

## Review

# Carbamoyl phosphate synthetase: an amazing biochemical odyssey from substrate to product

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**Abstract.** Carbamoyl phosphate synthetase (CPS) catalyzes one of the most remarkable reactions ever described in biological chemistry, in which carbamoyl phosphate is produced from one molecule of bicarbonate, two molecules of  $Mg^{2+}$  ATP, and one molecule of either glutamine or ammonia. The carbamoyl phosphate so produced is utilized in the synthesis of arginine and pyrimidine nucleotides. It is also employed in the urea cycle in most terrestrial vertebrates. Due to its large size, its important metabolic role, and the fact that it is highly regulated, CPS has been the focus of intensive investigation for nearly 40 years. Numerous enzymological, biochemical, and biophysical studies by a variety of investigators have led to a quite detailed

understanding of CPS. Perhaps one of the most significant advances on this topic within the last 2 years has been the successful X-ray crystallographic analysis of CPS from *Escherichia coli*. Quite unexpectedly, this structural investigation revealed that the three active sites on the protein are widely separated from one another. Furthermore, these active sites are connected by a molecular tunnel with a total length of approximately 100 Å, suggesting that CPS utilizes this channel to facilitate the translocation of reaction intermediates from one site to another. In this review, we highlight the recent biochemical and X-ray crystallographic results that have led to a more complete understanding of this finely tuned instrument of catalysis.

**Key words.** Carbamoyl phosphate synthetase; substrate channeling; pyrimidine metabolism; arginine biosynthesis; ATP-grasp enzyme; amidotransferase.

### Introduction

Carbamoyl phosphate synthetase (CPS) is one of those enzymes rarely discussed in detail in most introductory biochemistry courses offered on today's college campuses, especially those crammed into one semester of study. And yet, without this enzyme, life would not exist as we know it for there would be no synthesis of arginine or production of pyrimidine nucleotides in the

cell. Indeed, CPS catalyzes one of the most remarkable reactions ever described in biochemistry, as outlined in figure 1 [1].

Biochemical evidence for this catalytic mechanism has been obtained from partial reaction studies, kinetic investigations, and positional isotope exchange experiments [2–5]. In the first step of the reaction, bicarbonate is activated at the expense of a  $Mg^{2+}$  ATP molecule to yield the first intermediate, carboxyphosphate. This intermediate is calculated to have a half-life of approximately 70 ms [6]. Concurrent with the activa-

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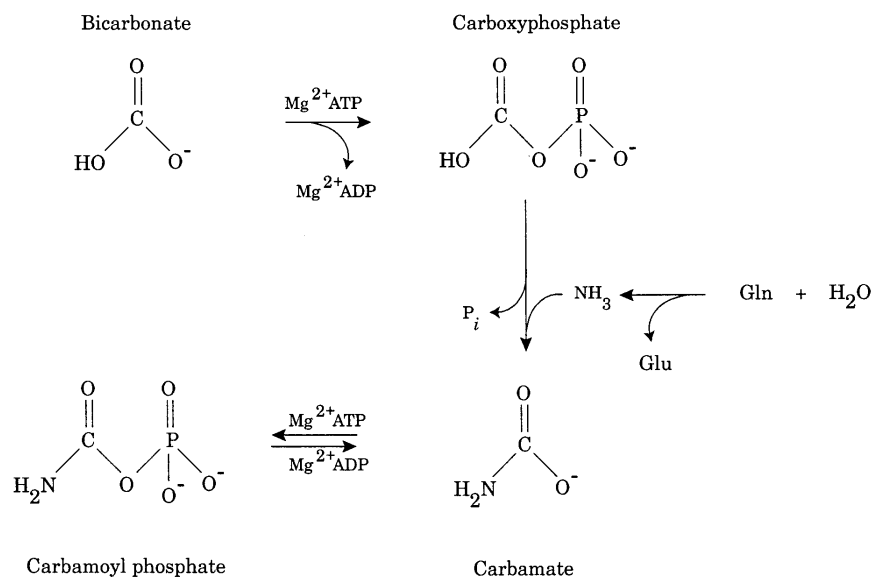


Figure 1. Scheme of the reaction catalyzed by carbamoyl phosphate synthetase.

tion of bicarbonate, glutamine is hydrolyzed to glutamate and ammonia. In the ensuing step, the ammonia conducts a nucleophilic attack on the carboxyphosphate intermediate to yield the third intermediate, carbamate which is even more unstable, with a half-life of 28 ms at neutral pH [7]. In the final step, the carbamate conducts a nucleophilic attack on the  $\gamma$ -phosphate of the second molecule of  $\text{Mg}^{2+}\text{ATP}$  to yield the product. Once formed, carbamoyl phosphate is employed in the production of pyrimidines and arginine in prokaryotes and eukaryotes and in the urea cycle in most terrestrial vertebrates [8, 9]. Note that in eukaryotic systems there are two distinct forms of CPS: a mitochondrial entity (CPS I) that participates in both arginine biosynthesis and the urea cycle and a cytosolic species (CPS II) involved in pyrimidine biosynthesis. The mitochondrial and cytosolic forms of CPS require free ammonia and glutamine, respectively, as nitrogen sources. In addition, CPS II occurs as a multienzymatic complex along with aspartate transcarbamoylase and dihydroorotase [10, 11]. Unlike the eukaryotic systems, prokaryotes have only one form of CPS for both metabolic pathways and this molecular species employs glutamine as the source of nitrogen. A comprehensive review of the fundamental biochemistry of CPS can be found in Meister [12]. For the production of pyrimidine nucleotides, carbamoyl phosphate condenses with aspartate to yield *N*-carbamoyl aspartate, a reaction catalyzed by aspartate transcarbamoylase as outlined in figure 2.

*N*-carbamoyl aspartate is ultimately converted to uridine monophosphate (UMP) by the subsequent action of four enzymes: dihydroorotase, dihydroorotate dehydrogenase, orotate phosphoribosyl transferase, and

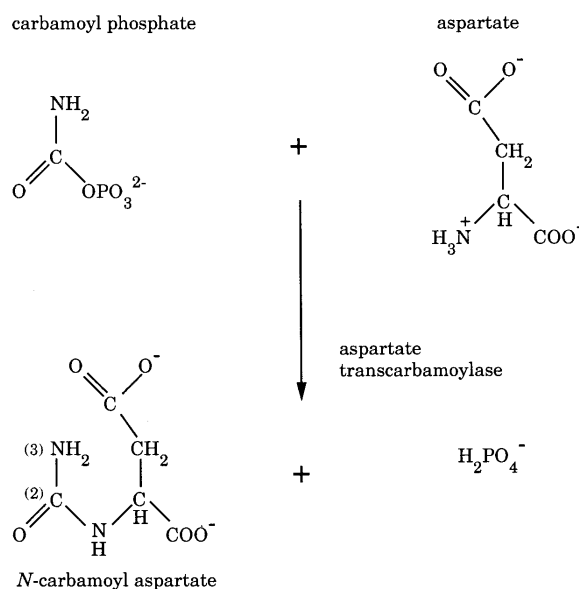


Figure 2. The production of *N*-carbamoyl aspartate from carbamoyl phosphate.

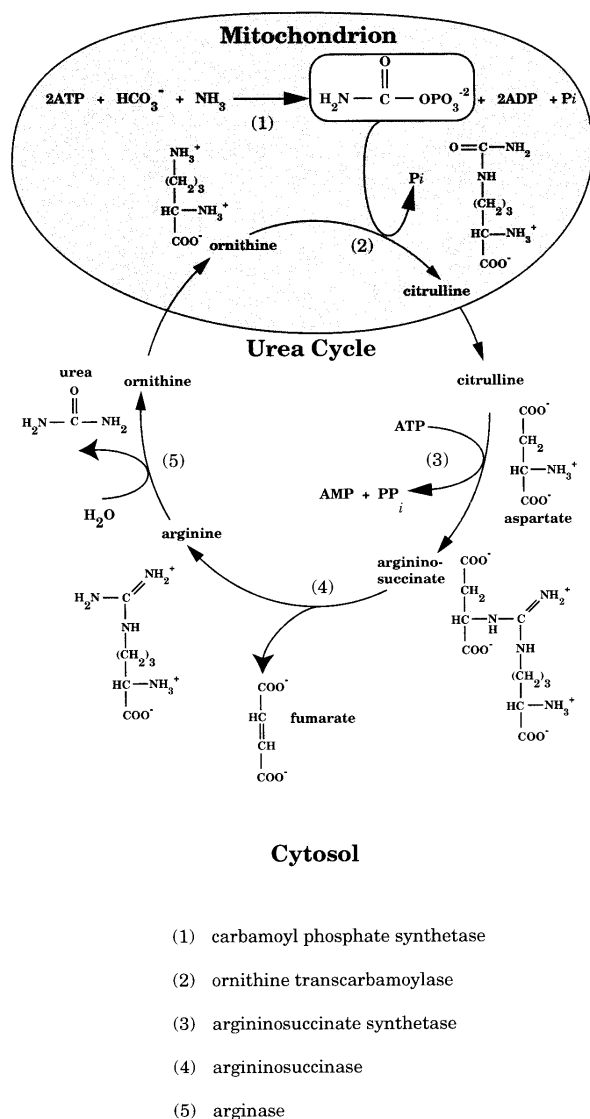


Figure 3. The urea cycle. As indicated, the carbamoyl phosphate synthesized in the mitochondrion condenses with ornithine to produce citrulline. Once in the cytosol, the citrulline reacts with aspartate to continue the urea cycle. The specific enzymes involved in this metabolic pathway are as listed. Adapted, with permission, from Voet and Voet [54]. © 1999 John Wiley & Sons, Inc., New York.

orotidine monophosphate decarboxylase. Note that when the pyrimidine moiety is formed in this de novo synthesis of UMP, the carbonyl functional group at position C-2 of the ring is ultimately derived from bicarbonate while N-3 of the ring is donated by the carboxamide group of glutamine.

The manner in which carbamoyl phosphate is utilized in the production of arginine and in the urea cycle in

eukaryotes is depicted in figure 3. Again, as indicated in figure 3, the mitochondrial form of CPS requires free ammonia as the nitrogen source. The carbamoyl phosphate produced in step 1 condenses with ornithine to yield citrulline which is subsequently transported to the cytosol. Once in the cytosol, citrulline reacts with aspartate to form argininosuccinate in step 3. The argininosuccinate so formed is subsequently broken down to fumarate and arginine in step 4. Fumarate can then enter the citric acid cycle, whereas arginine can be employed in the biosynthesis of proteins, for example, or can continue to participate in the urea cycle whereby it is cleaved to urea and ornithine. This metabolic pathway, as outlined in figure 3, functions to remove excess nitrogen in the cell.

By far the best characterized CPS species to date is that isolated from *Escherichia coli*. Long before the three-dimensional structure of the enzyme was successfully deduced by this laboratory in January 1997, an enormous wealth of information had already accumulated through the painstaking efforts of numerous laboratories. In keeping with the fact that the product of CPS is utilized in two competing metabolic pathways, the enzyme from *E. coli* is highly regulated by a variety of effector molecules including ornithine, which functions as an activator, and UMP which acts as an inhibitor [1, 13, 14]. Inosine monophosphate (IMP) is also known to bind to CPS but the actual effect of this ligand on the activity of the enzyme is dependent upon both temperature and assay conditions [15–18]. Furthermore, ornithine and either IMP or UMP can bind simultaneously to CPS but the activation properties of ornithine dominate the effects of either IMP or UMP [19].

As isolated from *E. coli*, CPS is an  $\alpha,\beta$ -heterodimer that readily converts to an  $(\alpha,\beta)_4$ -tetrameric species depending on the presence or absence of various effectors [20, 21]. Positive allosteric effector molecules such as ornithine,  $Mg^{2+}$  ATP, or potassium, for example, promote formation of the tetrameric species. In the presence of UMP, on the other hand, CPS exists as the  $\alpha,\beta$ -heterodimer. The  $\alpha$ - and  $\beta$ -subunits of the *E. coli* CPS (also referred to as the small and large subunits) are encoded by the *carA* and *carB* genes, respectively [22, 23]. The smaller of the two subunits contains 382 amino acid residues and is responsible for the hydrolysis of glutamine to glutamate and free ammonia, as indicated in figure 1 [22, 24]. The larger subunit provides the binding sites for the two  $Mg^{2+}$  ATP molecules required for the assembly of carbamoyl phosphate [25]. Additionally, the binding regions for the allosteric effector molecules are located within the large subunit. This larger subunit contains 1073 amino acid residues [23].

Sequencing of the *carAB* genes from *E. coli* by Dr. Carol Lusty and co-workers back in the early 1980s revealed two very intriguing features [22, 23]. Quite strikingly, the C-terminal half of the small subunit demonstrated significant homology to the N-terminal domains of the *trpG*-type amidotransferases. These types of enzymes employ an active site cysteine to initiate a nucleophilic attack on the carboxamide group of glutamine [26]. A recent review of the amidotransferase family can be found in Zalkin and Smith [27]. The second fascinating feature of CPS, as revealed from these sequencing studies, was the significant homology between residues Met 1 to Arg 400 and Ala 553 to Leu 933 of the large subunit. Indeed, the sequence identity was 40% leading Lusty to postulate that the present-day CPS arose by a gene duplication event from a more primitive enzyme displaying kinase activity [23]. The review presented here will focus on the recent three-dimensional structural investigations from this laboratory on the *E. coli* CPS and will discuss these analyses in light of the extensive biochemical literature that has accumulated over the last 40 years regarding this truly remarkable catalytic machine.

#### Quaternary structure of CPS from *E. coli*

The three-dimensional structure of CPS is elegant in both its simplicity and complexity. Overall, the molecule can be described in terms of quite simple domains and subdomains, many of which have been observed in other enzymes. It is the manner in which these domains function together that results in the complexity of CPS and leads to a finely tuned instrument of catalysis. Shown in figure 4 is a space-filling representation of the complete  $(\alpha,\beta)_4$ -heterotetramer [28, 29]. As can be seen, the four small subunits of the CPS  $(\alpha,\beta)_4$ -heterotetramer, displayed in magenta, are perched at either end of the protein. For the representation in figure 4, the four large subunits are broken down into domains and color-coded in green, yellow, blue, and red to correspond to those regions defined by Met 1 to Glu 403, Val 404 to Ala 553, Asn 554 to Asn 936, and Ser 937 to Lys 1073, respectively. From previous biochemical studies, it is known that the regions delineated by Met 1 to Glu 403, and Asn 554 to Asn 936 both contain a  $Mg^{2+}$ -ATP-binding site [30]. In addition, it has been demonstrated that the N-terminal half of the large

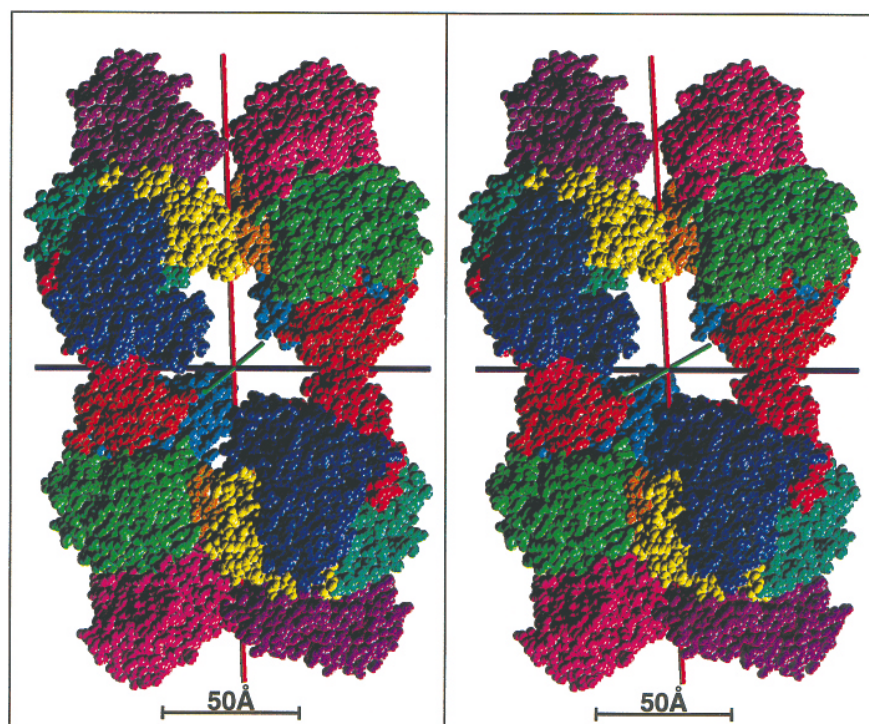


Figure 4. Space-filling representation of the CPS  $(\alpha,\beta)_4$ -heterotetramer from *E. coli*. The four small subunits, perched on either end of the heterotetramer, are color-coded in magenta. The large subunits are color-coded in green, yellow, blue, and red for those regions delineated by Met 1 to Glu 403, Val 404 to Ala 553, Asn 554 to Asn 936, and Ser 937 to Lys 1073, respectively. The CPS heterotetramer shows 222 symmetry as indicated by the three mutually perpendicular axes.

subunit catalyzes the formation of the carboxyphosphate intermediate (fig. 1) whereas the C-terminal half facilitates the phosphorylation of carbamate [30–33]. For this review, these regions will be referred to as the carboxyphosphate and carbamoyl phosphate domains, respectively. These domains can be further broken down into subdomains as discussed in more detail below.

As can be seen in figure 4, the yellow or oligomerization domains of the large subunits (Val 404 to Ala 553) are primarily involved in subunit:subunit interactions. Indeed, there are leucine residues contributed by each of the oligomerization domains that form hydrophobic patches which serve to maintain the proper quaternary structure of the tetrameric species. These oligomerization domains are quite simple in three-dimensional architecture, each containing seven  $\alpha$ -helices and two strands of anti-parallel  $\beta$ -sheet. The final motif of each large subunit, the red or allosteric domain, provides the binding sites for the effector molecules such as UMP and ornithine and a second molecular interface for maintaining the tetrameric organization of CPS as shown in figure 4. As observed in the intermolecular surface formed by the oligomerization domains, the subunit:subunit interactions between two allosteric domains in the tetramer are quite hydrophobic with a number of leucine and isoleucine residues pointing towards the interface. Additional details concerning the manner in which the effector molecules are accommodated within the allosteric domains are given below.

As is immediately obvious from figure 4, the tetrameric species of CPS is a large but rather open macromolecular assembly with overall dimensions of  $110 \times 200 \times 235 \text{ \AA}$  and showing 222 symmetry. As might be expected for a protein that readily interconverts between an  $\alpha, \beta$ -heterodimer and an  $(\alpha, \beta)_4$ -heterotetramer, the subunit:subunit interactions between one  $\alpha, \beta$ -species and another, while somewhat hydrophobic as discussed above, are not extensive. Indeed, the buried surface areas for those regions of polypeptide chain that do interact within the tetramer are between  $550$  and  $575 \text{ \AA}^2$ . As a point of reference, a systematic study of oligomeric proteins has revealed that buried surface areas typically range from about  $670 \text{ \AA}^2$  for superoxide dismutase to well over  $10,000 \text{ \AA}^2$  for catalase [34].

While space-filling representations are meaningful in terms of the overall molecular shapes of proteins, details concerning secondary and tertiary structural elements are lost. In an effort to appreciate more fully the engineering design of CPS, a ribbon representation of one  $\alpha, \beta$ -heterodimer is presented in figure 5a. The  $\alpha$ -helical and  $\beta$ -sheet regions, represented by the coils and arrows, respectively, are now immediately obvious. What is also absolutely clear is the extent of the subunit:subunit interface between the small subunit and the carboxyphosphate and oligomerization domains of the large subunit.

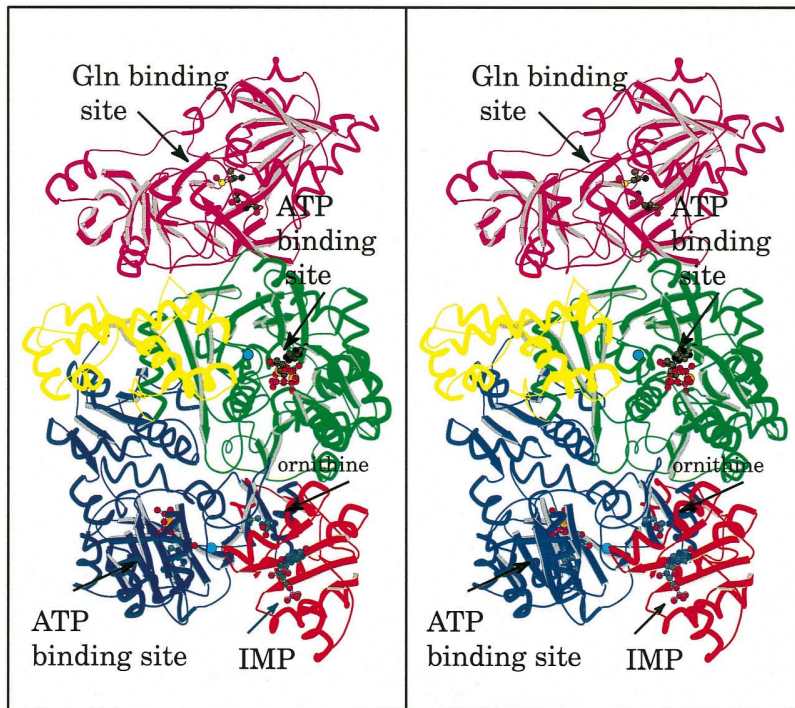
Indeed, the buried surface area is  $2100 \text{ \AA}^2$  and  $2250 \text{ \AA}^2$  contributed by the large and small subunits, respectively. There are 35 direct hydrogen bonds between protein atoms that link the large and small subunits together. This extensive interface is not surprising in light of the biochemistry that requires the ammonia produced in the small subunit to be transported to the first active site of the large subunit without release to the external solvent. For the sake of clarity, the following discussion of the architecture of CPS will be dissected into separate components. It must be kept in mind, however, that the proper functionings of these individual units are ultimately connected to one another.

### Structure of the small subunit

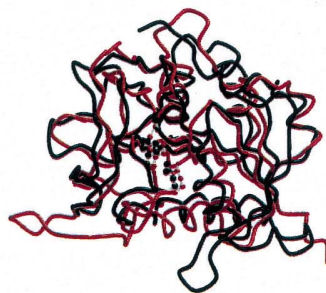
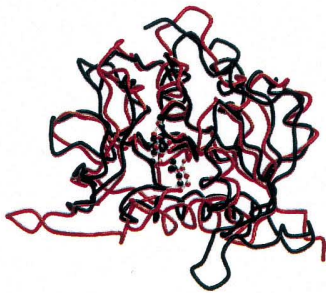
The small subunit or amidotransferase portion of CPS exhibits a distinctly bilobal appearance with the N- and C-terminal domains delineated by Leu 1 to Leu 153 and Asn 154 to Lys 382, respectively. While the three-dimensional motif of the N-terminal domain has not been crystallographically observed in other proteins thus far, the C-terminal domain is remarkably similar to the N-terminal domain of GMP synthetase, as originally predicted by amino acid sequence analyses [35]. Indeed, the amino acid sequences of these two glutaminase domains demonstrate 48% similarity and 27% identity. In CPS, the C-terminal domain of the small subunit (Asn 154 to Lys 382) is dominated by ten strands of mixed  $\beta$ -sheet flanked on either side by two and three  $\alpha$ -helices. A superposition of the CPS C-terminal domain onto the N-terminal domain of GMP synthetase is shown in figure 5b. These two motifs superimpose with a root-mean-square deviation of  $1.7 \text{ \AA}$  for 129 structurally equivalent  $\alpha$ -carbons. In addition to the close structural correspondence of their  $\alpha$ -carbons, the active site histidine and cysteine residues also align and are located at the interface formed by the N- and C-terminal domains. In both enzymes, the active site nucleophiles (Cys 269 in CPS) reside in so-called nucleophile elbows and adopt strained dihedral angles of approximately  $\phi = 60^\circ$  and  $\psi = -96^\circ$ . These dihedral angles are a hallmark for those enzymes belonging to the  $\alpha/\beta$ -hydrolase fold family [36].

### Reaction mechanism of the small subunit

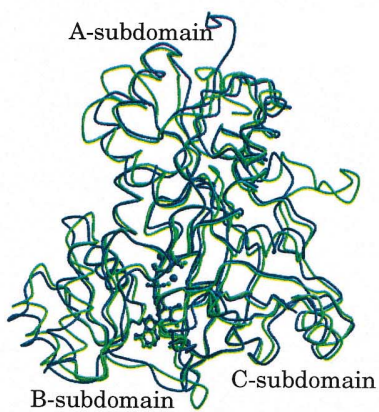
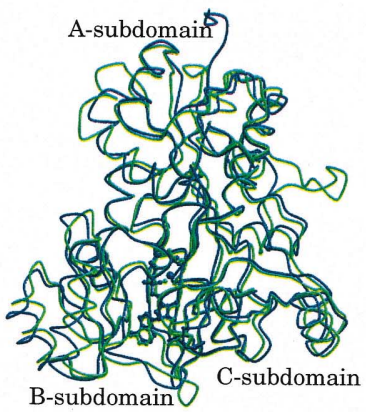
From previous biochemical studies, it is known that the catalytic mechanism of the small subunit proceeds through the reaction outlined in figure 6 [26, 37–41]. According to this model, the carbonyl carbon of the glutamine side chain is attacked by the thiolate group of Cys 269, leading to a tetrahedral intermediate. His 353 aids in the collapse of this intermediate by donating a proton to the  $\text{NH}_2$  group of the substrate thereby



(a)



(b)



(c)

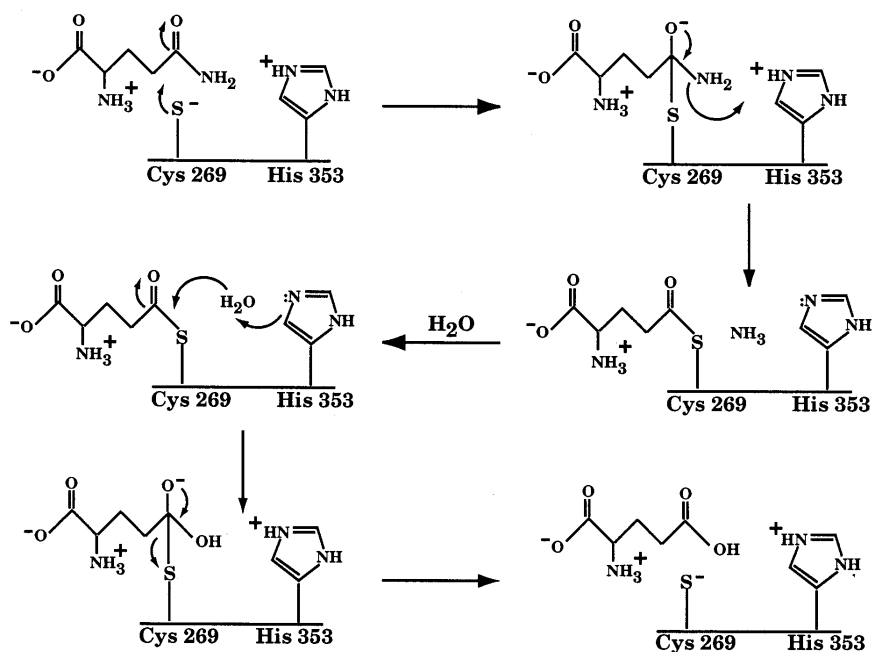


Figure 6. Catalytic reaction of the small subunit of CPS.

allowing ammonia to depart. The glutamyl thioester intermediate left behind is subsequently attacked by a water molecule which is activated by its close proximity to the imidazole ring of His 353. This nucleophilic attack leads to the second tetrahedral intermediate in the reaction pathway which finally collapses to yield free glutamate. By changing His 353 to an asparagine residue, it was possible to trap the glutamyl thioester intermediate in the active site of the small subunit and observe its structure crystallographically [40, 42]. A close-up view of the active site for the small subunit containing the bound glutamyl thioester intermediate is shown in figure 7. Those residues important for stabilizing the intermediate within the active site are as follows: the side chain functional groups of Ser 47 and Gln 273, the backbone carbonyl oxygens of Gly 241 and Gly 243, and the backbone amide groups of Gly 241, Gly 313, and Phe 314. Indeed, both O $\gamma$  of Ser 47 and the back-

bone amide hydrogen of Gly 241 serve not only to position the carbonyl carbon of the glutamine carboxamide group for nucleophilic attack by the thiolate of Cys 269 but also to aid in stabilization of the developing oxyanion.

#### Structure of the large subunit

As is obvious from figure 5a, the large subunit consists of four major components, all of which play an important biological role. Ignoring the oligomerization domain (in yellow) and the allosteric domain (in red), it is quite easy in figure 5a to observe the nearly exact twofold rotational axis (179.7 $^\circ$ ) lying perpendicular to the plane of the page and relating the carboxyphosphate (green) and carbamoyl phosphate (blue) domains. Indeed, these two halves of the large subunit superimpose

Figure 5. Stereo views of CPS. (a) A ribbon representation of one  $\alpha,\beta$ -heterodimer is displayed in the same color-coding described for figure 4. The active sites in the large subunit contain bound  $Mn^{2+}$ AMPPNP as indicated by the ball-and-stick representations. The positions of the potassium ions are indicated by the blue spheres and the binding pockets for the effector molecules, IMP and ornithine, are indicated by the arrows. (b) A superposition of the N-terminal domain of GMP synthetase (displayed in red) onto the C-terminal domain of the CPS small subunit (depicted in black). X-ray coordinates for the GMP synthetase model were obtained from the Brookhaven Protein Data Bank. The active site residues involved in catalysis, namely Cys 269 and His 353 in CPS, are displayed in ball-and-stick representations. Glu 355 (CPS numbering) is also included. There is some discrepancy in the literature regarding the role of this residue in catalysis. In GMP synthetase, it has been speculated that this glutamate is part of a catalytic triad [35]. However, a study of the glutaminase subunit of *p*-aminobenzoate synthetase has demonstrated that replacement of this residue with an alanine or aspartate results in little loss of catalytic activity [55]. (c) Superposition of the carboxyphosphate and carbamoyl phosphate domains of the CPS large subunit, depicted in green and blue, respectively.

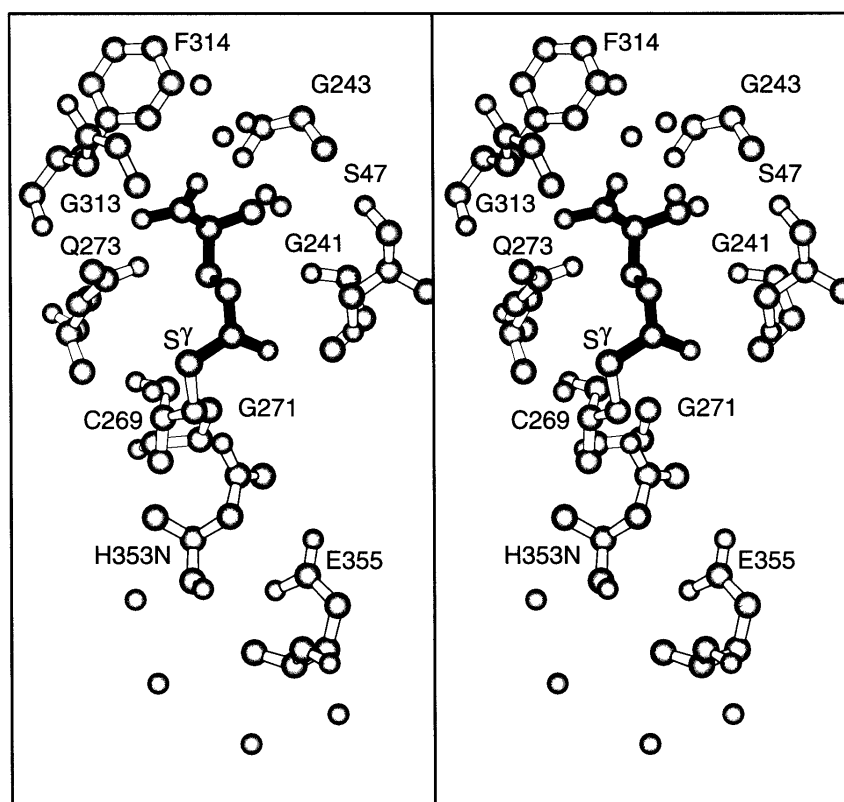


Figure 7. Stereo view of the active site pocket for the CPS small subunit. The glutamyl thioester intermediate is depicted in filled, black bonds. Ordered water molecules are indicated by the gray spheres.

with a root-mean-square deviation of 1.1 Å for 255 equivalent  $\alpha$ -carbons as shown in figure 5c. Each of these synthetase units can be further broken down into three smaller modules, referred to as the A-, B-, and C-subdomains. In both synthetase units, the A-subdomains are built from approximately 140 amino acid residues and contain five strands of parallel  $\beta$ -pleated sheet flanked on either side by  $\alpha$ -helices. The B-subdomains are somewhat smaller with approximately 65 amino acid residues and are dominated by four strands of anti-parallel  $\beta$ -sheet flanked on one side by two  $\alpha$ -helices. The C-subdomains are the most complicated of the three motifs with about 190 amino acid residues that wrap around to form seven-stranded anti-parallel  $\beta$ -sheets. The topological architectures of the carboxyphosphate and carbamoyl phosphate domains are reminiscent of those observed in biotin carboxylase and other members of the so-called ATP-grasp superfamily [43, 44]. For each enzyme in this superfamily, the nucleotide-triphosphate-binding regions are situated between the B- and C-subdomains as can be seen in figure 5c.

Recent studies with the nonhydrolyzable analog,  $\text{Mn}^{2+}$  AMPPNP [45], have revealed that the B-subdomain of the carbamoyl phosphate domain closes down over the active site pocket upon nucleotide triphosphate binding such that some atoms move by more than 7 Å relative to that observed with only bound  $\text{Mn}^{2+}$  ADP in the active site [46]. The trigger for this movement resides in the hydrogen-bonding interactions between two backbone amide groups (Gly 721 and Gly 722) and the  $\beta$ - and  $\gamma$ -phosphate groups of the nucleotide triphosphate. Note that the use of manganese rather than magnesium for all of the X-ray crystallographic studies conducted thus far was based simply on experimental considerations. Crystals of CPS grow somewhat better in the presence of manganese ions. The enzyme is fully active in the presence of  $\text{Mn}^{2+}$  ATP, however.

Close-up views of the two nucleotide-triphosphate-binding sites in the large subunit are shown in figure 8. It is immediately obvious that the binding pockets are, indeed, structurally similar with comparable chemical in-



teractions between the nucleotides and the protein. In addition to the nucleotides, bound manganese ions are observed in the structure of CPS complexed with AMPPNP, one located in the carboxyphosphate domain and two situated in the carbamoyl phosphate

domain as indicated by the gray spheres in figure 8. All of these ions are surrounded in an octahedral coordination sphere by phosphoryl oxygens contributed by the nucleotides, water molecules, and protein side chain functional groups. In the first structural analysis of CPS

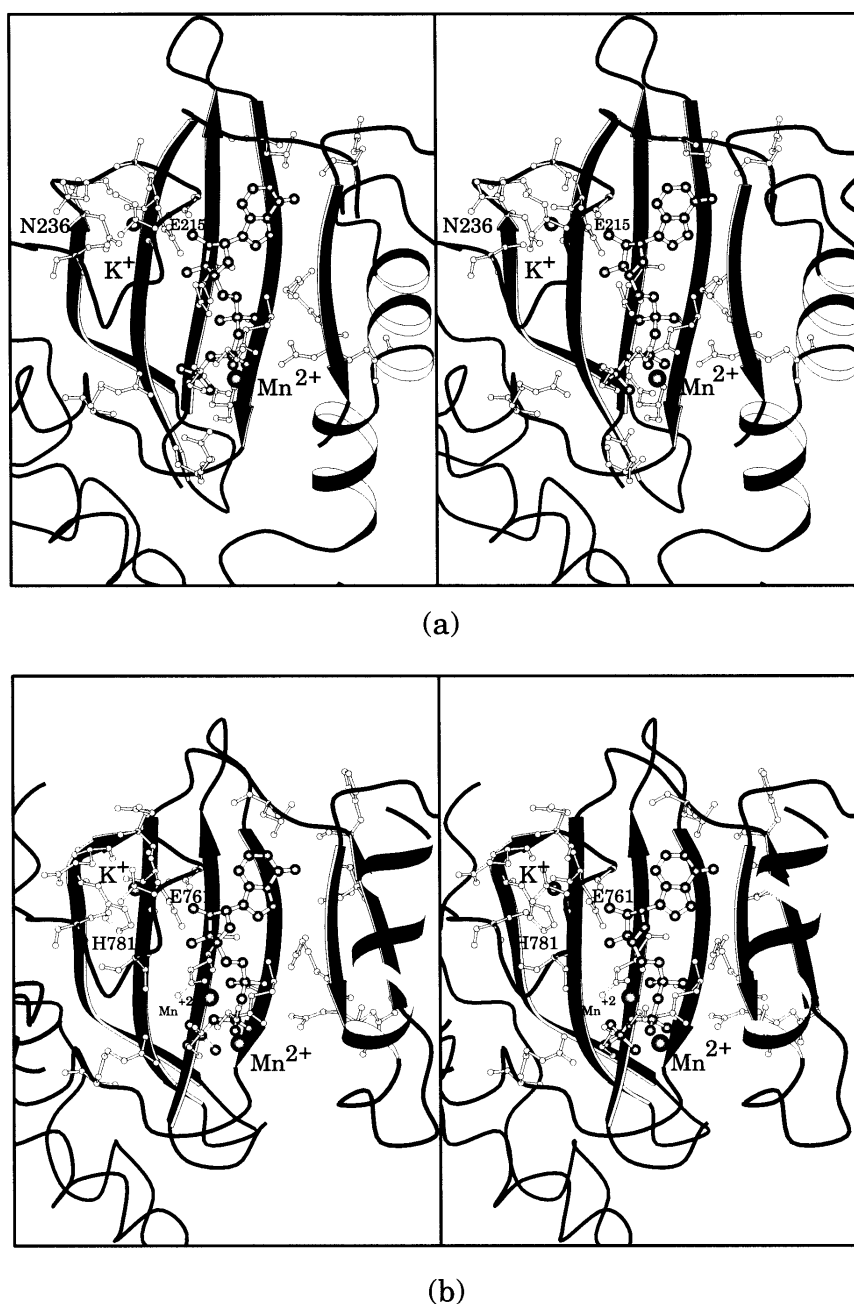


Figure 8. Close-up views of the active site pockets in the CPS large subunit. The binding pockets for the  $Mn^{2+}$ -AMPPNP moieties in the carboxyphosphate and carbamoyl phosphate domains are shown in (a) and (b), respectively. Those residues that are important for nucleotide binding are depicted in ball-and-stick representations. Note the close topological similarities between the two active sites. Both are primarily formed from four strands of anti-parallel  $\beta$ -sheet.

complexed with  $Mn^{2+}ADP$ , rather than  $Mn^{2+}AMPPNP$ , the two active sites contained the exact opposite in metal content: two in the carboxyphosphate domain and one in the carbamoyl phosphate unit. It is known from previous studies that in addition to the divalent cations needed to complex the nucleotides, a third metal is required for full catalytic activity [47]. Furthermore, it has been observed that with  $Mn^{2+}$  as the cation, excess metal is actually inhibitory. The exact role of the third metal will most likely become clear when the structure of CPS is solved in the presence of  $Mg^{2+}ATP$ . Regardless of the physiological significance in metal content between the structures of CPS solved with bound  $Mn^{2+}ADP$  or  $Mn^{2+}AMPPNP$ , it is absolutely clear that both active sites in the large subunit are capable of binding two metal ions.

CPS is known to be activated by the presence of potassium ions [1]. As indicated in figure 8, both active site regions contain bound potassium ions. In both cases, these ions are octahedrally coordinated by protein ligands. One difference between the two potassium-binding pockets is the substitution of Asn 236 in the carboxyphosphate domain with His 781 in the carbamoyl phosphate domain. In both active sites, there is a direct link between the potassium-binding pockets and the nucleotide-triphosphate-binding regions. Indeed, in the carboxyphosphate domain, the carboxylate group of Glu 215 serves to bridge the 2'- and 3'-hydroxyl groups of the ribose together and, in turn, acts as a ligand to the potassium ion, thereby providing a direct communication link between the cation and nucleotide-binding regions. The side chain carboxylate group of Glu 761 plays an identical role in the carbamoyl phosphate domain.

### Binding of the allosteric effector molecules

The manner in which CPS accommodates the allosteric effector molecules, ornithine and IMP, is now known from various crystallographic studies in this laboratory. Shown in figure 9 are close-up views of the binding regions for ornithine and IMP. The overall spatial relationship between these allosteric binding sites and the other active sites of the complete CPS  $\alpha,\beta$ -heterodimer can be seen in figure 5a.

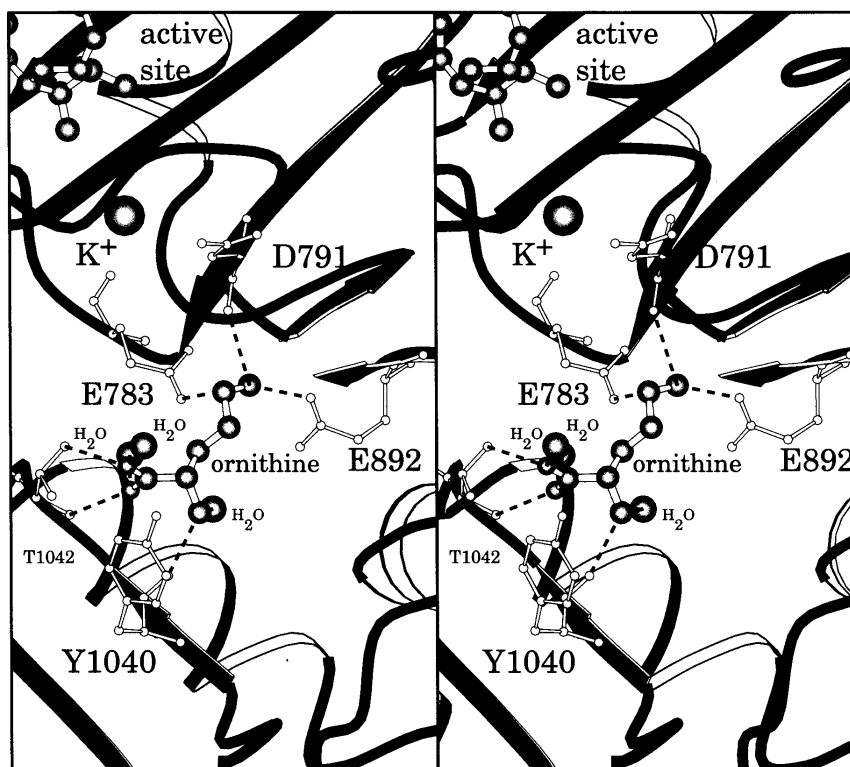
Ornithine binds to the CPS large subunit at the interface located between the allosteric and the carbamoyl phosphate domains. As indicated by the dashed lines in figure 9a, the carboxylate group of ornithine lies within hydrogen-bonding distance to two water molecules and to both the backbone amide group and the side chain hydroxyl group of Thr 1042. The  $\alpha$ -amino group of this activator is positioned within hydrogen-bonding distance to the carbonyl oxygen of Tyr 1040 and a water

molecule. Both Tyr 1040 and Thr 1042 are part of the allosteric domain. The side chain of ornithine bridges across and into the carbamoyl phosphate domain where its  $\delta$ -amino group interacts with the side chain carboxylate groups of Glu 783 and Glu 892 and the carbonyl oxygen of Asp 791. As can be seen, the ornithine is located fairly close to the potassium-binding pocket (8.7 Å) which in turn is bridged directly to the  $Mg^{2+}ATP$ -binding site via Glu 761, thereby establishing a direct connection between the allosteric effector-binding region and the active site of the carbamoyl-phosphate domain. Previous studies have demonstrated that ornithine affects the carbamoyl phosphate-dependent ATP synthesis reaction more so than the other two partial reactions catalyzed by CPS [48].

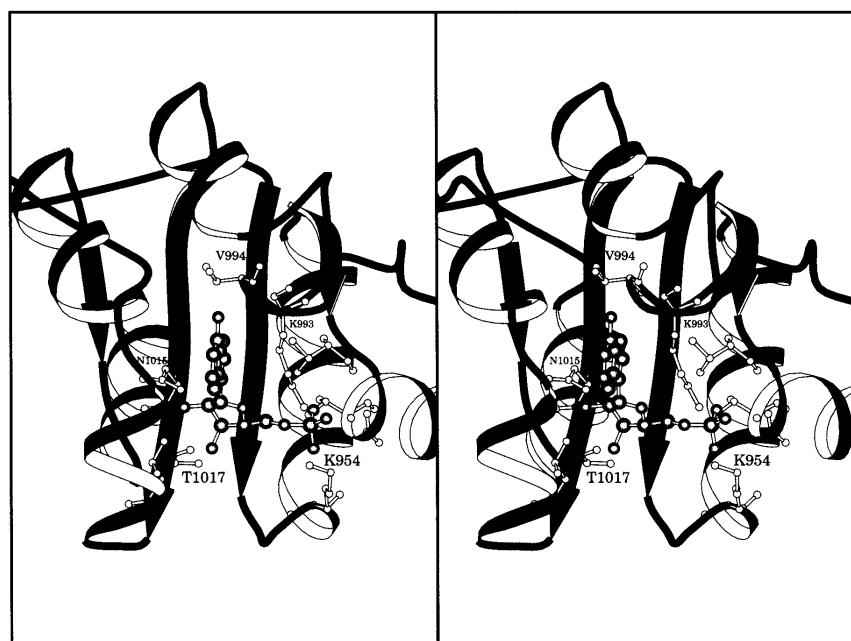
The manner in which IMP binds to the allosteric domain, as depicted in figure 9b, is somewhat intriguing. While CPS is known to discriminate between UMP and IMP there are few interactions between the base and the polypeptide chain [49]. In fact, the only direct electrostatic contact between the hypoxanthine base of IMP and the protein occurs between O-6 of the purine ring and the backbone amide group of Val 994. With respect to the rest of the effector molecule, the 2'- and 3'-hydroxyl groups of the IMP ribose are anchored to the protein via hydrogen bonds with the side chain groups of Asn 1015 and Thr 1017 and the backbone carbonyl group of Thr 1016. Both the side chain functional groups of Lys 954 and Lys 993 interact with the phosphoryl moiety of the IMP. Besides these electrostatic interactions with the lysine residues, there are additional hydrogen bonds formed between the phosphoryl oxygens of the nucleotide and the side chain groups of Thr 974 and Thr 977 and the backbone amide groups of Gly 976 and Thr 977. As can be seen in figure 9b, the IMP ligand is situated at the C-terminal end of a five-stranded parallel  $\beta$ -sheet with approximately 90% of its surface area buried within the protein [49]. The IMP-binding pocket is approximately 19 Å from the active site of the carbamoyl phosphate domain and it appears that these two binding regions are connected via a series of hydrogen bonds.

### The catalytic mechanism of CPS

The kinetic mechanism for CPS was first put forth over 20 years ago from research in the laboratory of Joseph J. Villafranca [3]. From this original investigation, the catalytic mechanism was established to be ordered Ter-Uni-Uni-Ter ping pong and is consistent with the chemical mechanism depicted in figure 1. The reaction sequence is initiated by the phosphorylation of bicarbonate from the  $Mg^{2+}ATP$  bound to the carboxyphosphate domain (colored in green in figure 5a). This event triggers the activation of the small subunit that enables



(a)



(b)

Figure 9. Binding pockets for the allosteric effector molecules. (a) The region of the large subunit responsible for harboring the ornithine ligand. Potential hydrogen bonds are indicated by the dashed lines. (b) Binding pocket for the IMP ligand. This nucleotide monophosphate binds across the C-terminal end of a parallel  $\beta$ -sheet. The ornithine- and IMP-binding sites are separated by approximately 12 Å.

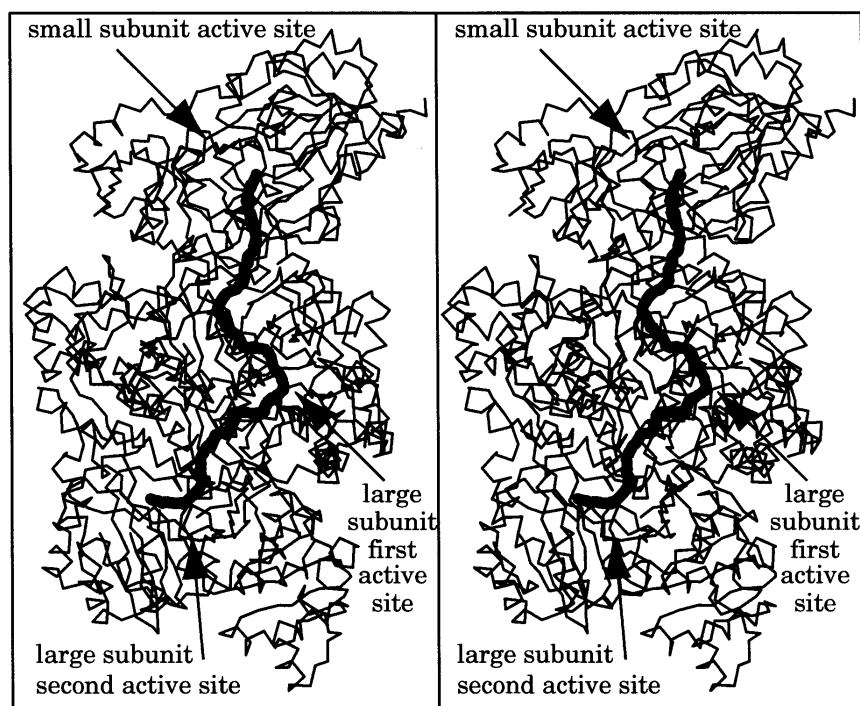


Figure 10. Molecular tunnel responsible for substrate channeling in CPS. This tunnel, represented by the thick black line, leads from the small subunit to the two active sites of the large subunit. Reprinted with permission from Thoden et al. [29], © 1999 IUCr England.

the amidotransferase domain to initiate the hydrolysis of glutamine. The ammonia product diffuses from its site of formation within the small subunit to the large subunit where it reacts with the carboxyphosphate intermediate. The carbamate product then diffuses towards the carbamoyl phosphate domain where it is phosphorylated by the second  $Mg^{2+}$ -ATP to produce the final product, carbamoyl phosphate.

Our current understanding of the reaction mechanism that occurs within the small subunit is now quite advanced from both the structural and biochemical investigations on CPS and on GMP synthetase, both members of the *trpG*-type amidotransferase family. It is absolutely clear that the active site nucleophile is a conserved cysteine residue and its close proximity to a histidine residue is critical for the hydrolysis of glutamine to glutamate and ammonia. It is also known that the reaction mechanism proceeds through two tetrahedral intermediates and a covalent glutamyl thioester intermediate.

Less is known regarding the actual reactions occurring in the two active sites of the large subunit. Indeed, the roles of individual amino acids in the facilitation of the catalytic events within the carboxyphosphate and carbamoyl phosphate domains have not been fully eluci-

dated. Those residues that serve to bind the nucleotides to these active sites have been identified by site-directed mutagenesis and X-ray crystallographic methods. However, structures have not yet been obtained that illuminate the manner in which bicarbonate or carbamate are bound to the protein. Nevertheless, His 243 is critical for the displacement of the phosphate from carboxyphosphate by ammonia [31]. It is likely that this histidine functions to accept a proton from ammonia during the attack on carboxyphosphate. The structures of additional complexes will be needed to ultimately define the molecular events occurring during the two phosphorylation reactions of the large subunit.

#### The tunnel

Perhaps the most surprising result from the initial X-ray crystallographic investigation of CPS was the location of the three active sites. Accordingly, the active site in the small subunit is located at  $\sim 45$  Å from the active site in the carboxyphosphate domain of the large subunit which, in turn, is situated at  $\sim 35$  Å from the active site in the carbamoyl phosphate domain. All the published biochemical data argue against any uncou-

pling of the three separate reactions. Furthermore, the individual reactions, as indicated in figure 1, must be exquisitely timed due to the formation of the reactive intermediates: ammonia, carboxyphosphate, and carbamate. Clearly some type of molecular channeling must be occurring in CPS. A visual search of the CPS model, in conjunction with software packages that attempt to locate cavities within the interior of proteins, has revealed the presence of a tunnel as indicated in figure 10. Other than the side chains donated by Asp 45, Lys 202, and His 353 in the small subunit, the tunnel leading from this amidotransferase component to the molecular interface with the large subunit is lined primarily with nonreactive side chains and backbone atoms. The portion of the tunnel that extends from the interface between the small and large subunits to the active site of the carboxyphosphate domain is also lined, for the most part, with nonreactive residues except for Glu 217 and Cys 232. The portion of the tunnel lying between the two active sites of the large subunit is somewhat less hydrophobic and includes side chains contributed by Glu 604, Glu 577, Arg 848, Lys 891, and Glu 916. While approximately 25 water molecules lying within 2 Å of the pathway have been identified in the structural analysis of CPS, their actual positions during catalysis are unknown [29]. Many of the residues lining the putative tunnel are absolutely conserved among 22 of 24 primary structural alignments of CPS [29] and many of the residues that are not strictly conserved are replaced with amino acid residues of comparable chemical reactivities.

The concept of channeling within proteins is not unique to CPS. It was first observed crystallographically in the three-dimensional X-ray analysis of tryptophan synthase from *Salmonella typhimurium* [50]. This enzyme, known to form stable  $(\alpha, \beta)_2$  complexes, catalyzes the last two reactions in L-tryptophan biosynthesis. As in CPS, the two active sites are widely separated (25 Å) but in this case are located on separate polypeptide chains. The tunnel connecting the two active sites is largely hydrophobic with molecular dimensions appropriate for the passage of the intermediate, indole.

Another striking example of substrate channeling has been observed in glutamine phosphoribosylpyrophosphate amidotransferase (GPATase) from *E. coli* [51–53]. This enzyme catalyzes the first step in the purine biosynthetic pathway and, like CPS, has two active sites located on the same polypeptide chain. Specifically, GPATase catalyzes the hydrolysis of glutamine to glutamate and ammonia and the subsequent coupling of the ammonia to phosphoribosylpyrophosphate yielding phosphoribosylamine and pyrophosphate. Interestingly, the molecular conduit in GPATase was only observed crystallographically when the enzyme structure was solved in the presence of both glutamine and phosphoribosylpyrophosphate analogs. The formation of the molecular

tunnel, with a length of approximately 20 Å, results from a kinking of the C-terminal helix and an ordering of a flexible surface loop [52]. Not unexpectedly, for the transport of ammonia from one active site to another, the tunnel in GPATase is lined primarily by hydrophobic amino acid residues [52].

#### Relationship between the prokaryotic and eukaryotic forms of CPS

Can anything be learned regarding the three-dimensional structures of eukaryotic carbamoyl phosphate synthetases from the model of the bacterial enzyme reviewed here? The answer is a decided yes based on the high degree of amino acid sequence conservation exhibited by these proteins and elegantly discussed in Zalkin [56]. Three different types of carbamoyl phosphate synthetase have been identified in eukaryotic systems thus far: (i) CPS I which functions in the production of arginine and urea and employs ammonia as the source of nitrogen, (ii) CPS II which is involved in pyrimidine biosynthesis and hydrolyzes glutamine for the production of the required ammonia, and (iii) CPS III which utilizes glutamine as the nitrogen source but functions in the arginine and urea cycles [57 and references therein]. In the case of CPS I, the glutaminase and synthetase domains have been fused into a single polypeptide chain [58, 59]. On the basis of amino acid sequence alignments, it has been demonstrated that the rat CPS I is approximately 42% identical along the entire length to both the small and large subunits of *E. coli* CPS. Interestingly, the active site cysteine in the small subunit of the bacterial enzyme has been replaced with a serine in rat CPS I and this change, along with other substitutions, abolishes the ability of the eukaryotic enzyme to hydrolyze glutamine. It appears that the 'small subunit' region in CPS I has been retained simply as a device for the delivery of ammonia to the 'large subunit' region. In higher eukaryotes, CPS II exists as a fused multifunctional protein that contains aspartate transcarbamoylase and dihydroorotase activities as well [10, 11]. Again, as indicated by numerous primary structural studies and discussed in Zalkin [56], there are significant amino acid sequence conservations between the enzyme from *E. coli* and those isolated from higher organisms such as hamster [60]. Like CPS I, CPS III (from invertebrates and fish) is a fused monofunctional enzyme. The amino acid sequence of CPS III from *Squalus acanthiase*, determined by Hong et al. [61], shows that the enzyme is more closely related to CPS I than to CPS II, despite the fact that it employs glutamine as its preferred nitrogen source. The active site cysteine residue is conserved in CPS III. Recent reviews on the evolutionary relationships of carbamoyl phosphate synthetase genes can be found in van den Hoff et al. [62] and Lawson et al. [63].

It is absolutely clear from the primary structural studies conducted thus far that CPSs from various sources show high degrees of amino acid sequence conservation particularly around those residues necessary for catalysis and ATP binding. It can thus be speculated that, for example, the mode of ATP binding, as observed in the *E. coli* protein, will be similar in other CPSs. Likewise, the manner in which the glutamyl-thioester intermediate is stabilized within the small subunit active site of the *E. coli* protein will be, for the most part, universal. Additionally, it is likely that the active sites within the various forms of CPS will be separated by significant distances. What will be different among these systems, however, is the mode in which effector molecules are bound to the respective enzymes and the manner in which the active sites communicate with one another. For example, CPS I and III, but not CPS II, require *N*-acetyl-L-glutamate for activity. Furthermore, CPS II from higher organisms exists as a multifunctional protein thereby adding an additional complication of channeling between different enzymatic species. Recently, crystals of CPS I from *Rana catesbeiana* have been obtained [64] and, indeed, this structure is eagerly awaited for it will add considerable new insight into the structural relationship between the eukaryotic and prokaryotic forms of CPS.

### Conclusion

With the various complexes of CPS that have now been solved to high resolution over the last several years, a quite detailed structural picture has emerged concerning the manner in which CPS catalyzes the production of carbamoyl phosphate from bicarbonate, glutamine and two molecules of  $Mg^{2+}$ -ATP. But these complexes are merely snapshots along the way towards a greater understanding of this remarkable molecular machine. Clearly many questions remain. For example, every structure of the enzyme that has been solved to date has always been of the activated form, i.e., with bound ornithine and  $Mn^{2+}$ -ADP or  $Mn^{2+}$ -AMPPNP moieties. What is the structure of the enzyme in the presence of UMP alone? What are the differences in the structure of CPS when it exists in the  $\alpha,\beta$ -heterodimeric form as opposed to the  $(\alpha,\beta)_4$ -heterotetrameric state? What is the structure of the enzyme in the absence of potassium ions? How is the carboxyphosphate intermediate stabilized? How does the enzyme orchestrate the synthesis of three separate intermediates? How are the conformational changes induced by the binding of the effector molecules transmitted to the active site of the carbamoyl phosphate domain? Is the tunnel for real? By the dedicated efforts of many researchers, the last 40 years have provided an enormous wealth of information

concerning CPS. For the next 20 years we can look forward to even more details, at an atomic level, as additional complexes of CPS from *E. coli* are solved to high resolution, site-directed mutants of the protein are constructed, and structures of the enzyme from other sources are determined. These analyses will not only increase our understanding of CPS but will also provide new molecular insight into both channeling in proteins and the allosteric control of highly regulated enzymes.

- 1 Anderson P. M. and Meister A. (1966) Control of *Escherichia coli* carbamyl phosphate synthetase by purine and pyrimidine nucleotides. *Biochemistry* **5**: 3164–3169
- 2 Anderson P. M. and Meister A. (1965) Evidence for an activated form of carbon dioxide in the reaction catalyzed by *Escherichia coli* carbamyl phosphate synthetase. *Biochemistry* **4**: 2803–2809
- 3 Raushel F. M., Anderson P. M. and Villafranca J. J. (1978) Kinetic mechanism of *Escherichia coli* carbamoyl-phosphate synthetase. *Biochemistry* **17**: 5587–5591
- 4 Raushel F. M. and Villafranca J. J. (1979) Determination of rate-limiting steps of *Escherichia coli* carbamoyl-phosphate synthase: rapid quench and isotope partitioning experiments. *Biochemistry* **18**: 3424–3429
- 5 Raushel F. M., Mullins L. S. and Gibson G. E. (1998) A stringent test for the nucleotide switch mechanism of carbamoyl phosphate synthetase. *Biochemistry* **37**: 10272–10278
- 6 Sauers C. K., Jencks W. P. and Groh S. (1975) The alcohol-bicarbonate-water system: structure-reactivity studies on the equilibria for formation of alkyl monocarbonates and on the rates of their decomposition in aqueous alkali. *J. Am. Chem. Soc.* **97**: 5546
- 7 Wang T. T., Bishop S. H. and Himoe A. (1972) Detection of carbamate as a product of the carbamate kinase-catalyzed reaction by stopped flow spectrophotometry. *J. Biol. Chem.* **247**: 4437–4440
- 8 Reichard P. (1959) The enzymic synthesis of pyrimidines. *Adv. Enzymol.* **21**: 263–294
- 9 Jones M. E. (1968) Amino acid metabolism. *Annu. Rev. Biochem.* **34**: 381–418
- 10 Jones M. E. (1980) Pyrimidine nucleotide biosynthesis in animals: genes, enzymes, and regulation of UMP biosynthesis. *Annu. Rev. Biochem.* **49**: 253–279
- 11 Coleman P. F., Suttle D. P. and Stark G. R. (1977) Purification from hamster cells of the multifunctional protein that initiates de novo synthesis of pyrimidine nucleotides. *J. Biol. Chem.* **252**: 6379–6385
- 12 Meister A. (1989) Mechanism and regulation of the glutamine-dependent carbamyl phosphate synthetase of *Escherichia coli*. *Adv. Enzymol. Relat. Areas Mol. Biol.* **62**: 315–374
- 13 Pierard A. (1966) Control of the activity of *Escherichia coli* carbamoyl phosphate synthetase by antagonistic allosteric effectors. *Science* **154**: 1572–1573
- 14 Anderson P. M. and Marvin S. V. (1968) Effect of ornithine, IMP, and UMP on carbamyl phosphate synthetase from *Escherichia coli*. *Biochem. Biophys. Res. Commun.* **32**: 928–934
- 15 Boettcher B. and Meister A. (1981) Conversion of UMP, an allosteric inhibitor of carbamyl phosphate synthetase, to an activator by modification of the UMP ribose moiety. *J. Biol. Chem.* **256**: 5977–5980
- 16 Boettcher B. and Meister A. (1982) Regulation of *Escherichia coli* carbamyl phosphate synthetase: evidence for overlap of the allosteric nucleotide binding sites. *J. Biol. Chem.* **257**: 13971–13976
- 17 Kasprzak A. A. and Villafranca J. J. (1988) Interactive binding between the substrate and allosteric sites of carbamoyl-phosphate synthetase. *Biochemistry* **27**: 8050–8056

- 18 Braxton B. L., Mullins L. S., Raushel F. M. and Reinhart G. D. (1996) Allosteric effects of carbamoyl phosphate synthetase from *Escherichia coli* are entropy-driven. *Biochemistry* **35**: 11918–11924
- 19 Braxton B. L., Mullins L. S., Raushel F. M. and Reinhart G. D. (1999) Allosteric dominance in carbamoyl phosphate synthetase. *Biochemistry* **38**: 1394–1401
- 20 Powers S. G., Meister A. and Haschemeyer R. H. (1980) Linkage between self association and catalytic activity of *Escherichia coli* carbamyl phosphate synthetase. *J. Biol. Chem.* **255**: 1554–1558
- 21 Anderson P. M. (1986) Carbamoyl-phosphate synthetase: an example of effects on enzyme properties of shifting an equilibrium between active monomer and active oligomer. *Biochemistry* **25**: 5576–5582
- 22 Piette J., Nyunoya H., Lusty C. J., Cunin R., Weyens G., Crabeel M. et al. (1984) DNA sequence of the *carA* gene and the control region of *carAB*: tandem promoters, respectively controlled by arginine and the pyrimidines, regulate the synthesis of carbamoyl-phosphate synthetase in *Escherichia coli* K-12. *Proc. Natl. Acad. Sci. USA* **81**: 4134–4138
- 23 Nyunoya H. and Lusty C. J. (1983) The *carB* gene of *Escherichia coli*: a duplicated gene coding for the large subunit of carbamoyl-phosphate synthetase. *Proc. Natl. Acad. Sci. USA* **80**: 4629–4633
- 24 Matthews S. L. and Anderson P. M. (1972) Evidence for the presence of two nonidentical subunits in carbamyl phosphate synthetase of *Escherichia coli*. *Biochemistry* **11**: 1176–1183
- 25 Trotta P. P., Burt M. E., Haschemeyer R. H. and Meister A. (1971) Reversible dissociation of carbamyl phosphate synthetase into a regulated synthesis subunit and a subunit required for glutamine utilization. *Proc. Natl. Acad. Sci. USA* **68**: 2599–2603
- 26 Rubino S. D., Nyunoya H. and Lusty C. J. (1986) Catalytic domains of carbamyl phosphate synthetase: glutamine-hydrolyzing site of *Escherichia coli* carbamyl phosphate synthetase. *J. Biol. Chem.* **261**: 11320–11327
- 27 Zalkin H. and Smith J. L. (1998) Enzymes utilizing glutamine as an amide donor. *Adv. Enzymol. Relat. Areas Mol. Biol.* **72**: 87–144
- 28 Thoden J. B., Holden H. M., Wesenberg G., Raushel F. M. and Rayment I. (1997) Structure of carbamoyl phosphate synthetase: a journey of 96 Å from substrate to product. *Biochemistry* **36**: 6305–6316
- 29 Thoden J. B., Raushel F. M., Benning M. M., Rayment I. and Holden H. M. (1999) The structure of carbamoyl phosphate synthetase determined to 2.1 Å resolution. *Acta Cryst. D* **55**: 8–24
- 30 Post L. E., Post D. J. and Raushel F. M. (1990) Dissection of the functional domains of *Escherichia coli* carbamoyl phosphate synthetase by site-directed mutagenesis. *J. Biol. Chem.* **265**: 7742–7747
- 31 Miles B. W., Mareya S. M., Post L. E., Post D. J., Chang S. H. and Raushel F. M. (1993) Differential roles for three conserved histidine residues within the large subunit of carbamoyl phosphate synthetase. *Biochemistry* **32**: 232–240
- 32 Javid-Majd F., Stapleton M. A., Harmon M. F., Hanks B. A., Mullins L. S. and Raushel F. M. (1996) Comparison of the functional differences for the homologous residues within the carboxy phosphate and carbamate domains of carbamoyl phosphate synthetase. *Biochemistry* **35**: 14362–14369
- 33 Stapleton M. A., Javid-Majd F., Harmon M. F., Hanks B. A., Grahmann J. L., Mullins L. S. et al. (1996) Role of conserved residues within the carboxy phosphate domain of carbamoyl phosphate synthetase. *Biochemistry* **35**: 14352–14361
- 34 Miller S., Lesk A. M., Janin J. and Chothia C. (1987) The accessible surface area and stability of oligomeric proteins. *Nature* **328**: 834–836
- 35 Tesmer J. J. G., Klem T. J., Deras M. L., Davisson V. J. and Smith J. L. (1996) The crystal structure of GMP synthetase reveals a novel catalytic triad and is a structural paradigm for two enzyme families. *Nat. Struct. Biol.* **3**: 74–86
- 36 Ollis D. L., Cheah E., Cygler M., Dijkstra B., Frolow F., Franken S. M. et al. (1992) The  $\alpha/\beta$  hydrolase fold. *Protein Eng.* **5**: 197–211
- 37 Khedouri E., Anderson P. M. and Meister A. (1966) Selective inactivation of the glutamine binding site of *Escherichia coli* carbamyl phosphate synthetase by 2-amino-4-oxo-5-chloropentanoic acid. *Biochemistry* **5**: 3552–3557
- 38 Pinkus L. M. and Meister A. (1972) Identification of a reactive cysteine residue at the glutamine binding site of carbamyl phosphate synthetase. *J. Biol. Chem.* **247**: 6119–6127
- 39 Anderson P. M. and Carlson J. D. (1975) Reversible reaction of cyanate with a reactive sulfhydryl group at the glutamine binding site of carbamyl phosphate synthetase. *Biochemistry* **14**: 3688–3694
- 40 Miran S. G., Chang S. H. and Raushel F. M. (1991) Role of the four conserved histidine residues in the amidotransferase domain of carbamoyl phosphate synthetase. *Biochemistry* **30**: 7901–7907
- 41 Lusty C. J. (1992) Detection of an enzyme bound  $\gamma$ -glutamyl acyl ester of carbamyl phosphate synthetase of *Escherichia coli*. *FEBS Lett.* **314**: 135–138
- 42 Thoden J. B., Miran S. G., Phillips J. C., Howard A. J., Raushel F. M. and Holden H. M. (1998) Carbamoyl phosphate synthetase: caught in the act of glutamine hydrolysis. *Biochemistry* **37**: 8825–8831
- 43 Waldrop G. L., Rayment I. and Holden H. M. (1994) Three-dimensional structure of the biotin carboxylase subunit of acetyl-CoA carboxylase. *Biochemistry* **33**: 10249–10256
- 44 Galperin M. Y. and Koonin E. V. (1997) A diverse superfamily of enzymes with ATP-dependent carboxylate-amine/thiol ligase activity. *Prot. Sci.* **6**: 2639–2643
- 45 Yount R. G., Babcock D., Ballantyne W. and Ojala D. (1971) Adenylyl imidodiphosphate, an adenosine triphosphate analog containing a P-N-P linkage. *Biochemistry* **10**: 2484–2489
- 46 Thoden J. B., Wesenberg G., Raushel F. M. and Holden H. M. (1999) Carbamoyl phosphate synthetase: closure of the B-domain as a result of nucleotide binding. *Biochemistry* **38**: 2347–2357
- 47 Raushel F. M., Rawding C. J., Anderson P. M. and Villafraña J. J. (1979) Paramagnetic probes for carbamoyl phosphate synthetase: metal ion binding studies and preparation of nitroxide spin-labeled derivatives. *Biochemistry* **18**: 5562–5566
- 48 Braxton B. L., Mullins L. S., Raushel F. M. and Reinhart G. D. (1992) Quantifying the allosteric properties of *Escherichia coli* carbamyl phosphate synthetase: determination of thermodynamic linked-function parameters in an ordered kinetic mechanism. *Biochemistry* **31**: 2309–2316
- 49 Thoden J. B., Raushel F. M., Wesenberg G. and Holden H. M. (1999) The binding of inosine monophosphate to *Escherichia coli* carbamoyl phosphate synthetase. *J. Biol. Chem.* **274**: 22502–22507
- 50 Hyde C. C., Ahmed S. A., Padlan E. A., Miles E. W. and Davies D. R. (1988) Three-dimensional structure of the tryptophan synthase  $\alpha_2\beta_2$  multienzyme complex from *Salmonella typhimurium*. *J. Biol. Chem.* **263**: 17857–17871
- 51 Kim J. H., Krahn J. M., Tomchick D. R., Smith J. L. and Azkin H. (1996) Structure and function of the glutamine phosphoribosylpyrophosphate amidotransferase glutamine site and communication with the phosphoribosylpyrophosphate site. *J. Biol. Chem.* **271**: 15549–15557
- 52 Krahn J. M., Kim J. H., Burns M. R., Parry R. J., Zalkin H. and Smith J. L. (1997) Coupled formation of an amidotransferase interdomain ammonia channel and a phosphoribosyltransferase active site. *Biochemistry* **36**: 11061–11068
- 53 Muchmore C. R., Krahn J. M., Kim J. H., Zalkin H. and Smith J. L. (1998) Crystal structure of glutamine phosphoribosylpyrophosphate amidotransferase from *Escherichia coli*. *Prot. Sci.* **7**: 39–51
- 54 Voet D. and Voet J. G. (1995) *Biochemistry*, Wiley, New York

- 55 Roux B. and Walsh C. T. (1993) *p*-Aminobenzoate synthesis in *Escherichia coli*: mutational analysis of three conserved amino acid residues of the amidotransferase PabA. *Biochemistry* **32**: 3763–3768
- 56 Zalkin H. (1993) The amidotransferases. *Adv. Enzymol. Relat. Areas Mol. Biol.* **66**: 203–309
- 57 Devaney M. A. and Powers-Lee S. G. (1984) Immunological cross-reactivity between carbamyl phosphate synthetases I, II, and III. *J. Biol. Chem.* **259**: 703–706
- 58 Nyunoya H., Broglie K. E. and Lusty C. J. (1985) The gene coding for carbamoyl-phosphate synthetase I was formed by fusion of an ancestral glutaminase gene and a synthetase gene. *Proc. Natl. Acad. Sci. USA* **82**: 2244–2246
- 59 Nyunoya H., Broglie K. E., Widgren E. E. and Lusty C. J. (1985) Characterization and derivation of the gene coding for mitochondrial carbamyl phosphate synthetase I of rat. *J. Biol. Chem.* **260**: 9346–9356
- 60 Simmer J. P., Kelly R. E., Rinker A. G. Jr, Scully J. L. and Evans D. R. (1990) Mammalian carbamyl phosphate synthetase (CPS): DNA sequence and evolution of the CPS domain of the Syrian hamster multifunctional protein CAD. *J. Biol. Chem.* **265**: 10395–10402
- 61 Hong J., Salo W. L., Lusty C. J. and Anderson P. M. (1994) Carbamyl phosphate synthetase III, an evolutionary intermediate in the transition between glutamine-dependent and ammonia-dependent carbamyl phosphate synthetases. *J. Mol. Biol.* **243**: 131–140
- 62 Hoff M. J. B. van den, Jonker A., Beintema J. J. and Lamers W. H. (1995) Evolutionary relationships of the carbamoylphosphate synthetase genes. *J. Mol. Evol.* **41**: 813–832
- 63 Lawson F. S., Charlebois R. L. and Dillon J. A. (1996) Phylogenetic analysis of carbamoylphosphate synthetase genes: complex evolutionary history includes an internal duplication within a gene which can root the tree of life. *Mol. Biol. Evol.* **13**: 970–975
- 64 Marina A., Bravo J., Fita I. and Rubio V. (1995) Crystallization, characterization, and preliminary crystallographic studies of mitochondrial carbamoyl phosphate synthetase I of *Rana catesbeiana*. *Prot. Struct. Funct. Genet.* **22**: 193–196