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## Perspectives in Biochemistry

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### The Amidotransferase Family of Enzymes: Molecular Machines for the Production and Delivery of Ammonia<sup>†</sup>

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**ABSTRACT:** The amidotransferase family of enzymes utilizes the ammonia derived from the hydrolysis of glutamine for a subsequent chemical reaction catalyzed by the same enzyme. The ammonia intermediate does not dissociate into solution during the chemical transformations. A well-characterized example of the structure and mechanism displayed by this class of enzymes is provided by carbamoyl phosphate synthetase (CPS). Carbamoyl phosphate synthetase is isolated from *Escherichia coli* as a heterodimeric protein. The smaller of the two subunits catalyzes the hydrolysis of glutamine to glutamate and ammonia. The larger subunit catalyzes the formation of carbamoyl phosphate using 2 mol of ATP, bicarbonate, and ammonia. Kinetic investigations have led to a proposed chemical mechanism for this enzyme that requires carboxy phosphate, ammonia, and carbamate as kinetically competent reaction intermediates. The three-dimensional X-ray crystal structure of CPS has localized the positions of three active sites. The nucleotide binding site within the N-terminal half of the large subunit is required for the phosphorylation of bicarbonate and subsequent formation of carbamate. The nucleotide binding site within the C-terminal domain of the large subunit catalyzes the phosphorylation of carbamate to the final product, carbamoyl phosphate. The three active sites within the heterodimeric protein are separated from one another by about 45 Å. The ammonia produced within the active site of the small subunit is the substrate for reaction with the carboxy phosphate intermediate that is formed in the active site found within the N-terminal half of the large subunit of CPS. Since the ammonia does not dissociate from the protein prior to its reaction with carboxy phosphate, this intermediate must therefore diffuse through a molecular tunnel that connects these two sites with one another. Similarly, the carbamate intermediate, initially formed at the active site within the N-terminal half of the large subunit, is the substrate for phosphorylation by the ATP bound to the active site located in the C-terminal half of the large subunit. A molecular passageway has been identified by crystallographic methods that apparently facilitates diffusion between these two active sites within the large subunit of CPS. Synchronization of the chemical transformations is controlled by structural perturbations among the three active sites. Molecular tunnels between distant active sites have also been identified in tryptophan synthase and glutamine phosphoribosyl pyrophosphate amidotransferase and are likely architectural features in an expanding list of enzymes.

A full accounting of the intimate relationship between structure and function in enzymatic systems remains largely

unresolved. This situation is particularly true for the architecturally and mechanistically rich family of amidotransferase enzymes. This class of enzymes is now known to initiate the hydrolysis of glutamine at one active site and to then deliver the ammonia product to another active site located within the same protein (*1*). The translocation of ammonia from the site of production to the site of utilization is facilitated in at least two cases (carbamoyl phosphate

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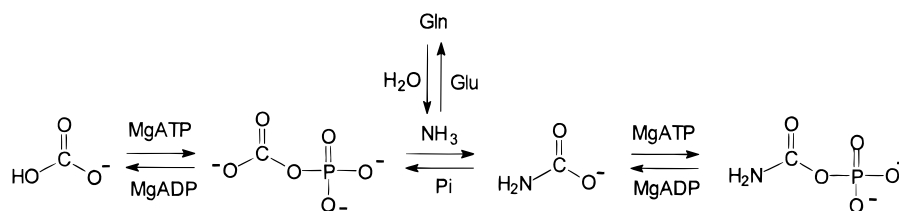
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Scheme 1

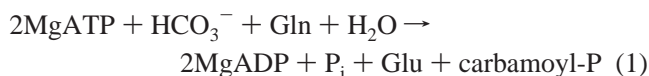


synthetase and phosphoribosyl pyrophosphate amidotransferase) by a molecular tunnel that runs through the interior of these proteins. However, the evolutionary path taken by these two enzymes for this common task is clearly different. The diffusion of intermediates from one active site to another, without release into the bulk solvent, serves as a model for the direct channeling of products from one enzyme to another within a single metabolic pathway.

In this perspective we attempt to provide a detailed account for how one member of this family of enzymes harnesses the hydrolysis of glutamine for a subsequent chemical event. The recent high-resolution X-ray crystal structure determination of carbamoyl phosphate synthetase (CPS)<sup>1</sup> from *Escherichia coli*, coupled with the associated biochemical and kinetic analyses, has begun to expose the inner workings of a remarkable molecular machine for the regulated assembly of carbamoyl phosphate (2, 3). Structurally, this protein establishes paradigms for two disparate superfamilies of enzymes. The amidotransferase component of CPS serves as a benchmark for how protein systems can initiate the capture and utilization of ammonia obtained directly from the hydrolysis of glutamine. Moreover, the synthetase component contains, within a single polypeptide chain, two related examples from the "ATP-grasp" superfamily of proteins (4). However, the most remarkable feature of CPS is the recent discovery that this protein contains three active sites that are separated from one another by ~45 Å and joined by a molecular tunnel that runs through the interior of the entire protein (5, 6). We also illustrate the complexities that can arise from systems that must coordinate and synchronize reactions at multiple catalytic and regulatory sites.

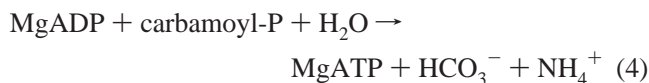
### MECHANISM OF ACTION FOR CARBAMOYL PHOSPHATE SYNTHETASE

CPS catalyzes the formation of carbamoyl phosphate from 2 mol of MgATP, bicarbonate, and glutamine in a reaction that is summarized in eq 1. The synthesis of carbamoyl



phosphate serves as the gateway for two distinct biosynthetic pathways. The carbamoyl moiety is either transferred to ornithine during arginine biosynthesis or, alternatively, this same group is transferred to the α-amino group of aspartate for the initiation of the synthesis of pyrimidine nucleotides. In simple organisms such as *E. coli* there is a common enzyme for both pathways, whereas in more complex systems there is a separate enzyme for each pathway. In addition to

the overall reaction, CPS also catalyzes three partial reactions at somewhat slower rates when one or more of the substrates are omitted from the reaction mixture. These partial reactions are summarized in eqs 2–4. The discovery of these reactions



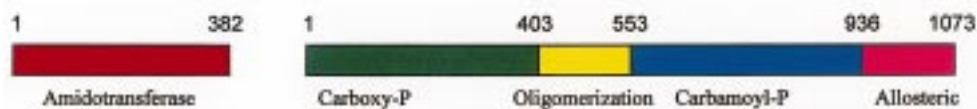
by Anderson and Meister was critical for the establishment of a self-consistent chemical mechanism (Scheme 1) for the formation of carbamoyl phosphate by CPS (7). These partial reactions have also been instrumental in the assignment of functional properties to the structural domains of this enzyme.

**Carboxy Phosphate.** The chemical mechanism presented in Scheme 1 postulates the existence of three independent reaction intermediates leading to the synthesis of carbamoyl phosphate. The first of these intermediates, carboxy phosphate, is experimentally supported by the observation of a bicarbonate-dependent ATPase reaction as shown in eq 3. In the absence of a nitrogen source (glutamine or ammonia), the enzyme will catalyze the hydrolysis of ATP to ADP and P<sub>i</sub> at a rate that is 1–10% of the rate of carbamoyl-P formation when all of the other substrates are present (eq 1). When the bicarbonate is labeled with oxygen-18, one atom of oxygen from the bicarbonate is transferred to the phosphate (8). This result is consistent with the formation of an intermediate between ATP and bicarbonate during the catalytic cycle. The estimated half-life for free carboxy phosphate is about ~70 ms, and thus the protein environment within the active site must protect this intermediate from attack by water and/or unimolecular collapse to CO<sub>2</sub> and phosphate (9). Positional isotope exchange (PIX) experiments (8, 10, 11) and rapid-quench kinetic studies by Villafranca have demonstrated that the carboxy phosphate intermediate is formed sufficiently fast to be a kinetically competent intermediate (12). An alternative proposal has suggested that CO<sub>2</sub>, rather than carboxy phosphate, may be the electrophile that subsequently reacts with the ammonia intermediate (9). However, recent kinetic studies have shown that CO<sub>2</sub> is not the product that is released into the bulk solution during the bicarbonate-dependent ATPase reaction (13). Thus, the breakdown of the carboxy phosphate intermediate during the bicarbonate-dependent ATPase reaction results from the attack of water (rather than ammonia) at the carbonyl carbon.

**Ammonia.** The second intermediate proposed in Scheme 1 is ammonia. The occurrence of this intermediate is supported by the observation of the hydrolysis of glutamine to glutamate and ammonia in the absence of either MgATP or bicarbonate (eq 2). The rate of this partial reaction is 0.1–

<sup>1</sup> Abbreviations: CPS, carbamoyl phosphate synthetase; GPATase, glutamine phosphoribosyl pyrophosphate amidotransferase; NEM, *N*-ethylmaleimide; PIX, positional isotope exchange.

Scheme 2



1% of the overall reaction depicted in eq 1. The intermediacy of ammonia is also supported by the fact that CPS can use ammonia as a direct source of nitrogen for the construction of carbamoyl phosphate. Recently, it has been shown that the ammonia produced from glutamine during the formation of carbamoyl phosphate does not mix with ammonia added directly to the bulk solvent (14). This observation demonstrates that the ammonia derived from glutamine does not dissociate from the enzyme surface and then rebind at an alternative location.

**Carbamate.** The last intermediate formed during the catalytic cycle is carbamate. Direct experimental support for this intermediate is not as conclusive as it is for either ammonia or carboxy phosphate. Nevertheless, the partial ATP synthesis reaction (eq 4) is consistent with a reversal of the last chemical event shown in Scheme 1. The kinetic significance of the carbamate intermediate in partial reaction 4 has been shown using isotopically labeled carbamoyl phosphate and measurement of a positional isotope exchange reaction that is four times faster than the net formation of ATP (11). The intermediacy of carbamate in partial reaction

4 has also been demonstrated through the measurement of the time course for proton release (13).

Recently, a novel “nucleotide switch” mechanism has been proposed for the formation of carbamoyl phosphate (15). In this mechanism carbamoyl phosphate is proposed to arise directly from the attack of ammonia on the carboxy phosphate intermediate without the intermediacy of carbamate. The proposed driving force for this thermodynamically unfavorable reaction is a protein conformational change that is the result of ATP hydrolysis at a distant location. In this mechanism the energy needed to synthesize carbamoyl phosphate is harnessed from the parallel hydrolysis of two ATP molecules. However, in the classic mechanism shown in Scheme 1, energy is abstracted in a linear fashion as mediated through a sequential production of carboxy phosphate and carbamate prior to the formation of carbamoyl phosphate. Recently, the nucleotide switch mechanism has been shown to be inconsistent with isotopic labeling experiments and pulse–chase experiments (16, 17). Two decades of kinetic investigations are fully consistent with the chemical mechanism depicted in Scheme 1.

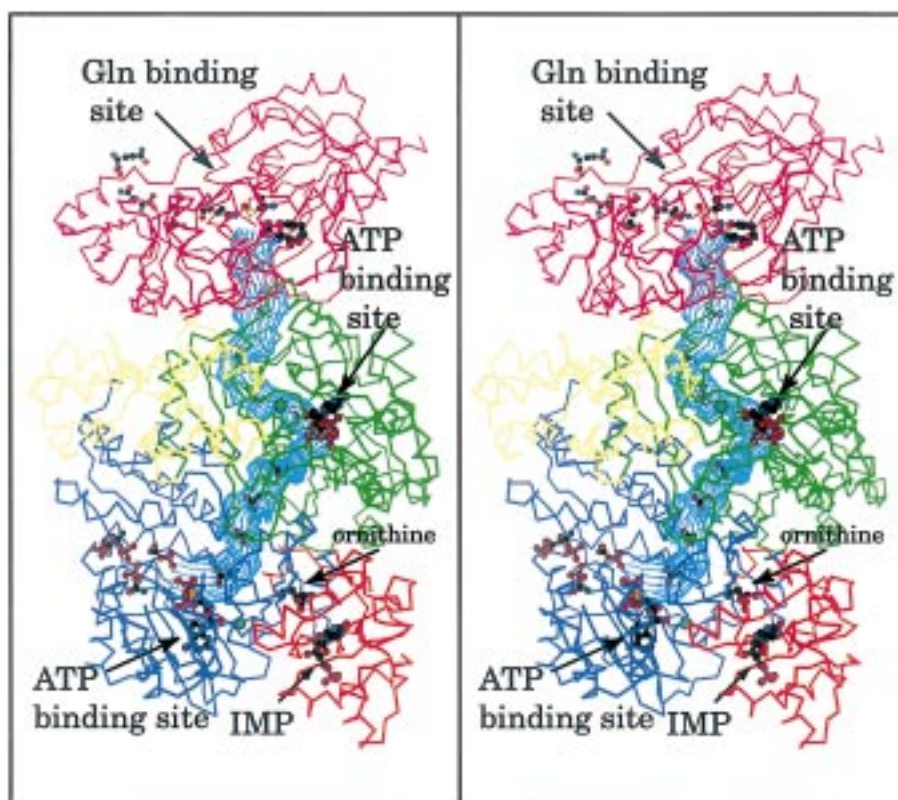


FIGURE 1:  $\alpha$ -Carbon trace of a CPS  $\alpha,\beta$ -heterodimer. The small subunit is color coded in magenta while the large subunit is depicted in green, yellow, blue, and red to indicate the positions of the carboxy phosphate, the oligomerization, the carbamoyl phosphate, and the allosteric domains, respectively. The course of the molecular tunnel leading from the small subunit active site to the two ATP binding sites in the large subunit is indicated by the blue “wire” representation. The locations of Cys-269 in the small subunit, the two ADP moieties, the potassium ions, the ornithine, and the IMP are indicated by the ball-and-stick representations. In addition, the ball-and-stick representations of the glutamines, the ammonia molecules, the carbamates, and the carbamoyl phosphates are meant to aid the viewer in following the course of the reaction from the small to the large subunit.



## STRUCTURAL PROTEIN DOMAINS

The CPS from *E. coli* is isolated as an  $\alpha,\beta$ -heterodimer. The smaller subunit of molecular weight  $\sim 42\ 000$  is encoded by the *carA* gene whereas the larger subunit of molecular weight  $\sim 118\ 000$  is encoded by the *carB* gene (18, 19). The individual subunits can be isolated through partial protein denaturation and separation on a size exclusion column. Alternatively, the two genes can be expressed while on separate plasmids. Characterization of the purified subunits has demonstrated that the small subunit can hydrolyze glutamine but cannot catalyze any other reaction. In contrast, the isolated large subunit is able to catalyze the formation of carbamoyl phosphate but only when ammonia is used as the nitrogen source. This subunit is also subjected to regulation by the two allosteric effectors, ornithine and UMP. Thus, the small subunit contains the active site for the binding of glutamine, whereas the large subunit contains the binding sites for ATP, bicarbonate, ammonia, and the two allosteric effectors (20).

**Sequence Comparisons.** Amino acid sequence analysis by Lusty and colleagues has shown that the C-terminal half of the small subunit is homologous to a small number of other amidotransferase enzymes that require glutamine as a source of ammonia (19). The amino acid sequence for the large subunit indicates that it can be subdivided in four major structural domains (18). Quite surprisingly, two of these domains show unequivocal evidence of a gene duplication event during the evolution of CPS. Residues 1–403 are  $\sim 40\%$  identical in sequence to the segment that extends from residue 553 to residue 936. These results immediately suggest that the overall structural fold and functional properties of these two domains within the large subunit of CPS are very similar to one another. Moreover, these two homologous domains are now known to be members of the ATP-grasp superfamily of proteins (4). The remaining two domains, which extend from 404 to 553 and from 937 to 1073, are not homologous to one another nor are they related in sequence to any other known protein. The approximate domain boundaries are illustrated in Scheme 2.

**Structural Comparisons.** The structural predictions based on the sequence of the large and small subunits of the bacterial CPS have been borne out by the monumental determination of the three-dimensional crystal structure at high resolution (5, 6). Shown in Figure 1 is an  $\alpha$ -carbon trace of the CPS  $\alpha,\beta$ -heterodimer. The small subunit, depicted in magenta, contains an N-terminal domain formed by Leu-1 to Leu-153 and a C-terminal domain delineated by Asn-154 to Lys-382. The N-terminal motif contains seven  $\beta$ -strands that fold into two layers of  $\beta$ -sheet oriented nearly perpendicular to one another while the C-terminal domain is dominated by a ten-stranded mixed  $\beta$ -sheet flanked on either side by  $\alpha$ -helical regions. As shown in Figure 1, the larger subunit can be envisioned as four structural units delineated by Met-1 to Glu-403, Val-404 to Ala-553, Asn-554 to Asn-936, and Ser-937 to Lys-1073 colored in green, yellow, blue, and red, respectively. As suggested from amino acid sequence comparisons (18), and subsequently confirmed by Thoden et al. (5), the two major domains are topologically equivalent, but the structures are not identical. Each of the homologous domains is responsible for binding one of the two MgATP molecules required for catalysis.

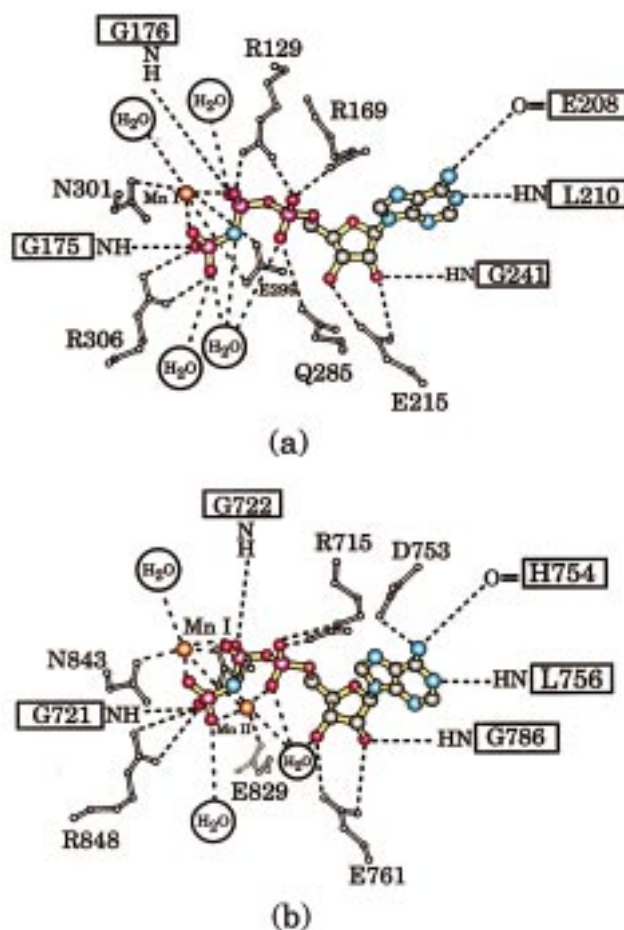


FIGURE 2: Cartoon of the binding modes exhibited by the MnAMPPNP moieties in the CPS large subunit. (a) Potential electrostatic interactions between the nucleotide triphosphate and the carboxy phosphate domain are shown. The dashed lines indicate distances equal to or less than  $3.0$  Å between atoms capable of participating in hydrogen-bonding interactions. (b) The interactions between the nucleotide triphosphate and the carbamoyl phosphate domain are displayed.

Both of these homologous synthetase units are further divided into three modules referred to as the A-, B-, and C-subdomains as also observed in biotin carboxylase and other members of the ATP-grasp superfamily (4, 21). In all of these enzymes, the binding sites for MgATP are wedged between the B- and C-subdomains (5, 6, 22). The mode of nucleotide binding within the active sites of these two CPS synthetase units is strikingly similar as shown in Figure 2. The carboxylate side chains (Glu-215 or Glu-761) which bridge the 2'- and 3'-hydroxyl groups of the ribose moieties also serve as ligands to the essential potassium ions. Recent studies with the nonhydrolyzable analogue, AMPPNP (23), have revealed that the B-subdomain of the carbamoyl phosphate domain closes down over the active site pocket upon binding of the nucleotide triphosphate. Some atoms move by more than  $7$  Å relative to that observed with only MnADP bound in the active site (22). 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 as indicated in Figure 2.

There are two other major structural domains within the large subunit of CPS. The domain which bridges the two homologous synthetase domains folds into seven  $\alpha$ -helices

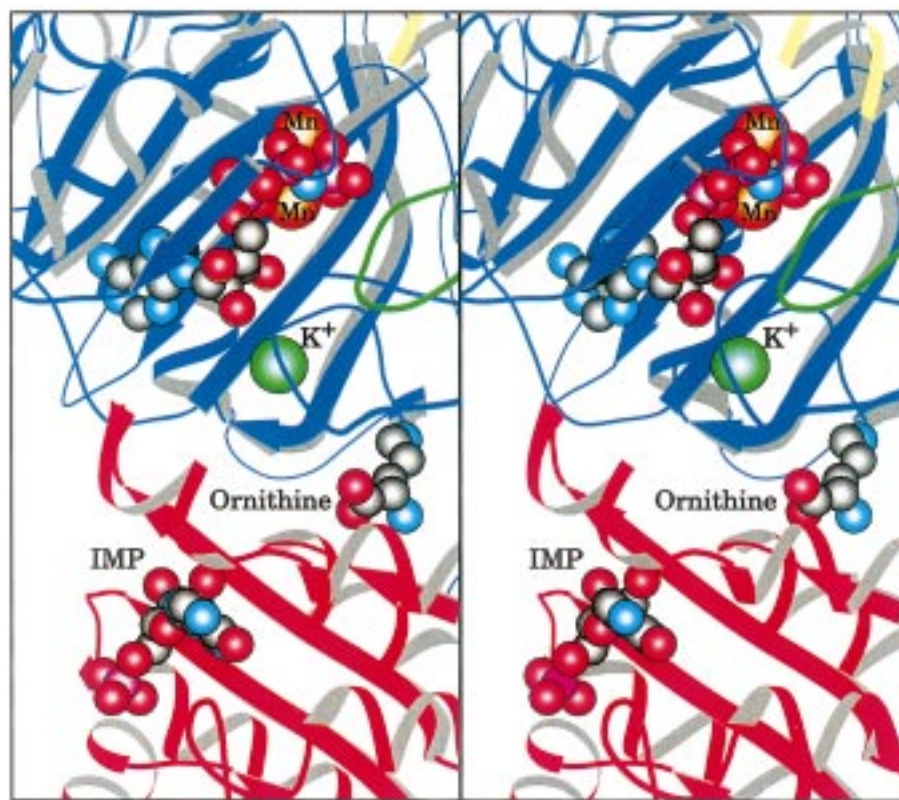


FIGURE 3: Relative disposition of the MnAMPPNP,  $K^+$ , ornithine, and IMP binding sites. The color coding of the ribbon is the same as described in Figure 1. The MnAMPPNP,  $K^+$ , ornithine, and IMP moieties are depicted in space-filling representations.

and two strands of antiparallel  $\beta$ -sheet. A significant fraction of the molecular interactions between the large and small subunit is mediated through this structural domain. Moreover, in the tetrameric  $(\alpha,\beta)_4$ -structure, formed by the oligomerization of the heterodimer, there is intermolecular contact between symmetry-related pairs of residues within this domain (5, 6). However, the number of molecular contacts is actually quite small, but these interactions may be critical for certain allosteric transitions at high enzyme concentrations. There is no known role for this domain in any of the catalytic events. This structural unit has been referred to as the “oligomerization” or “unknown” domain.

The structural domain that is located at the extreme terminus of the large subunit contains the binding sites for the two allosteric effectors. The central core of the allosteric domain is a modified “Rossmann” fold. It is formed from a five-stranded parallel  $\beta$ -sheet flanked on either side by two and three  $\alpha$ -helices. The function of the allosteric domain has been shown to harbor the binding sites for both ornithine and IMP/UMP (5, 6, 24–26). Specifically, ornithine bridges the interface between the allosteric and the carbamoyl phosphate domains such that its  $\alpha$ -amino group lies within hydrogen-bonding distance to  $O^\gamma$  of Tyr-1040 while the  $\delta$ -amino group is positioned within 3.0 Å from the carboxylate groups of Glu-783 and Glu-892 and O of Asp-791. In contrast to the ornithine binding region, the IMP nucleotide is contained wholly within the allosteric domain and is situated at the C-terminal portion of a five-stranded parallel- $\beta$  sheet (27). The precise location for the binding of UMP has not been structurally identified by crystallography. However, since the binding of UMP and IMP is mutually exclusive, it has been concluded that there is significant overlap in the

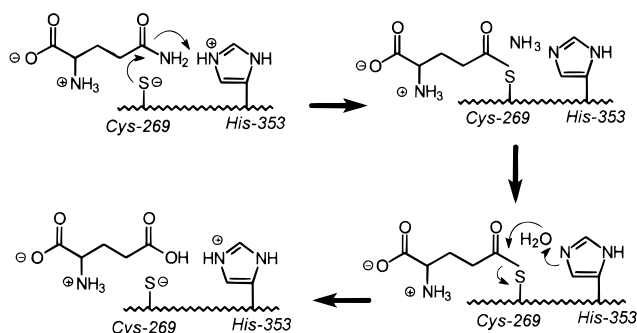
binding site for these effectors (28, 29). Those amino acid side chains responsible for anchoring the nucleotide monophosphate to the large subunit include Lys-954, Thr-974, Thr-977, Lys-993, Asn-1015, and Thr-1017. The ornithine and IMP ligands are situated at approximately 14 and 19 Å, respectively, from the active site of the carbamoyl phosphate domain. An image of the allosteric domain showing the binding sites for ornithine and IMP is presented in Figure 3 (27).

#### MAPPING OF THE FUNCTIONAL DOMAINS

The catalytic and allosteric roles that each of the five major components of CPS play in the assembly of carbamoyl phosphate have been elucidated through a combination of kinetic analyses with the wild-type enzyme and characterization of selected site-directed mutants. Analysis of the chemical mechanism for the hydrolysis of glutamine within the small subunit has demonstrated that the amide bond is cleaved via the formation of a covalent thioester intermediate with an active site cysteine residue. The synthetase domain within the N-terminal half of the large subunit is responsible for the phosphorylation of carboxy phosphate and the subsequent nucleophilic attack by ammonia during the formation of carbamate. The ATP bound at the synthetase domain within the C-terminal half of the large subunit is responsible for the phosphorylation of carbamate and the final formation of carbamoyl phosphate. Finally, kinetic investigations have shown that the ligands bound to the allosteric domain (ornithine and UMP) alter the catalytic properties of CPS primarily by modulating the affinity of the ATP that phosphorylates carbamate.



Scheme 3



**Amidotransferase Domain.** The hydrolysis of glutamine to glutamate and ammonia by the small subunit of CPS has been shown to proceed through the formation of a glutamyl thioester intermediate with Cys-269 (30). Sequence comparisons and site-directed mutagenesis have also identified His-353 as being critical for the activation of the thiolate nucleophile (31). Shown in Scheme 3 is a working model for the hydrolysis of glutamine using these two active site residues. The mechanistic details of this transformation are very similar to the hydrolysis of peptide bonds by the thiol proteases such as papain. The reaction is initiated by the attack of the thiolate anion of Cys-269 on the carbonyl carbon of glutamine. The tetrahedral intermediate collapses upon protonation of the amide nitrogen by the imidazole side chain of His-353 to produce ammonia and the thioester intermediate. The ammonia departs, and the thioester intermediate is subsequently hydrolyzed after attack by an activated water molecule. The active site is wedged between the N- and C-terminal domains of the small subunit with most of the substrate binding and catalytically essential amino acids, including Cys-269 and His-353, contributed by the C-terminal domain. It is the C-terminal domain of the small subunit that is homologous to the N-terminal domain of GMP synthetase and to other members of the *trpG* type (or triad) amidotransferases (32).

Recent X-ray crystallographic studies of a site-directed mutant of CPS in which His-353 was replaced with an asparagine have revealed the manner in which the glutamyl thioester intermediate is stabilized within the active site (33). Specifically, the side chain functional groups of Ser-47 and Gln-273, the backbone carbonyl oxygens of Gly-241 and Glu-243, and the backbone amide groups of Gly-241, Gly-313, and Phe-314 all lie within hydrogen-bonding distance to atoms of the glutamyl moiety. These interactions are graphically illustrated in Figure 4. The small subunit thus functions to hydrolyze glutamine and to deliver ammonia to the large subunit.

**Carboxy Phosphate Domain.** The X-ray crystal structure of CPS has confirmed that the domain within the N-terminal half of the large subunit contains the binding site for one of the two molecules of ATP that are needed to synthesize carbamoyl phosphate (5, 6). The specific function of this domain in the assembly of carbamoyl phosphate was identified by determining the catalytic properties of site-directed mutants that specifically perturb this nucleotide binding site (34). The disruptive effects of these mutations on the overall reaction and on the two partial reactions were used to assign the functional properties of the two homologous synthetase

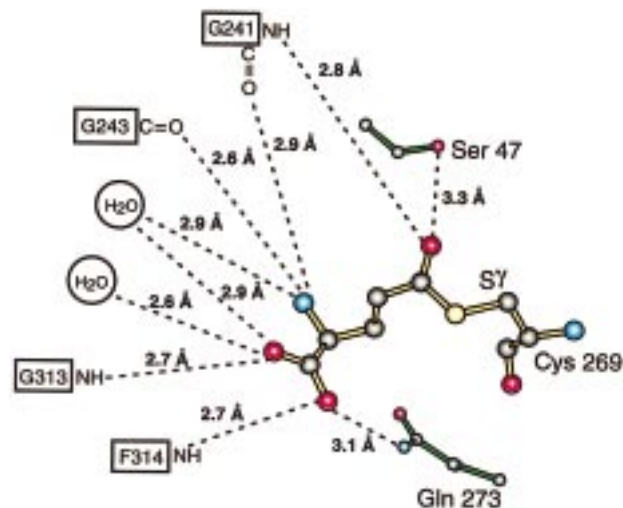


FIGURE 4: Electrostatic interactions between the amidotransferase unit of CPS and the glutamyl thioester intermediate. Potential hydrogen bonds are indicated by the dashed lines.

domains within the large subunit of CPS. For example, modification of two conserved glycine residues within the N-terminal domain (Gly-176 and Gly-180) causes a defect in the ability of these mutants to synthesize carbamoyl phosphate (34). However, the disruption of the catalytic properties of these two mutations is significantly greater on the bicarbonate-dependent ATPase reaction (eq 3) than on the partial ATP synthesis reaction (eq 4). Since the ATPase reaction is thought to represent the action of the nucleotide that phosphorylates bicarbonate, it was concluded that the domain that is found within the N-terminal half of the large subunit is responsible for the phosphorylation of bicarbonate. This conclusion has been confirmed by the construction of eight additional mutants at residues that are now known to serve as the active site residues for the binding of the substrates to this domain (Arg-129, Arg-169, Glu-215, Asn-283, Gln-285, Glu-299, Asn-301, and Arg-303) (35).

Mutagenesis of all conserved histidine residues within the large subunit of CPS has enabled the identification of the domain that is critical for the formation of carbamate from carboxy phosphate and ammonia (36). When His-243 is mutated to an asparagine residue, the resulting protein is unable to synthesize carbamoyl phosphate. However, the bicarbonate-dependent ATPase reaction and the partial ATP synthesis reactions are unaffected by this alteration, relative to the rates of the two partial reactions exhibited by the wild-type enzyme. Since neither of the reactions that involve ATP are affected by this mutation, but this mutant cannot make carbamoyl phosphate, it was concluded that the chemical step that links the two phosphorylation events was disrupted. These mutation studies have shown quite clearly that carboxy phosphate is formed and reacts with ammonia by the active site that is found within the N-terminal half of the large subunit of CPS. This segment of the large subunit (residues 1–403) has been tagged as the “carboxy phosphate domain”.

**Carbamoyl Phosphate Domain.** An identical set of homologous mutations was constructed and characterized for those residues that are localized in the ATP binding domain within the C-terminal half of the large subunit of CPS. The initial conclusion that this domain is responsible for the

phosphorylation of carbamate came from the mutation of a critical glycine residue that forms part of a flexible hinge. The modification of this glycine residue (Gly-722) to an isoleucine produced a protein that is differentially defective in the partial ATP synthesis reaction, whereas the bicarbonate-dependent ATPase reaction is relatively unperturbed (34). This initial assessment of the function of this domain has been furthered confirmed by the construction of eight additional mutants at the active site of this domain (Arg-675, Arg-715, Glu-761, Asn-827, Gln-829, Glu-841, Asn-843, and Arg-845) (37, 38). It is concluded that the ATP that binds to this domain is responsible for the phosphorylation of carbamate, and thus this segment of the large subunit has been labeled as the "carbamoyl phosphate domain".

**Allosteric Domain.** The domain found at the extreme C-terminus of the large subunit of CPS has been shown to contain the binding sites for the two significant allosteric effectors, ornithine and UMP (24, 25). The allosteric effects exerted by the binding of these compounds are primarily, but not exclusively, on the Michaelis constant for ATP (39). Since the allosteric effects are found on the partial back-reaction but barely measurable on the bicarbonate-dependent ATPase reaction, it has been concluded that the allosteric effectors primarily modulate the affinity of the ATP that binds to the carbamoyl phosphate domain. Ornithine activates CPS by lowering the  $K_m$  for ATP by about an order of magnitude whereas UMP has the opposite effect through elevation of the  $K_m$  for ATP. IMP binds to a site that overlaps with the binding of UMP, but the overall effects exhibited by IMP are rather small (28, 39). Reinhart has shown that the binding of IMP and UMP is mutually exclusive, but complexes of CPS can be made with the simultaneous binding of ornithine and UMP. However, with this ternary complex, the allosteric effects are dominated by ornithine (29). To date it has not been possible to determine the structural consequences of the binding of UMP on CPS relative to the structure of the protein bound with ornithine. It is also not possible to explain in structural terms how the binding of ornithine can dominate the effects normally exhibited upon the binding of UMP alone.

## MOLECULAR PASSAGEWAY FOR AMMONIA AND CARBAMATE

**Carbamoyl Phosphate Synthetase.** By far the most unexpected result from the first structural analysis of CPS was the molecular distances observed between the three active sites of the  $\alpha,\beta$ -heterodimer. Indeed, the active site in the small subunit is approximately 45 Å from the nearest active site within the large subunit, which in turn is 35 Å from the other ATP binding region. It is known that the ammonia product, derived from the hydrolysis of glutamine within the small subunit of CPS, is the nucleophile that reacts with the carboxy phosphate intermediate that is formed within the N-terminal half of the large subunit. Since the isotope labeling studies have demonstrated that the ammonia does not dissociate and then reassociate to the large subunit, this intermediate must migrate through the interior of the protein where it reacts with carboxy phosphate (14). Visual inspection of the CPS model and a computational search with the software package GRASP (40) reveal a potential molecular tunnel leading from the small subunit to both active sites of

the large subunit as depicted graphically in Figure 1. At the base of the small subunit there is a crown of charged amino acid side chains, Asp-45, Lys-202, and His-353, that face toward the interior of the small subunit. Other than these residues, the tunnel leading from the small subunit to the molecular interface with the large subunit is lined largely with nonreactive side chains and backbone atoms. As the tunnel extends from the interface between the small and large subunits toward the active site of the carboxy phosphate domain, the interior is once again lined, for the most part, with unreactive residues except for Glu-217 and Cys-232. This molecular passageway from the small subunit to the nearest active site of the large subunit leads directly to the  $\gamma$ -phosphate of ATP (22). It is this active site where bicarbonate reacts with ATP to form the carboxy phosphate intermediate, which subsequently reacts with ammonia to produce carbamate.

Furthermore, it has been shown that the carbamate intermediate that is formed within the N-terminal half of CPS is phosphorylated by the ATP that is bound to the phosphorylation domain within the C-terminal half of the large subunit. Since carbamate is very unstable at neutral pH, this intermediate must also be transported through the interior of the large subunit to the domain where it is phosphorylated by the second ATP to give the final product, carbamoyl phosphate. The portion of the tunnel lying between the two active sites within the large subunit is somewhat less hydrophobic. The side chain carboxylate group of Glu-604 points toward the pathway and a cluster of charges, Glu-577, Arg-848, Lys-891, and Glu-916, are located near the opening to the second active site of the large subunit. Approximately 25 water molecules have been located within 2 Å of the pathway, but their actual position during catalysis is unknown (6). It is important to note that many of the residues lining the putative tunnels are conserved in most of the sequences determined to date for CPS from bacterial sources (22) and many of the residues that are not strictly conserved are replaced with amino acid residues of comparable chemical reactivities. As more complexes of CPS are solved by X-ray analyses, the course of the molecular tunnel will undoubtedly become better defined. Thus far, all complexes of CPS have been solved in the presence of the known activators, ornithine and potassium. It will be highly instructive to observe what changes, if any, occur in the molecular tunnel when the structure of the enzyme is solved when other enzyme complexes are solved.

**Tryptophan Synthase.** Substrate channeling from one domain of an enzyme to another is not without precedence. To date, the most carefully analyzed molecular tunnel and the first to be crystallographically identified is that found in tryptophan synthase from *Salmonella typhimurium*, an enzyme known to form stable  $(\alpha,\beta)_2$ -complexes (41). This enzyme catalyzes the last two reactions in L-tryptophan biosynthesis. The two active sites in tryptophan synthase are located on separate polypeptide chains and positioned about 25 Å apart. Extensive and exquisite studies from the laboratories of Hyde and of Davies have demonstrated that the tunnel is largely hydrophobic with molecular dimensions appropriate for the passage of the intermediate, indole. A dramatic example of molecular changes that can occur within this tunnel is provided by the structural analyses of the enzyme in the presence of different monovalent cations. In

the native enzyme, originally solved in the presence of  $\text{Na}^+$ , Phe-280 of the  $\beta$ -subunit partially blocks the tunnel. When  $\text{Na}^+$  is replaced with either  $\text{K}^+$  or  $\text{Cs}^+$ , the aromatic ring of Phe-280 swings out of the tunnel, thus suggesting a possible control mechanism (42).

*Phosphoribosyl Pyrophosphate Amidotransferase.* Another remarkable example of substrate channeling in an amidotransferase enzyme is found in the elegant studies of Zalkin and of Smith on glutamine phosphoribosyl pyrophosphate amidotransferase (GPATase) from *E. coli* (43–45). GPATase belongs to the *purF*-type (also called Ntn) family of amidotransferases (1). This enzyme catalyzes the first step in the purine biosynthetic pathway by transferring an amide nitrogen derived from glutamine to phosphoribosyl pyrophosphate, thereby yielding phosphoribosylamine, pyrophosphate, and glutamate. Like tryptophan synthase, GPATase contains two separate active sites, although in this case they are located on the same polypeptide chain. The N-terminal domain contains the catalytic machinery required for the hydrolysis of glutamine to glutamate and ammonia while the C-terminal domain is responsible for the coupling of ammonia to phosphoribosyl pyrophosphate. In contrast to tryptophan synthase where the tunnel is “preformed”, the molecular conduit in GPATase is only observed when substrate analogues, and presumably substrates as well, are bound to both active sites. Specifically, when the structure of GPATase is solved in the presence of glutamine (6-diazo-5-oxonorleucine) and phosphoribosyl pyrophosphate (1 $\alpha$ -pyrophosphoryl-2 $\alpha$ ,3 $\alpha$ -dihydroxy-4 $\beta$ -cyclopentanemethanol 5-phosphate) analogues, the C-terminal helix formed from Asp-471 to Gln-492 becomes extensively kinked, and a flexible loop, defined by residues Val-325 to Arg-354, becomes well ordered. This surface loop closes down over the C-terminal active site in a quite dramatic fashion, and the combination of loop ordering and helix kinking results in the formation of a loosely packed molecular tunnel approximately 20 Å in length and lined primarily by hydrophobic amino acid residues (44).

*GMP Synthetase.* Another enzyme that might employ a molecular tunnel is GMP synthetase, a protein actively under investigation in the laboratories of Davisson and of Smith (32). As in CPS and GPATase, this enzyme uses glutamine as a source of ammonia. Once formed, the ammonia is employed in the amination of xanthosine 5'-monophosphate to yield GMP. Unlike CPS where the active sites for the hydrolysis of glutamine and the subsequent coupling of the ammonia to carboxy phosphate are on separate subunits, in GMP synthetase both active sites are located on a single polypeptide chain. As mentioned previously, the N-terminal domain of GMP synthetase is structurally similar to the C-terminal domain of the small subunit of CPS, and indeed, the catalytically important residues in these two glutaminase domains, namely, Cys-269 and His-353 (CPS numbering), are located in identical positions. It has been speculated that a substantial conformational change is required in GMP synthetase to couple the active sites together since the structure observed in the crystalline lattice is open and there is an apparent lack of an obvious pathway for the transfer of the ammonia to the synthetase active site (32). Clearly, the molecular strategies utilized by GMP synthetase and GPATase are structurally different from the one developed by CPS to deliver ammonia to its site of utilization.

## ALLOSTERIC COMMUNICATION AMONG ACTIVE SITES

The X-ray crystallographic and mutagenesis studies have quite clearly demonstrated that the ammonia that is produced via the hydrolysis of glutamine within the small subunit diffuses to the large subunit where it reacts with the product from the phosphorylation of bicarbonate. The carbamate intermediate then translocates to the C-terminal domain where it is phosphorylated for the last time. In order for this process to work efficiently, the catalytic activities within these three active sites must be coordinated with one another. Unfortunately, the molecular details for the communication among each of these three sites have not been fully elucidated. However, it is now known that the rate constants for the formation and hydrolysis of the thioester intermediate within the small subunit are stimulated by about 1000-fold when ATP is being hydrolyzed within the N-terminal half of the large subunit (46). A conformational change must therefore be transmitted from the large subunit to the small subunit that serves to optimize the orientation of specific active site residues. In contrast, the signal transduction from the small subunit to the large subunit is less intense. When glutamine is hydrolyzed by the small subunit, the steady-state rate of ATP hydrolysis is enhanced by about an order of magnitude. In preliminary studies we have shown that this stimulation is not on the rate constant for the phosphorylation of bicarbonate but rather is due to the enhanced rate of reaction for ammonia with the carboxy phosphate intermediate relative to water.<sup>2</sup> These results are in keeping with the small enhancement of the bicarbonate-dependent ATPase reaction when Cys-269 is covalently labeled with mimics of the thioester intermediate. We conclude from these preliminary studies that the phosphorylation of the bicarbonate within the carboxy phosphate domain acts as a gate keeper for the molecular tunnels in CPS. Thus, only after bicarbonate is phosphorylated is the hydrolysis of glutamine sufficiently fast enough to inject a molecule of ammonia into the tunnel. A mechanism of this type avoids the uncoupling of the two reactions initiated at separate reaction centers. Thus far we have not been able to detect any signal of communication between the two ATP sites that would synchronize the two phosphorylation events. This may be reasonable since the ATP bound to the carbamoyl phosphate domain does not appear to be hydrolytically unstable. Apparently the ATP bound at this site simply waits for carbamate to arrive. We have also not been able to detect any communication between this site and the binding site for glutamine. When this site is used to phosphorylate ADP with carbamoyl phosphate (eq 4), there is no measurable effect on the rate of hydrolysis of glutamine.<sup>2</sup>

Mutants of CPS have been identified where the chemical reactions are uncoupled from one another. These mutants require more than two molecules of ATP to synthesize one molecule of carbamoyl phosphate. Alternatively, these mutants require more than one molecule of glutamine to synthesize one molecule of carbamoyl phosphate. One of the more illustrative examples is the mutation of Cys-248 within the small subunit of CPS. This cysteine residue is located ~65 Å away from the nearest binding site for ATP

<sup>2</sup> Unpublished experiments by Bryant Miles at Texas A&M University.



on the large subunit (5, 6). However, the cysteine residue can only be labeled with *N*-ethylmaleimide (NEM) when ATP is simultaneously being hydrolyzed on the large subunit (47). A conformational change must therefore be transmitted over a span of  $\sim 65$  Å as the result of a chemical event on the large subunit. When this cysteine is labeled with NEM, the glutaminase activity is enhanced and the ATPase activity is diminished (47). Furthermore, when this residue is mutated to an aspartate, the glutaminase activity is accelerated by 2 orders of magnitude, but this mutant cannot make any carbamoyl phosphate whatsoever (48). We conclude that mutation of the cysteine to an aspartate has partially mimicked and stabilized the conformational change that is driven by the hydrolysis of ATP on the large subunit. This structural change forces the small subunit into a conformational state that is more active for the hydrolysis of glutamine. However, it would also appear that this conformational change is locked into place by the aspartate mutant. This conformational change must be in dynamic equilibrium during each catalytic turnover. Current efforts are directed at a greater understanding of the structural communication among the three active sites and the two allosteric sites.

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