cultures were centrifuged and the cell pellets were boiled 3 min in SDS–PAGE sample solution and were subjected to electrophoresis. The sample applied was adjusted to represent approximately the same number of cells (on the basis of OD_{600}) per track. For comparison, pure CK from *P. furiosus* or CK from *E. faecalis* were also subjected to electrophoresis.

with pCPS184 (Fig. 1B), even in the presence of added arginine and uracil, suggesting that the IPTG-promoted massive expression of these CKs has some toxic effect that delays growth. Consequently, IPTG was not used in further experiments, particularly since pilot assays demonstrated some expression of the plasmid-encoded proteins in the absence of IPTG (see below).

In the above experiments CP appeared to be synthesised in vivo by CK in the absence of a substantial source of carbamate, the substrate used by CK to make CP. At the concentrations in the medium of HCO_3^- (0.2 mM, derived from air [18]) and NH_3 (0.15 mM; derived from the ammonium sulphate present), carbamate should be present in trace amounts (0.06 μM, calculated from the equilibrium: $K = [NH_3] \times [HCO_3^-]/[\text{carbamate}] = 0.53$ M [3]) with respect to K_m values for carbamate of the CKs from *E. faecalis* (77 μM [3]) and *P. furiosus* (5–7 μM; [9] and unpublished results).

Thus, to increase the concentration of carbamate, 10–80 mM ammonium bicarbonate was added to the minimum medium (Fig. 3, top panel). This addition did not induce any growth (monitored after 18 h) of the non-transformed cells and it did not impair or promote the growth of the cells transformed with plasmid pLLK12, which encodes *E. coli* CPS, except at the highest salt concentration, at which growth was strongly inhibited. In contrast, the addition of the salt enabled the cells transformed with the CK-encoding plasmids to grow in minimum medium, although the growth was also inhibited at the highest salt concentration tested. The highest cell densities after 18 h of culture were attained at concentrations of ammonium bicarbonate of 20–40 mM with pCK41 and of 40–50 mM with pCPS184, and were approximately two-fold lower with pCPS184 than with pCK41, with which they were similar to those attained by transformation with pLLK12. A plot of the cell density of the pCK184-transformed cells versus the calculated carbamate concentration (Fig. 3, lower panel) suggests mid-saturation by approxi-

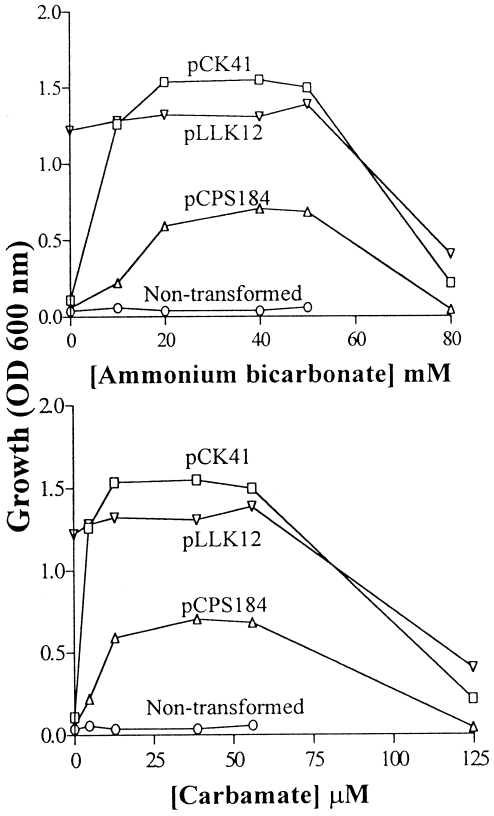

Fig. 3. Influence of supplementation of minimum medium with variable concentrations of ammonium bicarbonate (upper panel), and of the calculated resulting equilibrium concentration of carbamate (lower panel) on the growth of L814T7 *E. coli* cells, either non-transformed or transformed with pLLK12, pCK41 or pCPS184. Growth was carried out for 18 h at 37°C from identical inocula.

mately 7 μM carbamate and saturation by 50 μM carbamate, in agreement with the expectations for the carbamate dependency of pyrococcal CK. Thus, being saturated by carbamate, it appears that the rate of CP production by the expressed pyrococcal enzyme limits the growth of the pCPS184-transformed cells and causes the lower culture densities found with these cells than with the pCK41 and pLLK12-transformed cells.

To monitor the expression of the plasmid-encoded CKs, cell extracts were subjected to immunoblotting at the end of the 18 h incubation. Fig. 4 illustrates the lack of CK in non-transformed cells and the presence of a substantial amount of the expected CK in the pCK41 and pCPS184-transformed cells. In the former cells the enterococcal enzyme, quantified densitometrically in the blots (Fig. 5), represented approximately 6% of the protein at 10 mM ammonium bicarbonate and decreased to about 4% at 40 mM ammonium bicarbonate. In the pCPS184-transformed cells the pyrococcal enzyme accounted for 20% of the protein at 10 mM ammonium bicarbonate and decreased at higher salt concentrations to about 5% of the protein. These results suggest that at increasing cell density plasmid-encoded CK expression is decreased, but in any case they show that there is CK expression in the cells in which growth was observed. However, any connection between CK expression and growth would be invalidated if there were substantial endogenous background CK activity in *E. coli*. In fact, there are three putative endogenous CK genes in *E. coli* [10], and the expression of these cannot be excluded by the experiments shown thus far, since the corresponding protein products may not cross-react with our highly specific antibodies. CK activity assays were therefore used to rule out the existence of substantial endogenous CK. No substantial CK activity, assayed at either 37 or 60°C, was detected in the cell extracts used for immunoblotting of the non-transformed cells (detection limit, 0.03 U/mg protein in the extract), whereas substantial CK activity was found, paralleling the levels of immunodetected CK protein, in the pCK41- and pCPS184-transformed cells (Fig. 5, top and bottom panels, respectively) exhibiting, as expected, high thermostability in the pCPS184-transformed cell extracts (tested by heating the extract for 5 min at 90°C, data not shown). Furthermore, the ratio between the CK activity and CK protein levels yield estimates for the specific activities of the enterococcal and pyrococcal enzymes, respectively, of 668 (37°C) and 15.3 (60°C) U/mg, in excellent agreement with previous estimations with the purified enzymes of 630 [3] and 16.7 [9] U/mg protein. Thus, the activity observed is due to the expression of the plasmid-encoded enzyme, and is associated with the ability of the CPS-deficient cells to grow in the absence of arginine and uracil, thus allowing the conclusion that CK can replace CPS in vivo.

Our results are relevant in the context of the in vivo synthesis of CP in *P. furiosus* and *P. abyssi*. At the high temperatures of the normal living environment of these microorganisms the CK activity in these archaea was estimated to represent, in the direction of CP synthesis, approximately 10 μmol h^{-1} mg $protein^{-1}$ [9], a value not exceeding the activity of the pyrococcal enzyme that supported the growth of *E. coli* at 37°C in our experiments (10–3.5 μmol h^{-1} mg $protein^{-1}$ at 37°C, depending on the ammonium bicarbonate concentration, Fig. 5; applying a 13-fold reduction in the activity for the decrease in the temperature from 60 to 37°C [9]), supporting the possibility that a CK is responsible in these pyrococci for the synthesis of CP. In fact, as previously discussed [9], the concentration of carbamate may be relatively high in the habitat of these pyrococci, given the finding

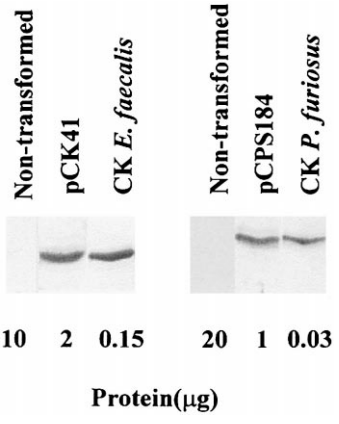

Fig. 4. Immunoblot illustrating CK expression in the absence of IPTG in L814T7 *E. coli* cells, either non-transformed or transformed with the plasmids pCK41 or pCPS184. The cultures illustrated in Fig. 3 in the presence of 20 mM ammonium bicarbonate were used to obtain cell extracts that were utilised for SDS–PAGE, Western blotting and immunostaining as described in Section 2. The amount of cell extract protein applied is given below each track. For comparison, the indicated amounts of pure CK from *P. furiosus* or of CK from *E. faecalis* were subjected to immunoblotting in parallel and are also depicted.

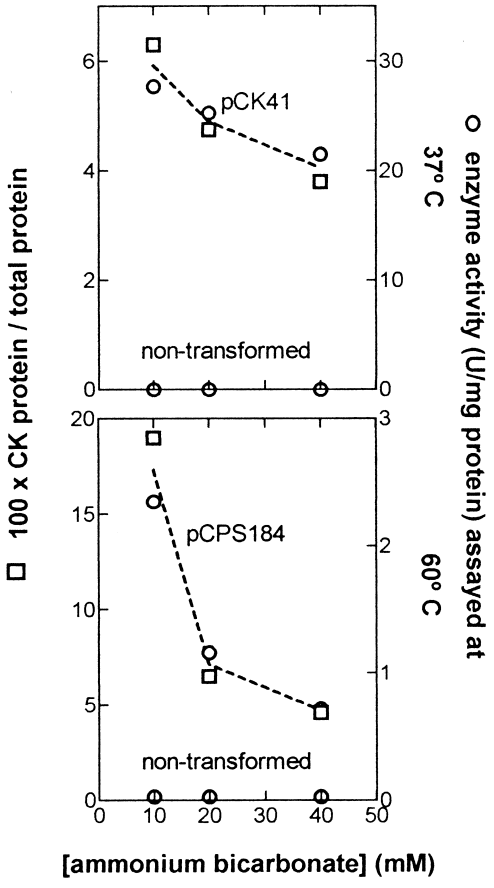

Fig. 5. Levels of CK protein and CK activity in non-transformed and in pCK41- or pCPS184-transformed *E. coli*. Cells collected at the end of the 18 h growth period from the cultures of Fig. 3 were used. The squares denote CK protein determined by densitometric analysis of immunoblots and the circles indicate CK activity measured at the specified temperature in the direction of CP synthesis. No CK protein was detected in non-transformed cells.

of high concentrations of CO_2 (the true reactant, rather than bicarbonate, in the chemical synthesis of carbamate) and of 0.6–1 mM ammonia in their environment. Furthermore, a CK gene but no classical CPS gene has been found in the entire genome of *Pyrococcus horikoshii* [19] (although a putative classical CPS gene has been identified in the genome of *P. furiosus*; http://www.genome.utah.edu/sequence.htlm). Pending a definitive answer on the role of pyrococcal CK that may possibly be provided only by knocking out the CK gene in these pyrococci, the present results stress the possibility that a physiological function of CK in some organisms may be to make CP for biosynthetic purposes rather than to use it for making ATP. To our knowledge the pyrococci would be the first organisms in which a CPS would not be utilised for the production of CP for biosynthetic purposes.

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References

[1] Rubio, V. (1994) in: Carbon Dioxide Fixation and Reduction in Biological and Model Systems (Brändén, C.I. and Schneider, G., Eds.), pp. 249–264. Oxford University Press, Oxford.
[2] Abdelal, A. (1979) Annu. Rev. Microbiol. 33, 139–168.
[3] Marshall, M. and Cohen, P.P. (1970) Methods Enzymol. 17, 229–234.
[4] Marina, A., Alzari, P.M., Bravo, J., Uriarte, M., Barcelona, B., Fita, I. and Rubio, V. (1999) Prot. Sci. 8, 934–940.
[5] Thoden, J.B., Holden, H.M., Wesenberg, G., Raushel, F.M. and Rayment, I. (1997) Biochemistry 36, 6305–6316.
[6] Durbecq, V., Legrain, C., Roovers, M., Piérard, A. and Glansdorff, N. (1997) Proc. Natl. Acad. Sci. USA 94, 12803–12808.
[7] Purcarea, C., Simon, V., Prieur, D. and Hervé, G. (1996) Eur. J. Biochem. 236, 189–199.
[8] Ramón-Maiques, S., Marina, A., Uriarte, M., Fita, I. and Rubio, V. (2000) J. Mol. Biol. 299, 463–476.
[9] Uriarte, M., Marina, A., Ramón-Maiques, S., Fita, I. and Rubio, V. (1999) J. Biol. Chem. 274, 16295–16303.
[10] Marina, A., Uriarte, M., Barcelona, B., Fresquet, V., Cervera, J. and Rubio, V. (1998) Eur. J. Biochem. 253, 280–291.
[11] Guillou, F., Liao, M., Garcia-España, A. and Lusty, C.J. (1992) Biochemistry 31, 1656–1664.
[12] Sandler, S.J. and Clarck, A.J. (1994) J. Bacteriol. 176, 3661–3672.
[13] Del Tito, B.J., Ward, J.M., Hodgson, J., Gershater, C.J.L., Edwards, H., Wysocki, L.A., Watson, F.A., Sathe, G. and Kane, J.F. (1995) J. Bacteriol. 177, 7086–7091.
[14] Mora, P., Rubio, V., Frequet, V. and Cervera, J. (1999) FEBS Lett. 446, 133–136.
[15] Penefsky, H.S. (1977) J. Biol. Chem. 252, 2891–2899.
[16] Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
[17] Meister, A. (1989) Adv. Enzymol. Relat. Areas Mol Biol. 62, 315–374.
[18] Anderson, P.M. and Meister, A. (1965) Biochemistry 4, 2803–2809.
[19] Kawarabayasi, Y., Sawada, M., Horikawa, Y., Hino, Y. and Yamamoto, S. et al. (1998) DNA Res. 5, 55–76.