Mechanism of allosteric modulation of *Escherichia coli* carbamoyl phosphate synthetase probed by site-directed mutagenesis of ornithine site residues

Lourdes Rochera[a], Vicente Fresquet[a], Vicente Rubio[b], Javier Cervera[a,*]

[a] *Instituto de Investigaciones Citológicas (FVIB), Amadeo de Saboya 4, Valencia 46010, Spain*
[b] *Instituto de Biomedicina de Valencia (CSIC), Jaime Roig 11, Valencia 46010, Spain*

Received 3 January 2002; revised 29 January 2002; accepted 31 January 2002

First published online 15 February 2002

Edited by Judith Ovádi

Abstract The role of residues of the ornithine activator site is probed by mutagenesis in *Escherichia coli* carbamoyl phosphate synthetase (CPS). Mutations E783A, E783L, E892A and E892L abolish ornithine binding, E783D and T1042V decrease 2–3 orders of magnitude and E892D decreased 10-fold apparent affinity for ornithine. None of the mutations inactivates CPS. E783 mutations hamper carbamate phosphorylation and increase K^+ and MgATP requirements, possibly by perturbing the K^+-loop near the carbamate phosphorylation site. Mutation E892A activates the enzyme similarly to ornithine, possibly by altering the position of K891 at the opening of the tunnel that delivers the carbamate to its phosphorylation site. T1042V also influences modulation by IMP and UMP, supporting signal transmission from the nucleotide effector to the ornithine site mediated by a hydrogen bond network involving T1042. Ornithine activation of CPS may be mediated by K^+-loop and tunnel gating changes.

Key words: Carbamoyl phosphate synthetase; Ornithine; Allosteric regulation; Site-directed mutagenesis; Pyrimidine biosynthesis; Arginine biosynthesis

1. Introduction

Carbamoyl phosphate synthetase (CPS), an αβ heterodimer of 40 and 120 kDa subunits, catalyzes in *Escherichia coli* the reaction 2ATP+glutamine+HCO_3^- → P_i + 2ADP + glutamate +$NH_2CO_2PO_3^{2-}$ (carbamoyl phosphate, CP) that is the first step of the routes of pyrimidine and arginine biosynthesis [1]. The small subunit hydrolyzes glutamine and transfers the ammonia to the large subunit, which uses it to synthesize CP in three steps: (1) ATP+HCO_3^- → ADP+$HOCO_2PO_3^{2-}$ (carboxyphosphate); (2) $HOCO_2PO_3^{2-}$+NH_3 → P_i+$NH_2CO_2^-$ (carbamate); and (3) $NH_2CO_2^-$+ATP → ADP+$NH_2CO_2PO_3^{2-}$ [1]. There is internal homology between the N- and C-terminal halves of the large subunit [2], and each homologous half consists of an N-terminal domain of 40 kDa and a C-terminal domain of 20 kDa. The 40-kDa domain of the N- and of the C-half phosphorylates bicarbonate and carbamate, respectively, and each hosts a separate ATP site [3]. A tunnel connecting these domains and the small subunit delivers the ammonia and allows the intramolecular migration of carbamate between the phosphorylation centers [3].

The enzyme is feedback inhibited by UMP and activated by ornithine and IMP [1]. IMP and UMP share the same site in the C-terminal 20-kDa domain of the large subunit, called the allosteric domain [4], and ornithine binds between this domain and the carbamate phosphorylation domain [3]. The nature of the allosteric signal or the mechanism of signal transmission to the catalytic machinery has not been clarified. CPS heterodimers associate into oligomers, in part through interactions mediated by the allosteric domain [3], but the possibility that the modulatory signals were transmitted between different CPS molecules was excluded recently using mutations that prevented association [5,6]. Thus, the regulatory signals emerging from the allosteric domain must be transmitted through the interface between this domain and the carbamate phosphorylating domain, which are adjacent in the enzyme structure [3]. As ornithine binds at the interdomain interface, its site may play a key role not only in triggering the changes associated with ornithine modulation, but also, as previously proposed [7], in the transmission of the signals elicited by the binding of the nucleotide effector in the allosteric domain. The present work probes the role of ornithine site residues, by mutating three such residues shown by X-ray crystallography to interact with bound ornithine [3]. The results provide experimental proof of the importance of these residues for ornithine binding and shed light on the mechanism of allosteric control of the activity of the enzyme.

2. Materials and methods

Site-directed mutagenesis using appropriate mutagenic primers, mutation corroboration by DNA sequencing, and expression and purification of wild type and mutant forms of CPS were carried out as described [6,8]. CPS activity was assayed at 37°C in a solution containing 0.1 M Tris–HCl pH 8.0, 0.1 M KCl, 20 mM $KHCO_3$, 10 mM glutamine, 5 mM ATP, 5 mM $MgSO_4$, and the indicated concentrations of the effectors, determining after 10 min the amount of CP or ADP produced [8]. When MgATP (as an equimolar mixture of ATP and $MgSO_4$) or bicarbonate (as $NaHCO_3$) were varied, other components were fixed at the concentrations of the standard assay. When K^+ (as KCl), was varied, ATP and bicarbonate were added as Na salts. The HCO_3^--dependent partial ATPase activity was assayed by omitting glutamine from the standard CPS assay, determining ADP

*Corresponding author. Fax: (34)-96-3601453.
E-mail addresses: rubio@ibv.csic.es (V. Rubio), cervera@ochoa.fib.es (J. Cervera).

Abbreviations: CPS, carbamoyl phosphate synthetase; CP, carbamoyl phosphate

0014-5793/02/$22.00
PII: S0014-5793(02)02392-X

production [8]. The partial reaction of ATP synthesis from ADP and CP was assayed at 37°C in a solution containing 0.1 M Tris–HCl pH 8.0, 0.1 M KCl, 5 mM $MgSO_4$, 5 mM CP, and 5 mM ADP. After 10 min the amount of ATP produced was determined with hexokinase and glucose-6-phosphate dehydrogenase [8]. One enzyme unit is the amount of enzyme producing 1 μmol product (CP, ADP or ATP, for the CPS, ATPase and ATP synthesis activities, respectively)/min. The program GraphPadPrism was used for curve fitting and estimation of the values of kinetic constants. Protein was determined by the method of Bradford [9], using bovine serum albumin as standard.

3. Results

3.1. Influence of the mutations on enzyme activity in the absence of effectors

Glu783 and Glu892, belonging to the carbamate phosphorylation domain, interact through their O^{ε} groups with the δ-amino group of ornithine [3] (Fig. 1), and were mutated to either Ala, Asp or Leu. Thr1042, belonging to the allosteric domain [3], which is hydrogen bonded through its O^{γ} to a carboxylate O atom of ornithine [3] (Fig. 1), was replaced by valine (T1042V), a residue that cannot form this hydrogen bond. Wild type and mutant proteins, overexpressed in *E. coli* (20–40% of the soluble bacterial protein), were purified to homogeneity (checked by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, SDS–PAGE) [8]. Mutation reversion always restored the wild type properties (data not shown).

Mutation E892A activates CPS to a similar degree as ornithine (Table 1). All other mutations reduce the activity to 1/3–2/3 of normal, or even to 1/10 (E783L; Table 1, first column). The mutations of Glu783 lower the activity more than those of Glu892 or Thr1042, and for both Glu783 and Glu892 the activity decreases with the amino acid series Ala, Asp, Leu. The stoichiometry of the reaction was normal (2 mol ADP per mol CP) [1] for all mutations (data not shown). The mutations of Glu783 and E892L decrease the CPS activity and the partial reaction of ATP synthesis (a reaction that reflects the reversal of the step of carbamate phosphorylation) [1] to a similar degree (Fig. 2), but not the ATPase partial activity (a partial reaction that reflects the step of bicarbonate phosphorylation) [1]. Thus, the step of carbamate phosphorylation may be selectively hampered by these mutations. Parallel increases in overall and partial ATPase activities are observed with the mutation E892A (Fig. 2).

At equimolar Mg and ATP, the plot of CPS activity versus MgATP concentration is sigmoidal, and ornithine lowers the $S_{0.5}$ value (Fig. 3B,C). Mutations E783A, E783D and E783L

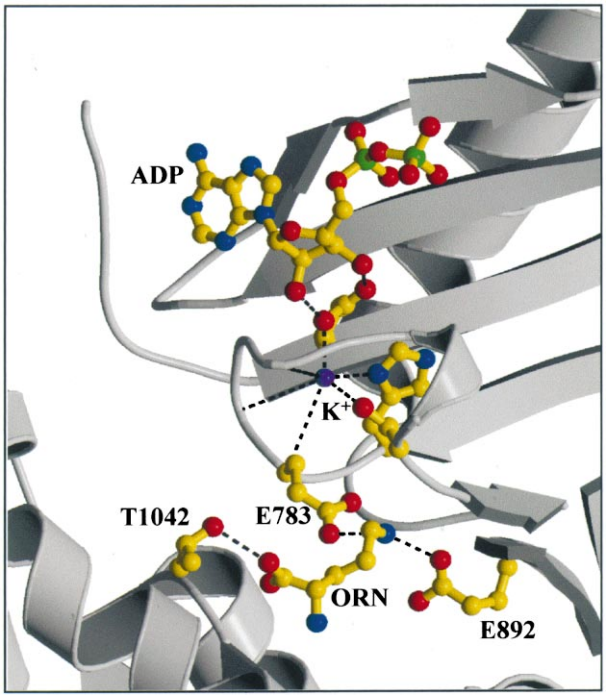

Fig. 1. Ornithine site, K^+-loop and nucleotide site of the carbamate phosphorylation domain of CPS. Drawn using MOLSCRIPT [17] and Raster3D [18] and the coordinates deposited for CPS (PDB 1ce8) [16].

increase substantially the $S_{0.5}$ for both K^+ (approximately to 2.5-, three- and five-fold, respectively) and MgATP (approximately to 1.5-, two- and three-fold, respectively), and the activity extrapolated at either K^+ saturation or MgATP saturation is substantially lower for these mutants (particularly for E783L) than for wild type CPS (Fig. 3A,B). However, their activity at saturation of both MgATP and K^+ might be comparable to that of wild type CPS. Other mutations increase little (E892L, approximately to 1.5- and two-fold), do not increase (E892D and T1042V) or even decrease (E892A; approximately to 1/3) the $S_{0.5}$ for K^+ and MgATP, yielding extrapolated activities at either K^+ saturation or MgATP saturation that only for the mutants E892D and T1042V are substantially lower than those of wild type CPS under the same conditions (Fig. 3A,C). Since the mutations E892D and T1042V do not change substantially the $S_{0.5}$ values for K^+ or MgATP, and none of the mutations changes the requirement for bicarbonate ($K_m^{Bic} = 1$ mM, data not

Table 1
Influence of the mutations on the CPS activity of the enzyme and on its modulation by ornithine, IMP and UMP[a]

Effector	None	+6 mM Orn		+6 mM IMP		+0.5 mM UMP	
	v	v^{Orn}	v^{Orn}/v	v^{IMP}	v^{IMP}/v	v^{UMP}	v^{UMP}/v
Wild type	3.15	7.18	2.28	5.73	1.82	0.65	0.21
E783A	1.40	1.40	1.00	3.63	2.59	0.35	0.25
E783D	1.00	1.24	1.24	2.36	2.36	0.65	0.65
E783L	0.32	0.31	0.98	0.83[b]	2.60[b]	0.25	0.79
E892A	6.08	6.38	1.05	8.15	1.34	4.20	0.69
E892D	1.89	3.65	1.93	3.89	2.06	0.70	0.37
E892L	1.56	1.56	1.00	6.33	4.06	0.58	0.37
T1042V	2.19	2.30	1.16	3.64	1.66	0.92	0.42

[a] v, velocity (U/mg) of the CPS reaction, either in the absence of effectors, or in the presence of the indicated concentration of the effector denoted by the superindex. $v^{effector}/v$ yields the activity at the indicated concentration of the effector, relative to the activity without effectors.
[b] Only 3 mM IMP was used.

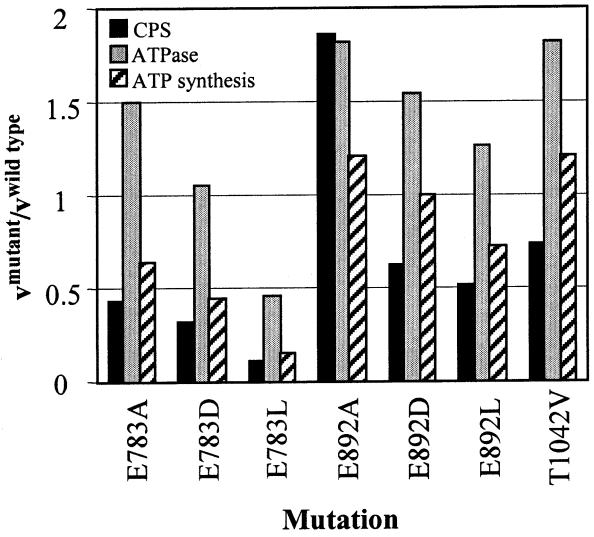

Fig. 2. Influence of the mutations on the velocity of the full CPS reaction (filled bars) and of the bicarbonate-dependent ATPase (open bars) and ATP synthesis (stripped bars) partial reactions.

shown), the lower activity of the E892D and T1042V mutants (Table 1) may indicate that these mutations render CPS intrinsically slower. Mutation E892A mimics the effect of ornithine on both the K^+ and MgATP requirement (Fig. 3A,C). Thus, the increased activity of this mutant in the standard assay at 5 mM MgATP in the absence of effectors (Table 1) is accounted fully by its increased apparent affinity for MgATP, compared with wild type CPS.

3.2. Influence of the mutations on effector modulation

Most mutations abolish ornithine activation (Table 1 and Fig. 4). High ornithine concentrations were found to be inhibitory (Fig. 4B,C), but D-ornithine, which was not an activator at low concentrations, was also inhibitory (data not shown), and thus the inhibition appears non-specific. Only the mutants E892D, E783D and T1042V are activated by L-ornithine, (Table 1, Fig. 4A,B), although with K_a^{Orn} values increased approximately 10-fold for E892D and 2–3 orders of magnitude for E783D and T1042V. The proportion by which the velocity was increased by saturating ornithine was normal for the E892D mutant, but the absolute activity of both the non-activated and the ornithine-saturated enzyme was lower than the corresponding activity of wild type CPS (Table 1, Fig. 4A), supporting the proposal (see above) that the E892D mutation intrinsically decreases the velocity of CPS. The high K_a^{Orn} and the inhibition at high ornithine concentrations (Fig. 4B) prevented accurate estimation of the velocity of the mutants E783D and T1042V at saturation of ornithine.

Similarly to the wild type enzyme, all the mutants are activated by IMP and inhibited by UMP (Table 1). In most cases the apparent affinities for these effectors are similar to those of wild type CPS, although the mutations E783D and T1042V increase K_a^{IMP} approximately 6.5- and 2.2-fold and K_i^{UMP} approximately 4.5- and 10-fold, respectively, and the mutations E783L and E892A increase K_i^{UMP} approximately 10- and 4.5-fold, respectively (Fig. 5A,B). The magnitude of the activation by IMP or of the inhibition by UMP differs substantially between the various mutants and the wild type enzyme

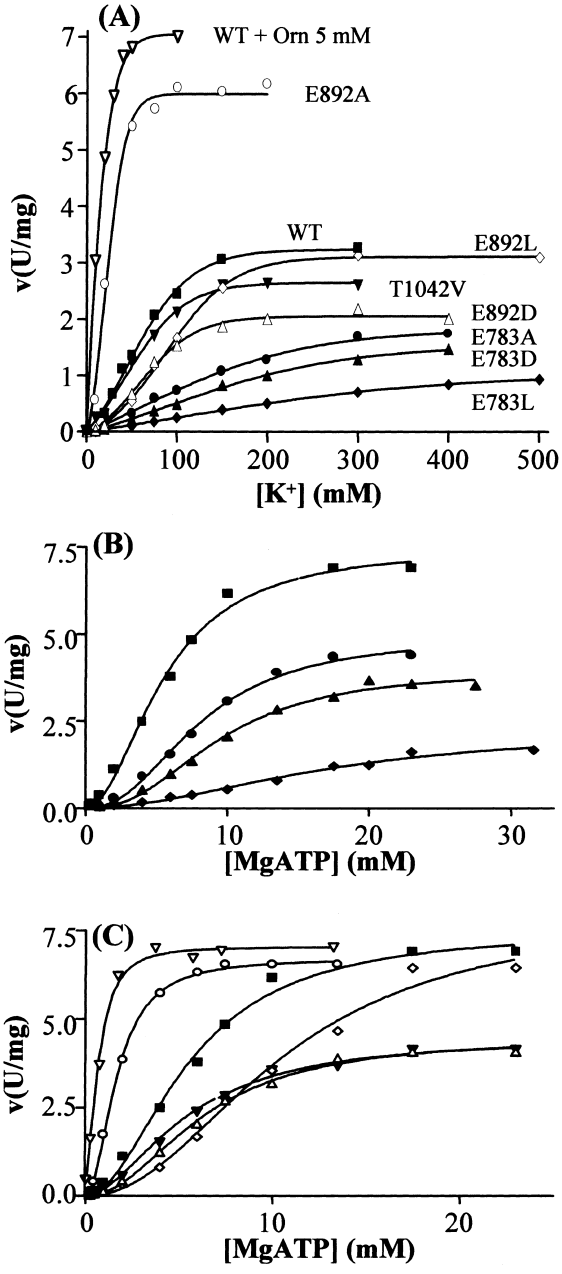

Fig. 3. Dependency of CPS activity on the concentrations of K^+ (A) or MgATP (B and C). (A) gives the key to the symbols used throughout. The lines are sigmoids ($v = V_{max}[S]^n/(K+[S]^n)$) corresponding to the following V_{max} (U/mg), $S_{0.5}$ (mM) and n (Hill number): (A) WT (wild type), 3.42, 61 and 2; WT in the presence of 5 mM ornithine, 7.36, 13 and 1.69; E783A, 2.16, 151 and 1.54; E783D, 1.87, 181 and 1.62; E783L, 1.38, 300 and 1.39; E892A, 6.11, 22 and 2.6; E892D, 2.12, 67 and 2.52; E892L, 3.19, 91 and 2.68; T1042V, 2.82, 57 and 1.96. (B) Wild type, 7.5, 5.3 and 2; E783A, 4.94, 8.1 and 2.3; E783D, 3.97, 9.5 and 2.44; E783L, 2.31, 17.3 and 1.92. (C) Wild type in the presence of 5 mM ornithine, 7.2, 0.95 and 2.2; E892A, 6.72, 1.7 and 2.09; wild type, same as for (B); E892D, 4.46, 6.2 and 2.12; E892L, 7.97, 10.7 and 1.95; T1042V, 4.44, 5.5 and 1.95.

(Table 1). The latter, under the present assay conditions, is activated nearly two-fold by 6 mM IMP and is inhibited approximately 80% by 0.5 mM UMP, whereas the constitutively activated E892A mutant is activated only 30% by IMP and is inhibited 30% by UMP, and the subactive E892L mutant is

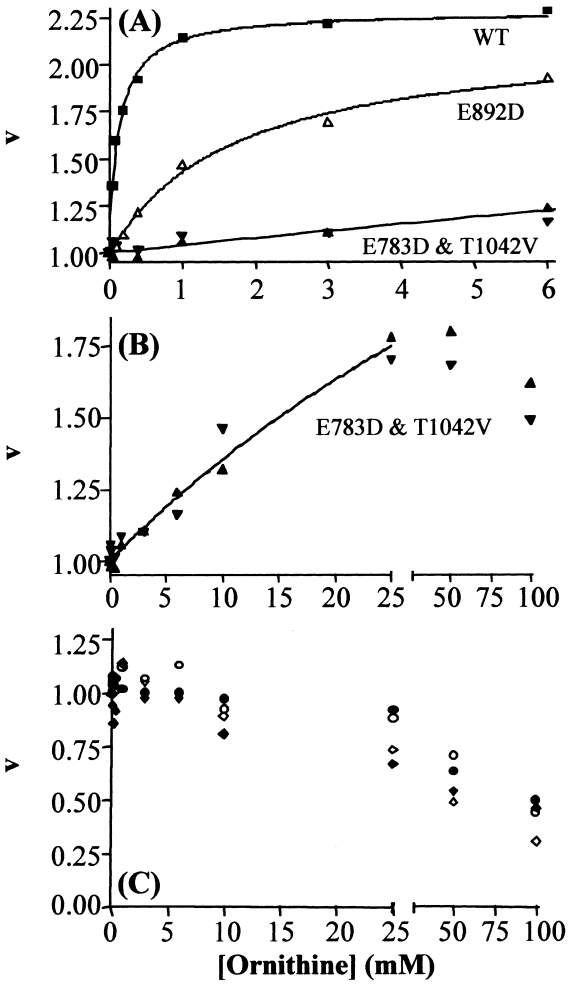

Fig. 4. Dependency of CPS activity on the concentration of ornithine. v is expressed relative to the velocity for the same enzyme form in the absence of ornithine. The symbols used are those of Fig. 3. The lines are the hyperbolae ($v = 1+(\Delta_{max}[Orn]/(K_a^{Orn}+[Orn])$) corresponding to the following Δ_{max} and K_a^{Orn} (mM) values: WT, 1.27, and 0.14; E892D, 1.16 and 1.64 mM; E783D and T1042V, 3 and 75.

activated approximately four-fold by IMP and inhibited approximately 60% by UMP. As a consequence of the abnormal degree of activation, mutants E892A and E892L exhibit at saturation of IMP similar activity as the IMP or ornithine-activated wild type CPS (Table 1). In contrast, the mutations E892D, T1042V and the three mutations affecting E783, decrease the absolute activity of the enzyme both in the absence of effectors and at saturation of IMP (and, in the case of E892D, at saturation of ornithine). The absolute activity of the different enzyme forms at saturation of the inhibitor UMP (Table 1) resembles that of the wild type enzyme, except for the constitutively activated mutant E892A, which has an activity at 0.5 mM UMP (essentially at UMP saturation) nearly seven-fold higher than wild type CPS.

3.3. Influence of the mutations on limited proteolysis of CPS

Limited tryptic digestion nicks CPS at Arg912 of the large subunit [10], and any of the three effectors, ornithine, UMP or IMP, protects the enzyme from this cleavage ([11] and Fig. 6, wild type). Mutation E892A protects the enzyme from tryptic cleavage, and thus this mutation mimics the effect of ornithine also in this assay. None of the other mutations prevents tryptic cleavage. Mutation E783L decreases CPS activity to a similar degree as UMP (Table 1), but its failure to protect CPS from tryptic cleavage indicates that mutation E783L does not trigger the same conformational changes as UMP. In agreement with the large decrease or null apparent affinity for ornithine, all of the mutations except E892D abolished the protection by 4 mM ornithine. With the mutation E892D, which increases approximately 10-fold the K_a^{Orn} (see above), there was slight ornithine protection. The specificity of the effect of the mutations on the allosteric effects of ornithine was confirmed by the preservation of the protection against tryptic cleavage effected by UMP and IMP on the different enzyme mutants. Only in two mutant forms exhibiting an increased K_i^{UMP} and K_a^{IMP}, E783D and T1042V, the protection by UMP and by IMP was less than that observed for the same effectors with the wild type enzyme.

3.4. Influence of the mutations on ornithine binding

The abolition by the mutations of ornithine activation or of ornithine protection against tryptic digestion may result from lack or decreased binding affinity for ornithine or from lack of transmission of the allosteric signal, with normal ornithine binding. Given the localization of the groups mutated [3], lack of binding is the most straightforward explanation. Nevertheless, ultracentrifugal [12] single point binding assays were carried out, using a mixture of 20 μM enzyme (wild type or mutant) and 1 μM [2,3-^{3}H]ornithine (170 000 cpm/ml) in

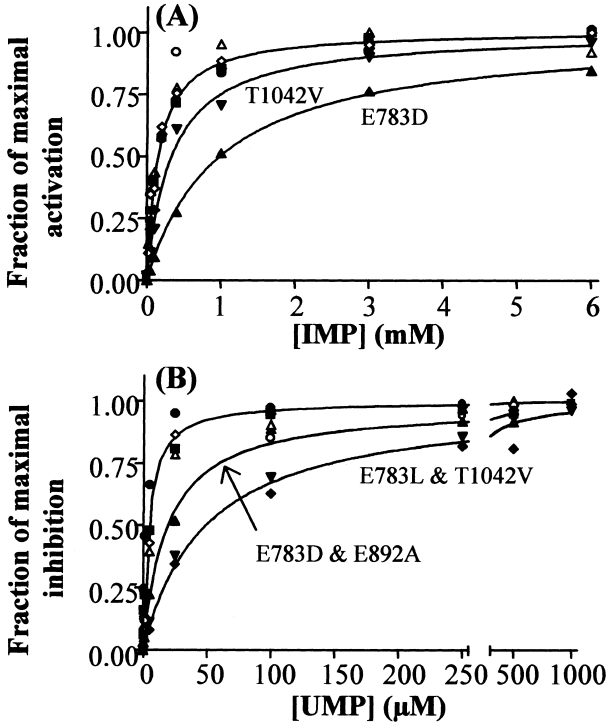

Fig. 5. Dependency of the change in CPS activity on the concentration of (A) IMP or (B) UMP. The change in activity is expressed as a fraction of the maximal change for a given enzyme form and effector. Symbols are those used in Fig. 3. The lines drawn in (A) are the hyperbolae ($v = [IMP]/(K_a^{IMP}+[IMP])$ for the following K_a^{IMP} values: E783D, 0.98 mM; T1042V, 0.33 mM; WT and other mutants, 0.15 mM. Similar hyperbolae are drawn in (B) for the following K_i^{UMP} values: E783L and T1042V, 49 μM; E783D and E892A, 19 μM; wild type and other mutants, 4 μM.

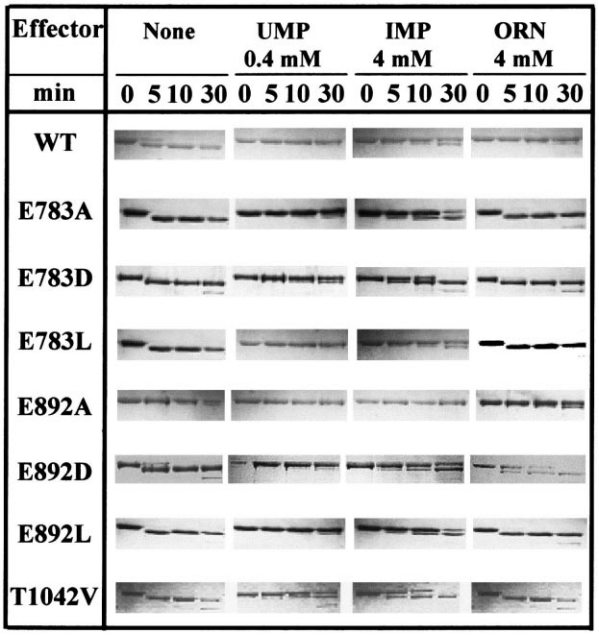

Fig. 6. Influence of the mutations and of UMP, IMP and ornithine on limited tryptic cleavage of CPS. Digestion of 1.2 mg/ml wild type or mutant CPS with 2 μg/ml trypsin in 0.1 M pH 7.6 phosphate buffer in the presence of the indicated type and concentration of effector was carried out as described [10]. After the indicated periods, samples were boiled in SDS and subjected to SDS–PAGE in 7.5% gels. The Coomassie-stained CPS large subunit and derived large cleavage product are shown.

0.1 M Tris–HCl pH 8.0, 0.1 M KCl, containing 2 mg/ml Dextran T10, and 10 mM MgADP and 7% glycerol (MgADP and glycerol decrease, respectively, the K_d and K_a values for ornithine [13,14]). The decrease in the radioactivity in the top of the tube upon ultracentrifugation reflects the ornithine carried down bound to the protein. With the wild type enzyme, the decrease in the radioactivity was $53 \pm 9\%$ (three replicate assays), consistent with a K_d^{Orn} of 18 μM, assuming a single binding site for ornithine per enzyme heterodimer. With the mutants no significant decrease in the radioactivity was found, indicating an increase in the K_d^{Orn} of at least 10-fold (a detection limit corresponding to a radioactive decrease of 10% would represent a K_d^{Orn} of 0.18 mM). Thus, the ultracentrifugation assays confirm the lack or drastically decreased binding of ornithine.

4. Discussion

The removal of the interacting γ-COO^- explains the lack of detectable ornithine effects in mutants E783A, E783L, E892A and E892L, since simply the loss of one typical hydrogen bond, by removing 5 kcal/mol [15] from the energy of ornithine binding, should increase K_d^{Orn} approximately 5000-fold (calculated from $\Delta(\Delta G^0) = -RT\ln(K_d^{Orn}(\text{mutant})/K_d^{Orn}(\text{wild type}))$. The increase of 2–3 orders of magnitude in K_a^{Orn} for mutations T1042V and E783D, corresponding to a decrease of 3–4 kcal/mol in ornithine binding energy, is still compatible with the loss of a weaker hydrogen bond [15]. However, mutation E783D and also mutation E892D are more likely to weaken rather than to abolish the normal interactions with ornithine, since the interacting side-chain –COO^- is preserved, although with altered geometry. This would explain the relatively small increase (10-fold) in K_a^{Orn} for the E892D mutation, corresponding to the loss of only < 1.5 kcal/mol from the energy of binding of ornithine, much too little even for the loss of a weak hydrogen bond [15].

The increases in K_a^{IMP} and/or K_i^{UMP} caused by mutations T1042V, E783D, E783L and E892A, although small ($\leq$10-fold) when compared with the corresponding increases in K_a^{Orn}, indicate that changes in the ornithine site can propagate to the nucleotide effector site. Previously the mutation T1042I, identified in *E. coli* having altered regulation of arginine biosynthesis, was shown to induce in CPS very similar effects to those found here for mutation T1042V, including the triggering of substantial changes in IMP and UMP modulation [7]. In addition, the mutations S948F [7] and T974A [8], affecting the nucleotide effector site and having major effects on UMP and IMP binding and modulation, were shown to affect also ornithine modulation. Thus, the present evidence provides additional support to the proposal [7] of coupling effects between the ornithine and the nucleotide effector sites. This coupling may be mediated by hydrogen bond networks linking these sites, such as the network identified already that involves centrally Thr1042 [16] and that extends through bound ornithine to Glu783. The functionality of this network is supported by the effects of the T1042I [7] and T1042V (present work) mutations.

Glu783 is coordinated through its backbone O atom with a bound K^+ ion in the neighborhood of the site for the ATP that phosphorylates carbamate, being a part of the K-loop that coordinates the K^+ (Fig. 1) [3]. This flexible loop contours bound ATP, and the K^+ is coordinated also with Glu761, a residue making hydrogen bonds through its γ-COO^- with the 2′- and 3′-OH groups of the ribose [3]. It is therefore understandable that both ornithine, by binding to Glu783, and the mutations at this residue, by perhaps altering the conformation of the K^+-loop as an indirect consequence of aberrant contacts of the mutated side-chain, may affect the K^+-dependency of CPS activity and the requirement of the enzyme for ATP, as observed here.

Mutations E892A and E892L may affect the allosteric equilibrium of CPS. E892L is less active than wild type CPS but can be activated fully by IMP, whereas E892A resembles ornithine-activated CPS in having high activity and high apparent affinities for K^+ and MgATP, in being little inhibited by UMP, and in being refractory to tryptic cleavage. Thus, Glu892 may be highly involved in the conformational changes that trigger allosteric activation of CPS. Lys891, the residue adjacent in the sequence to Glu892, is at the opening at the carbamate phosphorylation site of the intramolecular tunnel that delivers the carbamate [3]. Changes in the geometry of Glu892 caused by ornithine binding or by mutation may influence the position of Lys891 and the patency of the tunnel and have an important effect on enzyme activity. Thus, conformational changes affecting this tunnel, and changes in the K^+-loop, at the carbamate phosphorylation site, are likely to be the main effector mechanisms of allosteric control of *E. coli* CPS activity.

Acknowledgements: Supported by grants PM97-0134-CO2 and BMC2001-0251 from the Dirección General de Enseñanza Superior (DGES). L.R. is a Predoctoral Fellow of the Fundación Ferrer and V.F. was a recipient of a predoctoral fellowship from the FVIB. We thank Dr. Carol Lusty for providing *E. coli* strains and plasmids and Dr. Santiago Ramón-Maiques for help with Fig. 1.

References

[1] Meister, A. (1989) Adv. Enzymol. Relat. Areas Mol. Biol. 62, 315–374.
[2] Nyunoya, H. and Lusty, C.J. (1983) Proc. Natl. Acad. Sci. USA 80, 4629–4633.
[3] Thoden, J.B., Raushel, F.M., Benning, M.M., Rayment, I. and Holden, H.M. (1999) Acta Crystallogr. D 55, 8–24.
[4] Bueso, J., Cervera, J., Fresquet, V., Marina, A., Lusty, C.J. and Rubio, V. (1999) Biochemistry 38, 3910–3917.
[5] Kim, J. and Raushel, F.M. (2001) Biochemistry 40, 11030–11036.
[6] Mora, P., Rubio, V. and Cervera, J. (2002) FEBS Lett. 511, 6–10.
[7] Delannay, S., Charlier, D., Tricot, C., Villeret, V., Pierard, A. and Stalon, V. (1999) J. Mol. Biol. 286, 1217–1228.
[8] Fresquet, V., Mora, P., Rochera, L., Ramón-Maiques, S., Rubio, V. and Cervera, J. (2000) J. Mol. Biol. 299, 979–991.
[9] Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
[10] Bueso, J., Lusty, C.J. and Rubio, V. (1994) Biochem. Biophys. Res. Commun. 203, 1083–1089.
[11] Rubio, V., Cervera, J., Lusty, C.J., Bendala, E. and Britton, H.G. (1991) Biochemistry 30, 1068–1075.
[12] Howlett, G.J., Yeh, E. and Schachman, H.K. (1978) Arch. Biochem. Biophys. 190, 809–819.
[13] Anderson, P.M. (1977) Biochemistry 16, 587–593.
[14] Rubio, V. and Llorente, P. (1982) Biochem. Biophys. Res. Commun. 107, 1400–1405.
[15] Fersht, A. (1977) Enzyme Structure and Mechanism, WH Freeman and Co., San Francisco, CA.
[16] Thoden, J.B., Raushel, F.M., Wesenberg, G. and Holden, H.M. (1999) J. Biol. Chem. 274, 22502–22507.
[17] Kraulis, P.J. (1991) J. Appl. Crystallogr. 24, 946–950.
[18] Merritt, E.A. and Murphy, M.E.P. (1994) Acta Crystallogr. D 50, 869–873.