Localization of the site for the nucleotide effectors of *Escherichia coli* carbamoyl phosphate synthetase using site-directed mutagenesis

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Abstract Replacement by alanine of Ser-948, Thr-974 and Lys-954 of *Escherichia coli* carbamoyl phosphate synthetase (CPS) shows that these residues are involved in binding the allosteric inhibitor UMP and the activator IMP. The mutant CPSs are active in vivo and in vitro and exhibit normal activation by ornithine, but the modulation by both UMP and IMP is either lost or diminished. The results demonstrate that the sites for UMP and IMP overlap and that the activator ornithine binds elsewhere. Since the mutated residues were found in the crystal structure of CPS near a bound phosphate, Ser-948, Thr-974 and Lys-954 bind the phosphate moiety of UMP and IMP.

Key words: Carbamoyl phosphate synthetase; Allosteric regulation; Site-directed mutagenesis; Arginine synthesis; Pyrimidine synthesis; *car*B

1. Introduction

Carbamoyl phosphate synthetase (CPS) catalyzes the first committed step in the routes of biosynthesis of pyrimidines, arginine and urea [2ATP+HCO_3^-+NH_3 (glutamine) $\rightarrow$2ADP+carbamoyl phosphate+P_i (+glutamate)], and is in many cases regulated allosterically [1,2]. The effectors vary with the source and the physiological function of the CPS. CPS I is involved in the synthesis of urea and is activated by *N*-acetyl-L-glutamate, whereas the *Escherichia coli* enzyme, which is involved in the synthesis of arginine and of pyrimidines, is activated by ornithine and IMP and is inhibited by UMP, and CPS II, which participates in the synthesis of pyrimidines in animals, is activated by phosphoribosyl pyrophosphate and is inhibited by UTP.

The structural bases of the allosteric modulation of CPS are currently being elucidated. Different CPSs exhibit a similar structural plan [3,4], epitomized by the *E. coli* enzyme. This enzyme is composed of a small subunit of 41.4 kDa, which binds and cleaves glutamine, and a large subunit of 117.7 kDa, which synthesizes carbamoyl phosphate from NH_3 and binds the effectors [2] in its C-terminal domain of approximately 20 kDa [4]. We carried out photoaffinity labeling experiments with the inhibitor UMP and the activator IMP that revealed the cross-linking of these effectors to Lys-993 [5] and His-995 [6], respectively, two amino acid residues that are found approximately at the middle of the amino acid sequence of this domain. Based on the photoaffinity labeling by UMP and IMP at these residues and on the effects of replacing His-995 by alanine on the binding of the two nucleotide effectors [6], we confirmed that these effectors bind at overlapping sites, and we proposed the involvement of specific sequence regions around Lys-993 in the formation of the binding site for the nucleotide effectors [5,6]. Recently the three-dimensional structure of *E. coli* CPS was determined and a P_i ion was localized binding to the crystal in the region assigned by us to the effector nucleotide site [7]. We have used the position of this P_i and the sites of photoaffinity labeling by UMP and IMP to model the bound effector nucleotides [6] (Fig. 1). We now subject to experimental testing this model by replacing by alanine three key polar residues that interact with the P_i. If such P_i truly represents the phosphate group of the nucleotide effectors, the negative effect expected to be caused on the binding of the P_i by the replacement of the polar groups of these residues by the apolar and smaller side chain of alanine should selectively hamper the modulation by UMP and IMP. The results reported here confirm the importance of these residues in the binding of the nucleotide effectors.

2. Materials and methods

2.1. Site-directed mutagenesis and expression of mutant enzymes

Standard protocols were used for transformation of *E. coli* and for isolation, digestion and ligation of DNA [8]. Plasmid DNA from pLLK12 [9] (which contains the *car*AB gene inserted in the *Bam*HI site of pUC19; *car*A and *car*B encode the small and large CPS subunits) was digested with *Sma*I, and the DNA fragment (897 bp) encoding the C-terminal region of *car*B was isolated electrophoretically and was ligated with the *Sma*I site of bacteriophage M13mp19. Site-directed mutagenesis was carried out [10], using uracil-containing single-stranded DNA generated in *E. coli* strain CJ236 [10] and the antisense oligonucleotides 5′-GCCTTCGCGTACGGCAAGCAGC-G-3′, 5′-CGTTCGGCGTCGCCTTCG-3′ and 5′-GCCGTGCCATG-GGCCGCATCCAGC-3′, in which the original triplets GGA, TTT and GGT (complementary to the TCC, AAA and ACC codons encoding Ser-948, Lys-954 and Thr-974 of *car*B, respectively) are replaced by the underlined triplet (complementary to the GCC codon for alanine). The mutations were corroborated by manual sequencing [11] of single-stranded DNA isolated from individual colonies of DH5α cells (from Clontech) transformed with the M13 vector containing the mutated insert.

The mutant *car*AB genes were constructed by replacement of the wild-type *Sma*I fragment of *car*B with the corresponding mutant fragment carrying the desired mutation. The resultant plasmids pLLK12S948A, pLLK12K954A and pLLK12T974A were sequenced again to confirm the presence of the mutation and the correctness of the construction, and were used to transform *E. coli* strain L814 (*car*AB$^-$) [9]. The mutant CPSs were expressed in *E. coli* L814 strain and grown overnight in LB medium. After spinning down the cells and resuspension of the precipitate in 6 ml/g cell paste of K-phosphate buffer 0.12 M, pH 7.6 containing 1 mM EDTA, the cell suspensions were sonicated and centrifuged at 4°C and the supernatant was used for protein [12] and enzyme activity assays, SDS-PAGE [13], blotting

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Abbreviations: CPS, carbamoyl phosphate synthetase; EDTA, ethylene diamine tetraacetic acid; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate

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and immunophosphatase staining [14] using a monoclonal mouse IgG1 (MabCPSEc1) raised [14] against wild-type *E. coli* CPS.

2.2. Enzyme activity assays

Samples of the supernatants were desalted by centrifugal gel filtration [15] through Sephadex G-50 equilibrated with the assay buffer. CPS activity was determined by measuring as hydroxyurea at 450 nm [16] the carbamoyl phosphate formed during 10 min incubation at 37°C in a solution of 0.1 M Tris-HCl, pH 8, 0.1 M KCl, 10 mM glutamine, equimolar ATP and $MgCl_2$ (5 or 10 mM), 20 mM $NaHCO_3$ and the indicated concentration of either ornithine, UMP or IMP. When testing the activation by ornithine, the assay solution was supplemented with 25 U/ml ornithine transcarbamylase (from Sigma) and the citrulline formed was determined identically to the hydroxyurea at 450 nm. Controls made by adding carbamoyl phosphate to the solutions confirmed [16] that the color yield is the same with hydroxylamine conversion to hydroxyurea and with ornithine transcarbamylase conversion to citrulline.

3. Results

E. coli strain L814 has no CPS activity [9] and cannot grow in minimal medium [17] unless uracil and arginine are added (proline and thiamine are also needed by L814 cells, and are added when using minimal medium, in amounts of 0.1 mg/ml and 1 μg/ml, respectively). L814 cells transformed with plasmid pLLK12, which carries the wild-type *car*AB genes, regain the ability to grow in minimal medium. L814 cells transformed with plasmids pLLK12S948A, pLLK12K954A or pLLK12T974A, which carry the *car*AB genes with the *car*B mutations Ser-948-Ala, Lys-954-Ala or Thr-974-Ala respectively, also grew similarly in minimal medium. Therefore, the mutant enzymes are expressed and are functional in vivo. The amount of CPS produced, estimated densitometrically from SDS-PAGE of extracts of the transformed cells, stained with Coomassie or immunostained in Western blots (Fig. 2), represented 16.5% of the protein in the extract for wild-type CPS and 8.8%, 6% and 13% for the CPSs with the mutations Ser-948-Ala, Lys-954-Ala and Thr-974-Ala, respectively. Enzyme activity (assayed by coupling with ornithine transcarbamylase as indicated in Section 2, in the presence of 10 mM ATP, 20 mM $MgCl_2$ and 4 mM ornithine) was, in μmol/min/mg protein in the extract, 0.79, 0.13, 0.07 and 1.9

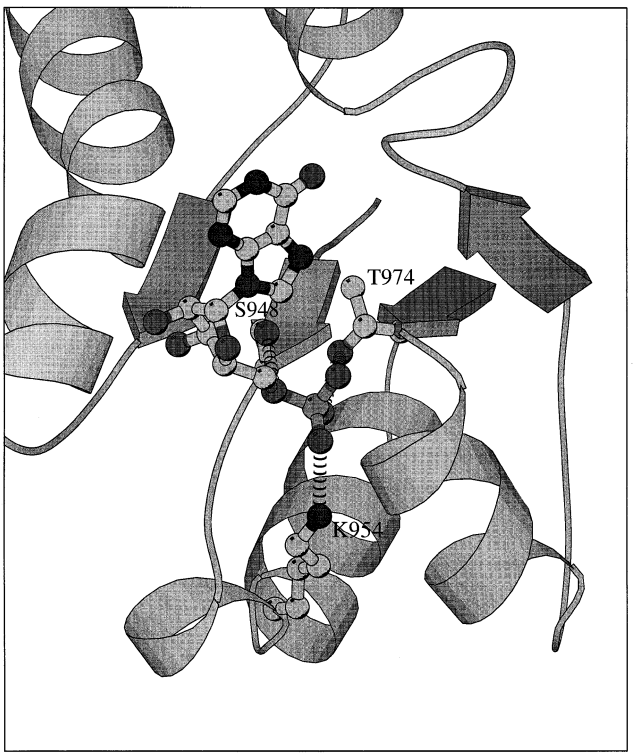

Fig. 1. Ribbon representation of the putative nucleotide effector binding site of *E. coli* CPS, with IMP shown in ball and stick representation. The side chains of Ser-948, Lys-954 and Thr-974 are shown explicitly in ball and stick representation. Broken sticks represent hydrogen bonds with the phosphate group of IMP (shown in a darker shade of gray). The model was built using program O and the coordinates deposited for carbamoyl phosphate synthetase (PDB 1JDB) [7]. IMP structure was taken from the library of heterocompounds of Uppsala University (http://alpha2.bmc.uu.se/hicup/). The nucleotide was positioned manually with the purine ring properly positioned to account for the photoaffinity labeling of His-995 [6] and with the phosphate at the position of the phosphate ion present in the reported enzyme structure [7], keeping all atoms of the nucleotide at a distance of at least 2 Å from any atom of the protein, to avoid potential steric problems.

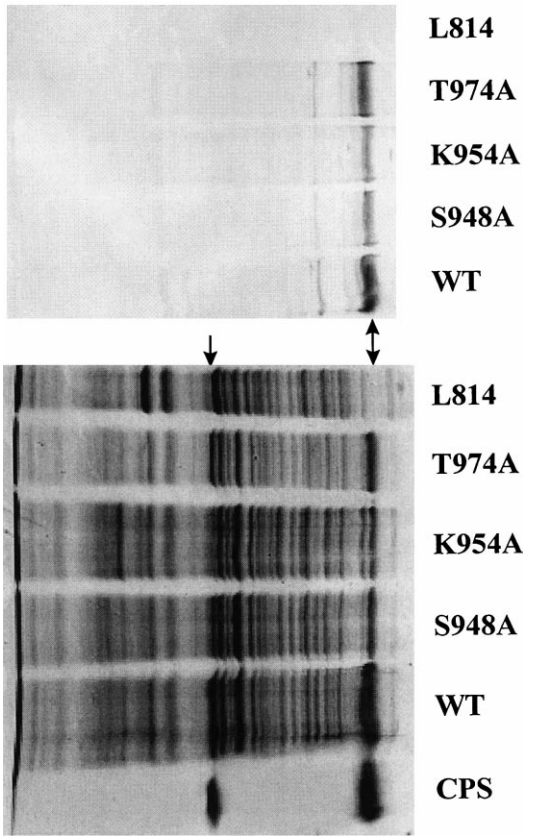

Fig. 2. SDS-PAGE analysis (9% polyacrylamide) of the expression of CPS (either wild-type, WT, or mutant, represented by the one-letter convention for amino acid substitutions) in the bacterial cell extracts used in the present studies. L814 denotes extracts of the bacterial cells used for transformation. CPS denotes the purified wild-type *E. coli* CPS. The double-pointed and single-pointed arrows signal the large and small CPS subunits. Left panel: Immunoblot analysis using as primary antibody MabCPSEc1 (0.01 mg/ml) and alkaline phosphatase immunostaining with a conjugate (from Promega) of rabbit anti-mouse IgG. Right panel: Coomassie staining. CPS was quantitated densitometrically by comparison with immunoblots and Coomassie-stained gels of appropriate amounts of purified *E. coli* CPS, using for immunoblots a computer scanner and the analysis program Sigmagel (from Sigma), and for gels an LKB Ultroscan densitometer.

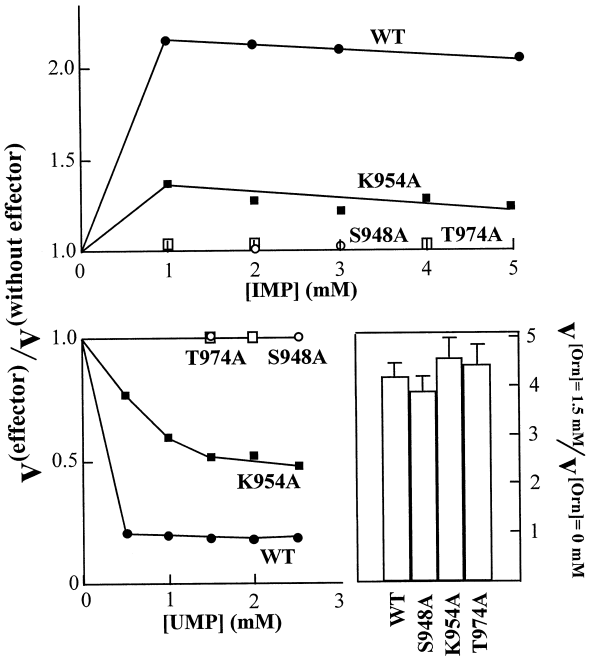

Fig. 3. Influence of IMP, UMP and ornithine on enzyme activity of wild-type (WT) or mutant CPS. The mutations are indicated in single-letter notation. ATPMg concentration was 10 mM in the inhibition assays with UMP and 5 mM in the activation assays with IMP and ornithine. Ornithine was used at a concentration of 1.5 mM.

for the extracts of cells expressing wild-type, Ser-948-Ala, Lys-954-Ala and Thr-974-Ala CPS forms, respectively. Using the densitometric estimates of CPS content in the corresponding extracts, the estimated specific activity of the pure enzyme is 4.76 μmol/min/mg for wild-type CPS and 1.45, 1.20 and 1.46 μmol/min/mg for the Ser-948-Ala, Lys-954-Ala and Thr-974-Ala mutant forms, respectively. The specific activity estimated for the wild-type enzyme is within the expected range for pure *E. coli* CPS [18,19]. The lower estimated specific activity of the mutant forms of CPS does not reflect substantial differences in enzyme solubility, since the mutant forms, similarly to wild-type CPS, appeared mainly in the supernatant after centrifugation of the sonicates of the transformed cells.

Fig. 3 shows that the Ser-948-Ala and Thr-974-Ala mutations abolish the ability of the enzyme to be activated or inhibited by IMP or UMP, respectively. The CPS with the Lys-954-Ala mutation is still activated by IMP and inhibited by UMP, but the degree of activation or inhibition attained is considerably smaller than for the wild-type enzyme, and the concentrations of UMP needed for inhibition are approximately two orders of magnitude higher than for wild-type (K_i for UMP $\cong$ 5 μM for wild-type CPS [6]). In contrast with the detrimental effects of these mutations on the modulation by the nucleotide effectors, the mutants are activated by ornithine similarly to wild-type CPS (Fig. 3).

4. Discussion

The large CPS subunit has been long known [18] to host the sites for the effectors. The sites for UMP and IMP have been localized more recently [19,20] in the C-terminal 20 kDa domain, which is known as the regulatory or allosteric domain because it is involved in the different CPSs in the regulatory processes triggered by the various effectors of these enzymes [21]. The fact that the three residues mutated here are found in this domain and that the mutations abolish or hamper the modulation of the enzyme by UMP and IMP provides additional proof for the localization of the sites for these nucleotide effectors in this domain. Boettcher and Meister [22,23] proposed on the basis of experiments with analogs of UMP and IMP that these two effectors bind at overlapping sites. Confirmation of this view is provided by the present results and by our recent findings that UMP and IMP are cross-linked by UV light to Lys-993 [5] and His-995 [6] and that the mutation His-995-Ala [6] selectively affects the modulation by both nucleotide effectors. However, the present effects are much more dramatic than those triggered by the His-995-Ala mutation, which only decreased the apparent affinity for the nucleotide effectors by less than one order of magnitude, whereas even in the less deleterious of the present mutations, Lys-954-Ala, the apparent affinity for UMP is decreased not less than two orders of magnitude. In agreement with our findings, the enzyme with the mutation Ser-948-Phe was reported [24] to be insensitive to purine and pyrimidine nucleotides. Thus, the present results constitute conclusive proof that UMP and IMP share the same site on the enzyme. In addition, given the lack of effect of the present mutations on the activation by ornithine, our results agree with the binding in different sites of ornithine and of the nucleotide effectors. This solves doubts on the localization of the site for ornithine that were raised by the report of ornithine binding at two sites in the crystal structure of the enzyme, one in the neighborhood of the phosphate, within the putative effector nucleotide site, and the other at the fringe between the C-terminal domain and the remainder of the large subunit [7]. The present results agree with the latter localization of the site for ornithine, an activator that was previously shown to bind to the enzyme with an stoichiometry of only an ornithine molecule per enzyme molecule [25].

Another question solved by the present studies is the demonstration that the P_i found in CPS crystals occupies the site for the phosphate group of the effectors UMP and IMP. P_i itself was also reported (see [2]) to be an effector of *E. coli* CPS, having a role in the 'fine tuning' of the allosteric regulation of the enzyme. Interference of the present mutations with the binding of such effector P_i would be expected to cause only modest changes in the responses of the enzyme to the nucleotide effectors rather than the dramatic changes observed here. Thus, in agreement with the model shown in Fig. 1, the three residues that have been mutated here appear to participate in the site for the effector nucleotide and, more precisely, of the subsite for the nucleotide phosphate. The fact that the mutations Ser-948-Ala and Thr-974-Ala abolish and the mutation Lys-954-Ala has a partial effect on both activation by IMP and inhibition by UMP suggests that in enzyme-bound UMP and IMP the position of the phosphate group, relative to the enzyme, is identical. Therefore, the different modulatory effects exerted by UMP and IMP are due to differences in the binding to the same site of other parts of the effector molecule that deserve further investigation.

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References

[1] Rubio, V., Britton, H.G. and Grisolia, S. (1983) Mol. Cell. Biochem. 53/54, 279–298.
[2] Meister, A. (1989) Adv. Enzymol. Relat. Areas Mol. Biol. 62, 315–374.
[3] Simmer, J.P., Kelly, R.E., Rinker, A.G., Scully, J.L. and Evans, D.R. (1990) J. Biol. Chem. 265, 10395–10402.
[4] Rubio, V. (1994) in: Carbon Dioxide Fixation and Reduction in Biological and Model Systems (Bränden, C.-I. and Schneider, G., Eds.), pp. 249–264, Oxford University Press, Oxford.
[5] Cervera, J., Bendala, E., Britton, H.G., Bueso, J., Nassif, Z., Lusty, C.J. and Rubio, V. (1996) Biochemistry 35, 7247–7255.
[6] Bueso, J., Cervera, J., Fresquet, V., Marina, A., Lusty, C.J. and Rubio, V. (1999) Biochemistry (in press).
[7] Thoden, J.B., Holden, H.M., Wesenberg, G., Raushel, F.M. and Rayment, I. (1997) Biochemistry 36, 6305–6316.
[8] Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A. and Strühl, K. (1997) Current Protocols in Molecular Biology, Wiley Interscience, New York.
[9] Guillou, F., Liao, H., García-España, A. and Lusty, C.J. (1992) Biochemistry 31, 1656–1664.
[10] Kunkel, T.A., Roberts, J.D. and Zakour, R.A. (1987) Methods Enzymol. 154, 367–382.
[11] Sanger, F., Nieklen, S. and Coulson, A.R. (1977) Proc. Natl. Acad. Sci. USA 74, 5463–5467.
[12] Bradford, M. (1976) Anal. Biochem. 72, 248–254.
[13] Laemmli, U.K. (1970) Nature 227, 680–685.
[14] Harlow, E. and Lane, D. (1988) Antibodies. A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
[15] Penefsky, H.S. (1977) J. Biol. Chem. 252, 2891–2899.
[16] Pierson, D.L. (1980) J. Biochem. Biophys. Methods 3, 31–37.
[17] Rebollo, J.E., Moreau, P.L., Blanco, M. and Devoret, R. (1984) Mol. Gen. Genet. 195, 83–89.
[18] Trotta, P.P., Pinkus, L.M., Haschemeyer, R. and Meister, A. (1974) Biochemistry 249, 492–499.
[19] Rubio, V., Cervera, J., Lusty, C.J., Bendala, E. and Britton, H.G. (1991) Biochemistry 30, 1068–1075.
[20] Bueso, J., Lusty, C.J. and Rubio, V. (1994) Biochem. Biophys. Res. Commun. 203, 1083–1089.
[21] Cervera, J., Conejero-Lara, F., Ruiz-Sanz, J., Galisteo, M.L., Mateo, P.L., Lusty, C.J. and Rubio, V. (1993) J. Biol. Chem. 17, 12504–12511.
[22] Boettcher, B. and Meister, A. (1981) J. Biol. Chem. 256, 5977–5980.
[23] Boettcher, B. and Meister, A. (1982) J. Biol. Chem. 257, 13971–13976.
[24] Delannay, S., Tricot, C., Charlier, D., Stalon, V. and Piérard, A. (1996) Arch. Physiol. Biochem. 104, B5.
[25] Anderson, P.M. (1977) Biochemistry 16, 587–593.