

Carbamoyl phosphate synthetase: a crooked path from substrates to products

Frank M Raushel*, James B Thoden†, Gregory D Reinhart‡ and Hazel M Holden†

The formation of carbamoyl phosphate is catalyzed by a single enzyme using glutamine, bicarbonate and two molecules of ATP via a reaction mechanism that requires a minimum of four consecutive reactions and three unstable intermediates. The recently determined X-ray crystal structure of carbamoyl phosphate synthetase has revealed the location of three separate active sites connected by two molecular tunnels that run through the interior of the protein. It has been demonstrated that the amidotransferase domain within the small subunit of the enzyme from *Escherichia coli* hydrolyzes glutamine to ammonia via a thioester intermediate with Cys269. The ammonia migrates through the interior of the protein, where it reacts with carboxy phosphate to produce the carbamate intermediate. The carbamate intermediate is formed by the phosphorylation of bicarbonate by ATP at a site contained within the amino-terminal half of the large subunit. The carbamate intermediate is transported through the interior of the protein to a second site within the carboxy-terminal half of the large subunit, where it is phosphorylated by another ATP to yield the final product, carbamoyl phosphate. The entire journey from substrate to product covers a distance of nearly 100 Å.

Addresses

*Department of Chemistry, Texas A&M University, College Station, TX 77843, USA; e-mail: raushel@tamu.edu

†Institute for Enzyme Research, Department of Biochemistry, University of Wisconsin, Madison, WI 53706, USA; e-mail: jim@enzyme.wisc.edu
e-mail: holden@enzyme.wisc.edu

‡Department of Biochemistry and Biophysics, Texas A&M University, College Station, TX 77843, USA; e-mail: gdr@tamu.edu

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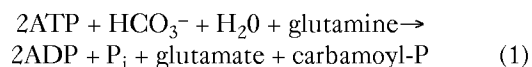
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Abbreviations

ATCase	aspartate transcarbamoylase
carbamoyl-P	carbamoyl phosphate
CPS	carbamoyl-P synthetase
IMP	inosine monophosphate
OTCase	ornithine transcarbamoylase
UMP	uridine monophosphate

Introduction

Carbamoyl phosphate synthetase (CPS) catalyzes one of the most complex reactions in biological chemistry. This enzyme requires five separate substrates to construct carbamoyl phosphate (carbamoyl-P) and four other products as shown below in Equation 1.



Amazingly, this transformation requires a minimum of three distinct reactive intermediates. The catalytic machinery

that has evolved to carry out this reaction has recently been identified through the elucidation of the three-dimensional X-ray structure of CPS from *E. coli* [1]. This monumental structure has revealed the location of three active site pockets joined together by two molecular tunnels! These results indicate that CPS operates very much like a molecular machine in which the products of one active site are channeled directly to the next active site without dissociation into the solvent. The ammonia derived from the hydrolysis of glutamine must therefore make a journey of nearly 100 Å from the point of origin to the site of production of the ultimate product, carbamoyl-P.

In most cells, carbamoyl-P is required for the initiation of two biosynthetic pathways. The assembly of pyrimidine nucleotides starts with the carbamoylation of aspartate in a reaction catalyzed by the enzyme aspartate transcarbamoylase (ATCase), while the detoxification of ammonia and the biosynthesis of arginine begin with the transfer of the carbamoyl group from carbamoyl-P to ornithine. This is catalyzed by the enzyme ornithine transcarbamoylase (OTCase). In lower organisms there is generally a single CPS isozyme responsible for producing carbamoyl-P for both pathways, while in higher organisms there is usually a separate enzyme for each pathway. This review will emphasize the intimate relationship between mechanism and structure of the CPS from *E. coli*, since what is known about the enzyme from this source will be applicable to CPS from other sources as well.

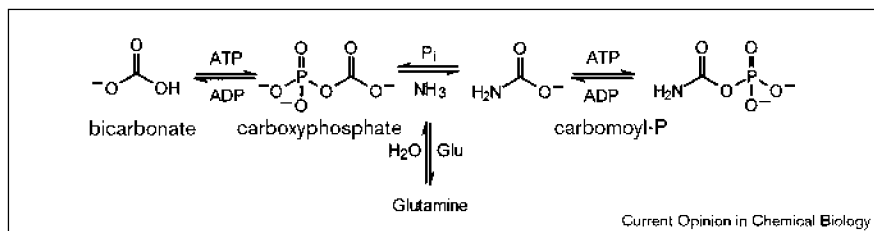
Regulation by allosteric effectors

CPS from *E. coli* is subject to allosteric activation by ornithine and inhibition by uridine monophosphate. These ligands bind to separate sites on the large subunit. Although the heterodimer of CPS aggregates to form tetramers of dimers at high concentration, the allosteric influence of these ligands is clearly apparent in the absence of this aggregation. Indeed, all of the effects are manifested exclusively within the large subunit. Thus, CPS represents one of the few examples of a monomeric allosteric enzyme that is physiologically relevant.

A minor controversy exists over the actions of inosine monophosphate (IMP), which, in competition with uridine monophosphate (UMP), binds to the nucleotide allosteric site. Originally, it was reported that IMP activates CPS, thereby providing a means by which CPS activity would help to maintain a balance between the purine and pyrimidine nucleotide pools [2]. It was later realized that these early studies were performed with limiting Mg^{2+} concentrations and in the presence of phosphate, which is another

Figure 1

The chemical mechanism for assembly of carbamoyl-P by CPS.



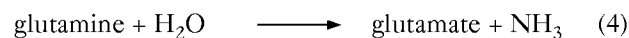
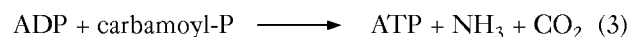
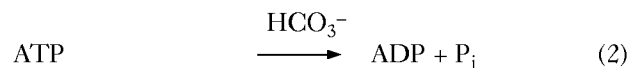
ligand with some affinity for various sites on CPS. Subsequent studies of the effects by IMP in the absence of these complications have indicated that IMP has virtually no effect by itself, although it does bind with good affinity to the enzyme [3]. Indeed, the small effect that IMP does have changes from activation to inhibition if the temperature is lowered below $\sim 35^\circ\text{C}$ [4].

With the structure of CPS solved, attention is now being focused on using this information to understand the molecular basis for these allosteric effects. The potential limitations of this approach have been underscored by an energetic analysis of the allosteric properties of CPS [5]. The allosteric ligands primarily modify the affinity of CPS for its nucleotide substrate, but the effects on ΔG (the change in the Gibbs free energy) of binding are not reflected quantitatively or qualitatively in the ΔH (change in enthalpy) for binding. In fact, both ornithine activation and UMP inhibition are the result of enhancing or diminishing the ΔS (change in entropy) for binding, respectively, with the effects on ΔH favoring the opposite allosteric action for each ligand. These observations suggest that other factors, such as solvation and/or fluctuations in protein structure, may have a greater impact on the nature of the allosteric effects than static structural features, such as charge proximity or steric relationships.

Chemical mechanism

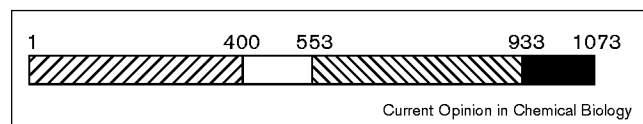
The reaction mechanism that is most consistent with all of the experimental observations conducted on the CPS from *E. coli* is shown below in Figure 1 [2]. This minimal mechanism postulates the existence of three independent reaction intermediates: ammonia, carboxy phosphate and carbamate. This mechanism is supported in part by three partial reactions catalyzed by CPS. In the absence of a nitrogen source, CPS will catalyze the bicarbonate-dependent hydrolysis of ATP. When the bicarbonate is labeled with ^{18}O one of the three oxygen atoms is found in the inorganic phosphate product, indicating that water attacks this highly reactive intermediate at the carbonyl carbon atom. The kinetic competence of the carboxy phosphate intermediate has been established by positional isotope exchange and rapid-quench experiments [6–8]. CPS also catalyzes the phosphorylation of ADP from carbamoyl-P. This partial reaction is consistent with the reversal of the last step of the mechanism depicted in Figure 1. Finally, in the absence of ATP or

bicarbonate, the enzyme will catalyze the relatively slow hydrolysis of glutamine to glutamate and ammonia. These three partial reactions are summarized below in equations 2–4. An alternative chemical mechanism for CPS has recently been proposed by Kothe *et al.* [9 \bullet]. This ‘nucleotide switch’ mechanism suggests that the two phosphorylation events are coupled through protein conformational changes rather than the migration of carbamate from one site to the other. This novel proposal awaits experimental verification.



Protein domains

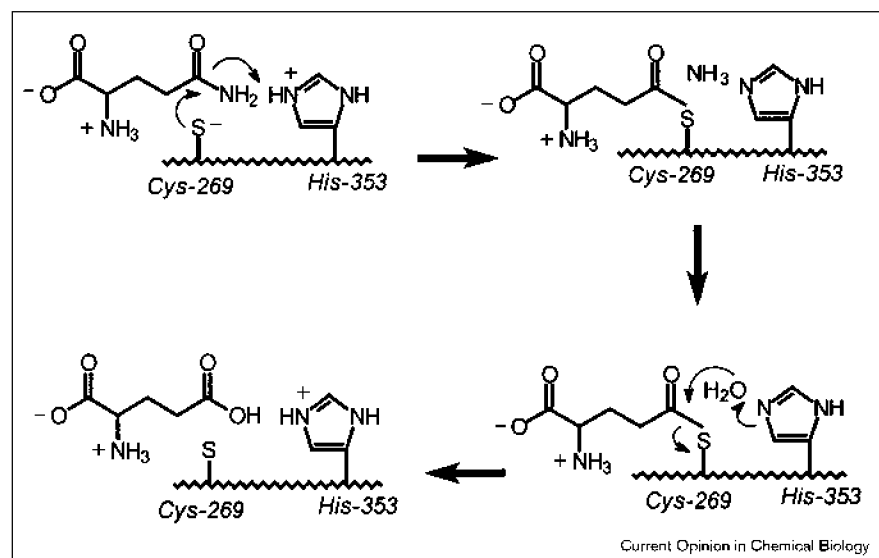
The CPS from *E. coli* is a heterodimer. The smaller of the two subunits can be separated from the large subunit using either genetic or physical methods. Alone, the small subunit is able to catalyze the hydrolysis of glutamine, whereas the isolated large subunit can catalyze the formation of carbamoyl-P using ammonia as a nitrogen source. In many other organisms, the large and small subunits can be found fused into a single polypeptide chain or conjugated to ATCase and dihydroorotase.

Figure 2

Domain boundaries for the large subunit of CPS. The hatched region from residues 1–400 represents the ATP-binding domain, which is used in the phosphorylation of bicarbonate. The white region is the unknown domain. The hatched region from residues 553–933 represents the ATP-binding domain used in the phosphorylation of carbamate. The black domain is the allosteric domain.

The first amino acid sequence determined for CPS was completed by Lusty and co-workers [10,11]. The small and large subunits from *E. coli* are encoded by the *carA* and *carB* genes, respectively. As expected, the small

Figure 3

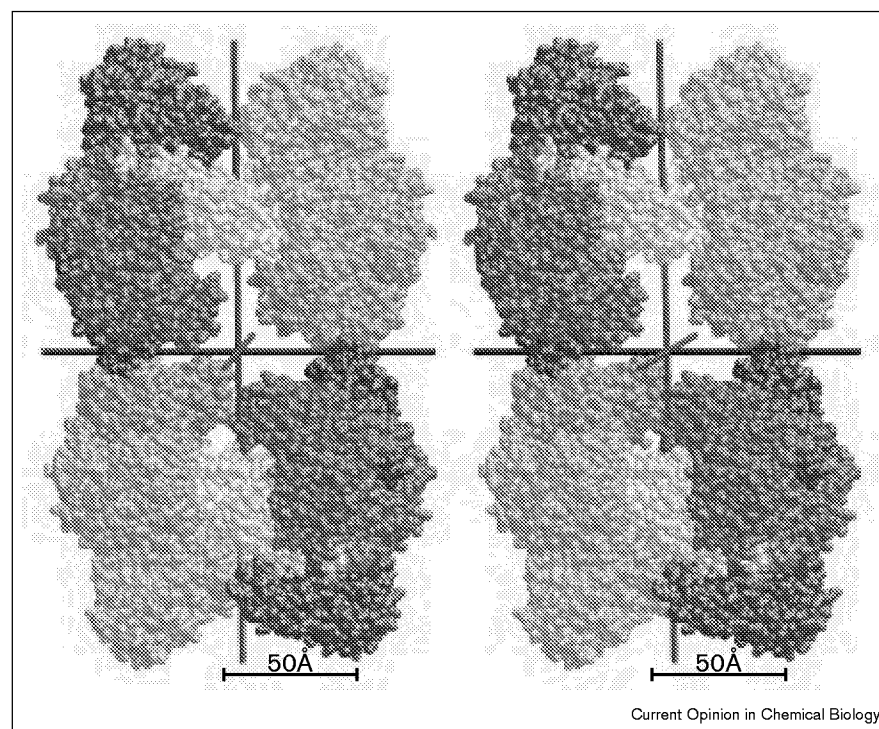


The chemical mechanism for the hydrolysis of glutamine using the active-site residues of the small subunit of CPS. The reaction is initiated by a nucleophilic attack of the thiolate anion of Cys269 on the carbonyl group of glutamine. This reaction is assisted by proton donation from His353 during the formation of the thioester intermediate and ammonia. The ammonia departs the active site and is replaced by a water molecule. His353 abstracts a proton from the incoming water molecule to initiate a nucleophilic attack on the thioester intermediate. The thioester intermediate is hydrolyzed and the formation of glutamic acid is complete.

subunit is homologous to a family of enzymes that utilize glutamine as a source of ammonia. The large subunit was found to be a member of a group of enzymes that use ATP to activate carboxylate functional groups for condensation with a nitrogen or sulfur nucleophile [12*]. Most unusual, however, is the internal sequence identity between the amino-terminal and carboxyl-terminal halves of the large subunit. Residues 1–400 are

40% identical in sequence to residues 553–933 [10]. Lusty and co-workers have postulated that the present-day enzyme arose from a gene duplication event followed by the independent mutation of the domains for more specialized functions. On the basis of the amino acid sequence of the large subunit a domain map can be constructed for the *E. coli* enzyme as illustrated in Figure 2.

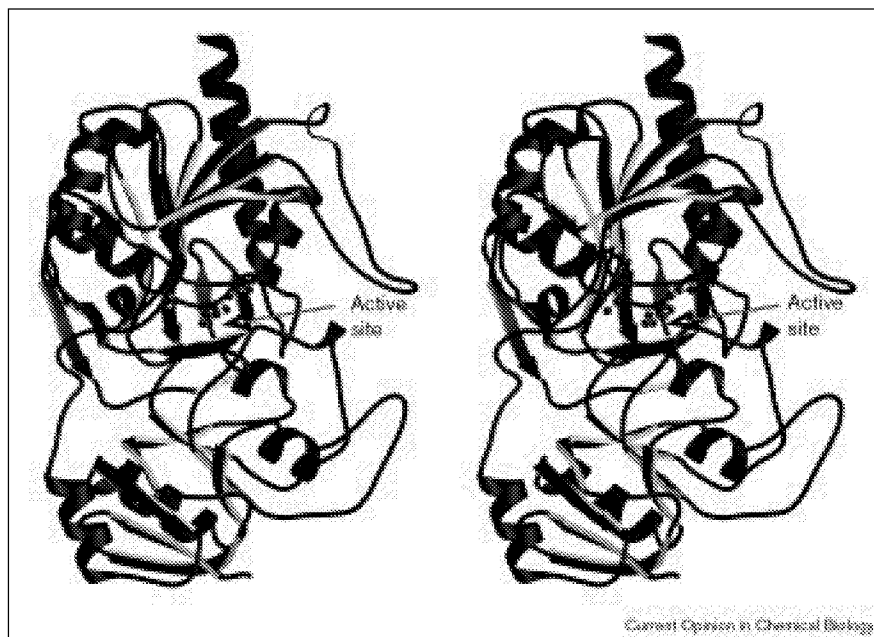
Figure 4



Space-filling stereorepresentation of the CPS (α,β)₄ heterotetramer. The CPS heterotetramer displays 222 symmetry, as indicated by the mutually perpendicular axes. Each heterodimer consists of five structural units: the small subunit is shown as magenta, the carboxy phosphate domain displayed is green, the oligomerization domain is depicted in yellow, the carbamoyl phosphate domain is shown in blue and the allosteric domain is drawn in red. The tetramer is a large open macromolecular assembly with dimensions of 110 × 200 × 235 Å, as measured along the symmetry axes.

Figure 5

Ribbon stereorepresentation of the CPS small subunit. Residues Glu355, His353 and Cys269, which form part of the active site, are displayed in ball-and-stick representations.



Mapping of functional domains

The catalytic and regulatory functions of the hypothetical structural domains depicted in Figure 2 have been identified. It was initially anticipated that each of the two major domains (residues 1–400, 553–933) within the large subunit of CPS from *E. coli* would contain a binding site for one of the two molecules of ATP required for the overall synthesis of carbamoyl-P. Steady state and rapid quench experiments had previously demonstrated that each of the two ATP molecules were bound to physically distinct sites [13]. In the absence of a three-dimensional structure for this protein, the overall strategy for this endeavor was to select potentially critical residues on the basis of established ATP-binding sites in structurally related proteins. Most of the mutants constructed within these sites were fully characterized with regard to their ability to catalyze each of the two partial reactions involving ATP and the full biosynthetic reaction. The bicarbonate-dependent ATPase reaction was used as a direct probe of the nucleotide site required for the phosphorylation of bicarbonate, while the ATP synthesis reaction was used to monitor the site required for the phosphorylation of carbamate.

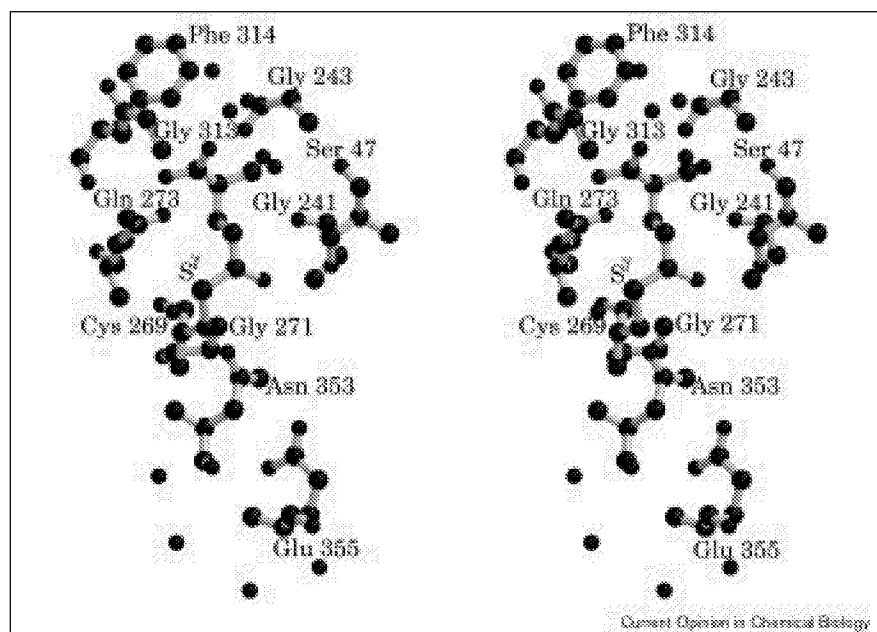
Over 15 different mutations were constructed within each of the two catalytic domains of the large subunit of CPS from *E. coli* and other organisms [14–16]. In nearly every case where effects on the catalytic properties were obtained, the mutations within the amino-terminal domain predominantly affected the bicarbonate-dependent ATPase reaction, while the homologous mutations within the carboxy-terminal domain were deficient in the synthesis of ATP from ADP and carbamoyl-P. Thus, it has been concluded that the amino-terminal domain (now designat-

ed as the carboxy phosphate domain) functions to phosphorylate bicarbonate, whereas the carboxy-terminal domain (now designated as the carbamoyl-P domain) functions to phosphorylate carbamate [15]. These investigations have also indicated that the formation of carbamate, initiated by the attack of ammonia on the carboxy phosphate intermediate, occurs within the amino-terminal domain [17].

The extreme carboxyl terminus of the large subunit has been shown to harbor the binding sites for the two allosteric effectors. Truncations and site-directed mutations have demonstrated that the ornithine and UMP allosteric binding sites are distinct and reside in the domain formed between amino acid residues 933 and 1073 [18]. These results were consistent with chemical labeling studies using radioactive UMP by Rubio *et al.* [19]. Mutants were unable to define the role of the 'unknown' domain between amino acid residues 400 and 553.

The catalytic mechanism of the small subunit has also been extensively explored. The role of this domain is to hydrolyze glutamine for the sole purpose of supplying ammonia to the large subunit. The most critical residue within the active site of the small subunit of CPS from *E. coli* appears to be Cys269. This residue was first identified by covalent labeling with a chloroketone analog of glutamine and later by mutagenesis to a serine residue [20]. The serine mutation renders the enzyme catalytically inactive but the mutant will still bind glutamine. Lusty [21] has also demonstrated that incubation of CPS with labeled glutamine results in the formation of an adduct that can be trapped by precipitation with acid.

Figure 6



A close-up stereoview of the region surrounding the glutamyl-thioester intermediate observed in the CPS small subunit. Ordered water molecules are indicated by the black spheres. S γ represents the sulfur atom of Cys269.

The chemical properties of the adduct are consistent with a thioester intermediate. Additional mutations of residues His353 and His312 have implicated these residues in the activation of the thiolate anion and binding of glutamine, respectively [22]. The working model for the hydrolysis of glutamine and generation of ammonia is presented in Figure 3.

X-ray crystal structure

The overall molecular architecture of the CPS (α,β)₄ tetramer is displayed as a space-filling representation in Figure 4 [1]. The four small subunits of the (α,β)₄ tetramer are perched at the ends of the molecule. Each large subunit in the tetramer can be envisioned as containing four distinct components: two units referred to as the carboxy phosphate and carbamoyl-P domains, an oligomerization domain and the allosteric domain. The molecular interactions between the small and large subunits of the CPS heterodimer are quite extensive but only those residues in the carboxy phosphate and the oligomerization domains of the large subunit contribute to the formation of this dimeric interface. In contrast to the subunit-subunit interface of the α,β heterodimer, the number of interactions between one α,β heterodimer and another within the complete tetramer are minimal. The three-dimensional structure of CPS is remarkable in both its complexity and, yet, its simplicity. Each of the component parts is quite simple, but the manner in which these individual units form the complete enzyme is exceedingly complicated.

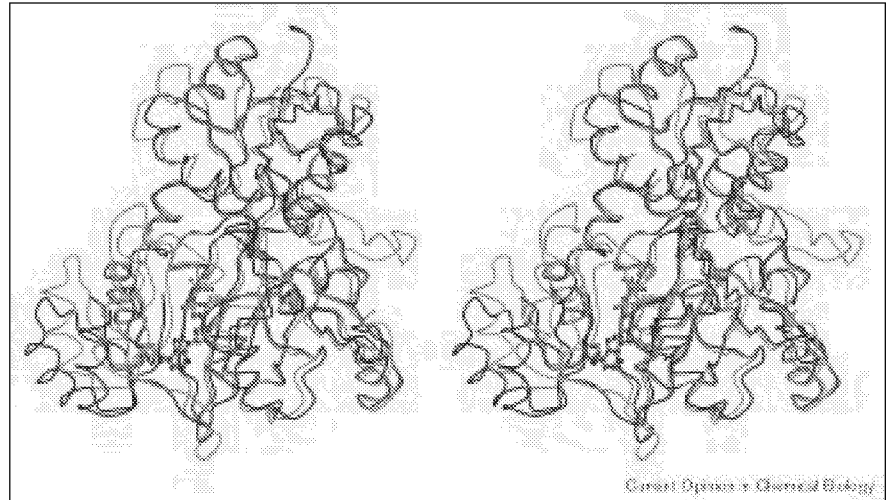
A ribbon representation of the small subunit is displayed in Figure 5, and, as can be seen, the polypeptide chain folds into two distinct structural motifs. Three-dimensional struc-

tural searches have thus far failed to reveal any significant homology between the amino-terminal domain of the CPS small subunit and other proteins of known structure; however, it is absolutely clear that the carboxy-terminal domain of the small subunit is highly homologous to the amino-terminal domain of guanosine monophosphate synthetase and to other members of the *trpG*-type amidotransferases [23]. Recent X-ray crystallographic analyses of the His353→Asn mutant have confirmed the existence of the glutamyl thioester intermediate, which was trapped in the active site [24**]. A close-up view of the region surrounding the intermediate is shown in Figure 6. The hydroxyl group of Ser47 and the backbone amide hydrogen of Gly-241 are in ideal locations to position the carbonyl carbon atom of the substrate for nucleophilic attack by the thiolate anion of Cys269 and to stabilize the developing oxyanion. This work represents the first structural evidence for a glutamyl thioester intermediate in the amidotransferase family of proteins.

The large subunit is even more complex. As anticipated the carboxy phosphate and carbamoyl-P domains are topologically, but not structurally, equivalent, as can be seen from the superposition shown in Figure 7. These structural units are related by a nearly exact twofold rotation axis within the large subunit, thereby suggesting that CPS has evolved by a gene duplication from a primordial α_2 homodimeric enzyme. Each of the two synthetase catalytic domains can be broken down into three smaller motifs referred to as the A, B and C subdomains, which are similar in overall structures to those observed in biotin carboxylase [25], D-alanine : D-alanine ligase [26], glutathione synthetase [27] and succinyl-CoA synthetase [28]. In all of these enzymes, the active sites are

Figure 7

Stereo superpositions of the carboxy phosphate and carbamoyl-P domains of the CPS large subunit, displayed in green and blue, respectively. The observed ADP moieties and manganese ions are depicted in ball-and-stick representations.



wedged between the B and C subdomains with the B subdomains showing conformational flexibilities that are dependent upon the nature of the molecular species occupying the active sites.

The active sites for both the carboxy phosphate and carbamoyl-P domains contain bound MnADP. In addition, an inorganic phosphate has been observed to bind only in the active site of the carboxy phosphate domain. As a

Figure 8

Close-up stereo views of the active sites of the CPS large subunit. **(a)** The active site for the carboxy phosphate domain. The ADP and the manganese and potassium ions are displayed in ball-and-stick representations. Residues responsible for binding the nucleotide and the ions are indicated (Arg169 and Glu215). **(b)** The active site for the carbamoyl-P domain. Residues responsible for binding the nucleotide are shown (Arg715 and Glu761).

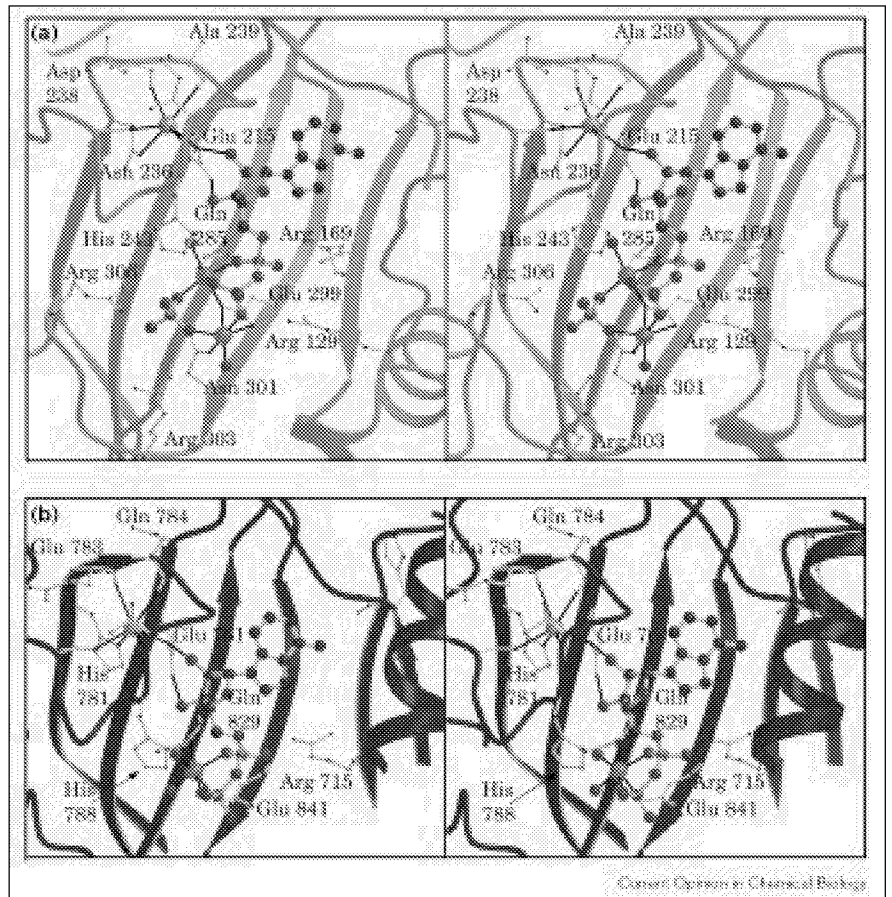
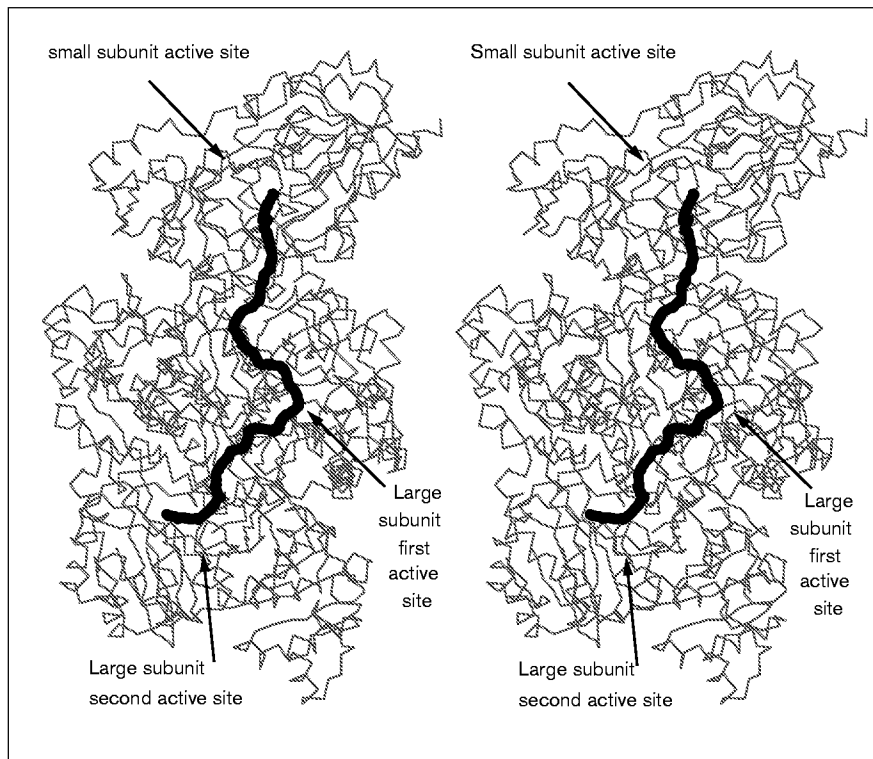


Figure 9



A possible molecular tunnel from the CPS small subunit active site to the two active sites of the large subunit, shown as a stereoview. The α,β -heterodimer is shown as an α -carbon trace while the pathway of the putative molecular tunnel is indicated by the thick black line. Reproduced, with permission, from [32].

result of this inorganic phosphate, the B-subdomain in the carboxy phosphate domain, relative to that in the carbamoyl-P synthetic component, is closed down, as shown in Figure 7. Close-up views of the two active sites in the large subunit are depicted in Figure 8. The manner by which the nucleotides are linked to the protein is strikingly similar for each active site. For example, Arg169 (in the carboxy phosphate domain) and Arg715 (in the carbamoyl-P domain) play similar roles by interacting with the α -phosphate moieties of the ADP molecules. Likewise, both Glu215 (in the carboxy phosphate domain) and Glu761 (in the carbamoyl-P domain) serve similar functions by anchoring the 2'- and 3'-hydroxyl groups of the nucleotide riboses and acting as bridges to the potassium binding sites. In both synthetase units, the potassium ions are octahedrally coordinated by three carbonyl oxygen atoms and three sidechain ligands.

The two nucleotide-binding sites (Figure 8) differ primarily by the presence of an inorganic phosphate and a second manganese ion in the carboxy phosphate domain. In this domain the manganese ions are octahedrally coordinated by oxygen-containing ligands and are bridged by the carboxylate sidechain of Glu299. In the carbamoyl-P domain the sole manganese ion is surrounded in an octahedral coordination sphere by two phosphoryl oxygen atoms, a water molecule and the sidechains of Gln829 and Glu841.

The one undeniable fact to emerge from the recent structural investigations of CPS is that the three active sites contained within this α,β heterodimer are separated by a linear distance of nearly 100 Å. The intermediates, carboxy phosphate, ammonia and carbamate, are highly reactive and thus the catalytic cycles among the three active sites must be coordinated with one another. Visual inspection of the CPS model indicates a possible tunnel connecting the three active sites in the α,β heterodimer. This tunnel, as depicted in Figure 9, extends from the base of the small subunit active site towards the carboxy phosphate domain and is lined, for the most part, with unreactive side chains and backbone atoms. The portion of the tunnel lying between the two active sites of the large subunit is somewhat less hydrophobic. In support of the molecular tunnel, it should be noted that a majority of the residues lining the pathway depicted in Figure 9 are conserved throughout the evolution of CPS. Interestingly, a channel of 20 Å has also recently been found in glutamine phosphoribosylpyrophosphate amidotransferase [29**].

Domain interconversion

The carboxy phosphate and carbamoyl-P domains of CPS from *E. coli* are very similar in amino acid sequence and are structurally similar, but not identical. The structural differences are directly due to the specialized functions of these domains. For example, the carboxy phosphate domain must provide for a site of interaction with the

small subunit, in addition to forming a molecular tunnel for the assimilation of ammonia after the hydrolysis of glutamine. In contrast, only the carbamoyl-P domain needs to be receptive to the effects induced by the allosteric ligands; therefore, it is not surprising that a molecular tunnel for ammonia cannot be identified within the carbamoyl-P domain. Moreover, the amino acid residues required for the association of the small subunit to the carboxy phosphate domain are not conserved in the carbamoyl-P domain and thus it is unlikely that the small subunit could associate with both domains. These observations make it difficult to understand the catalytic activities recently reported by truncated and fused versions of the large subunit of CPS [30*,31*]. For example, it has been reported that a miniprotein, comprised only of residues 96–363, is more catalytically active than the wild type protein. The formation of carbamoyl-P by this truncated protein has been interpreted to occur through the formation of a homodimer, where the domains are functionally equivalent for the phosphorylation of either bicarbonate or carbamate; however, every one of the amino acid residues required for intradomain contact in the native enzyme has been removed during the truncation process. Therefore, it is very difficult to imagine how a homodimer is able to form with sufficient affinity. Similar problems occur in attempting to understand, on the basis of the current X-ray model of CPS, the manner in which a truncated variant formed from the carbamoyl-P domain (composed of residues 507–899) is able to catalyze the formation of carbamoyl-P using glutamine as a nitrogen source. The binding site for the association of the small subunit is not maintained in the carboxy-terminal half of CPS and an analogous tunnel for the utilization of ammonia cannot be located in this half of the large subunit. In order for these many uncertainties to be clarified, the truncated and fused proteins must be purified to homogeneity and fully characterized for their ability to catalyze all of the partial and full biosynthetic reactions.

Conclusions

CPS has been shown to catalyze the formation of carbamoyl-P via a reaction mechanism that utilizes ammonia, carboxy phosphate and carbamate as reaction intermediates. The high resolution X-ray crystal structure of CPS has revealed the relative locations of three active sites that are interconnected by two molecular tunnels that run through the interior of the protein. It is likely that the products of one active site are channeled to the next active site. It remains to be determined how the three active sites communicate with one another in order to optimize the efficiency of this spectacular molecular machine.

Acknowledgements

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