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Tunneling of intermediates in enzyme-catalyzed reactions

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A fascinating group of enzymes has been shown to possess multiple active sites connected by intramolecular tunnels for the passage of reactive intermediates from the site of production to the site of utilization. In most of the examples studied to date, the binding of substrates at one active site enhances the formation of a reaction intermediate at an adjacent active site. The most common intermediate is ammonia, derived from the hydrolysis of glutamine, but molecular tunnels for the passage of indole, carbon monoxide, acetaldehyde and carbamate have also been identified. The architectural features of these molecular tunnels are quite different from one another, suggesting that they evolved independently.

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Introduction

The tunneling of reaction intermediates through the interior of proteins with multiple active sites is found in an increasing number of enzymes [1]. Recent advances in molecular modeling and an expanding structural database have led to a greater understanding of the mechanisms for the migration of activated reaction intermediates from the site of production to the site of utilization. We define the molecular *tunneling* of reaction intermediates as the translocation of a product from one active site to another active site in the same enzyme where it is utilized as a substrate for a subsequent enzymatic reaction. This contrasts with molecular *channeling* which we define as the transfer of a reaction product from one enzyme to another enzyme without diffusion of the reaction product into the bulk solution. Such transfers are generally thought to occur through the *transient* formation of protein–protein complexes without the participation of a well-defined and physically constrained intramolecular tunnel [2].

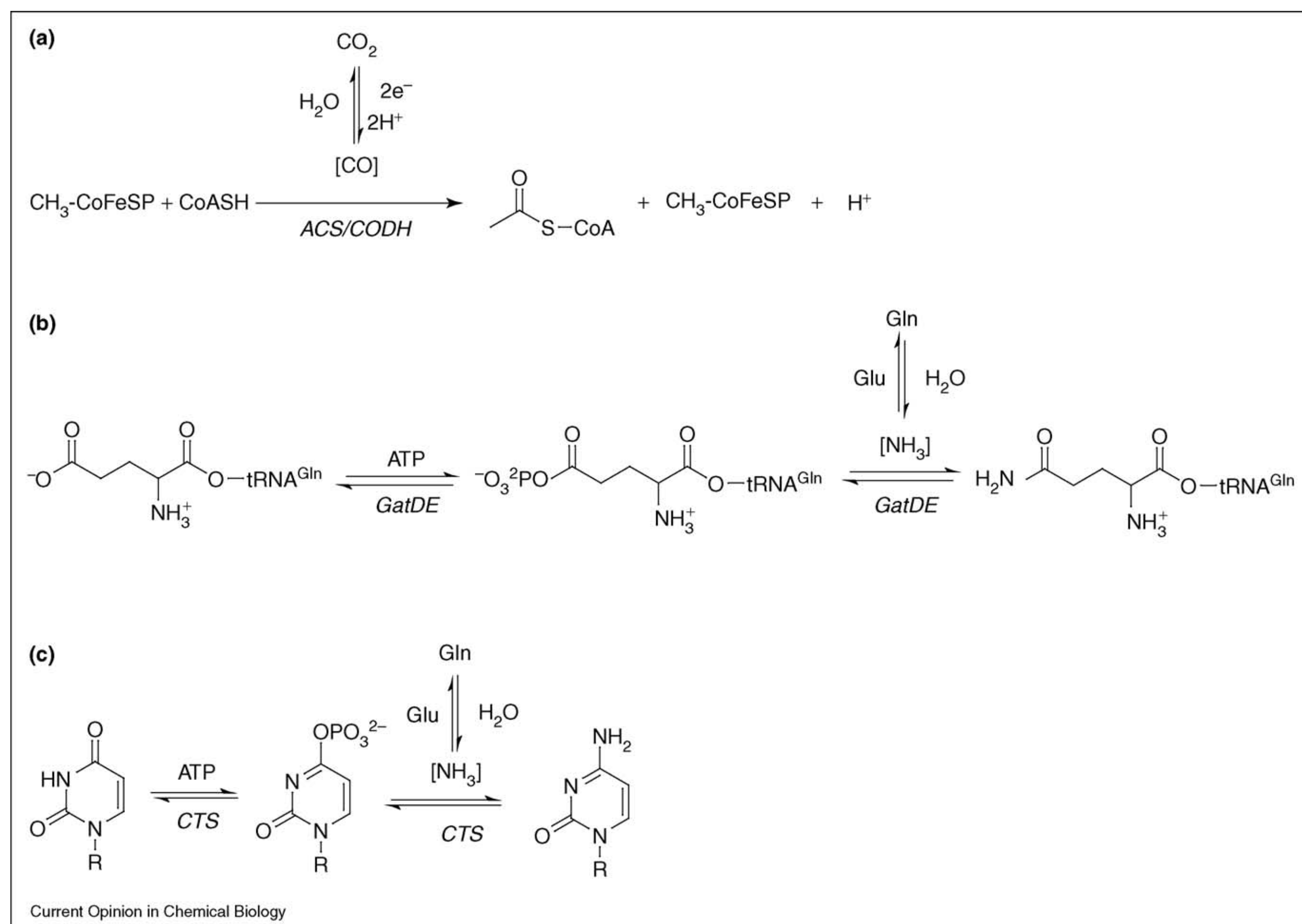
A significant number of protein tunnels have been found connecting distinct active sites in multifunctional enzymes. The sheltering of unstable reaction intermediates and the facilitated one-dimensional diffusion between active sites can have kinetic and thermodynamic advantages in biosynthetic processes. The most common reactive intermediate that tunnels between successive active sites is ammonia. Thus far, eight such enzymes have been crystallized and the physical structures of these proteins determined to high resolution. These enzymes include carbamoyl phosphate synthetase [3], glutamine phosphoribosylpyrophosphate amidotransferase [4], asparagine synthetase [5], glutamate synthase [6], imidazole glycerol phosphate synthase [7], glucosamine 6-phosphate synthase [8,9•], a tRNA-dependent amidotransferase [10•] and cytidine triphosphate synthetase [11]. All utilize the nucleophile ammonia that is derived from the hydrolysis of glutamine. Other small molecules have been found to be translocated between multiple active sites using similar mechanisms. Tryptophan synthase, which has a tunnel for an indole intermediate, was the first enzyme for which an intramolecular tunnel was verified by crystallographic methods [12]. Acetyl-CoA synthase/carbon monoxide dehydrogenase (ACS/CODH) tunnels carbon monoxide, formed from the reduction of carbon dioxide [13,14]. A bifunctional aldolase/dehydrogenase, DmpFG, has been found to tunnel acetaldehyde [15], and formiminotransferase-cyclodeaminase tunnels N^5-formiminotetrahydrofolate [16]. The most intriguing aspect of the molecular tunnels identified thus far is that they seem to be structurally different and apparently evolved independently from one another. However, the catalytic machinery for the generation and utilization of these reactive intermediates (especially the hydrolysis of glutamine) has been derived from common ancestral enzymes.

Intramolecular enzyme tunnels

Acetyl-CoA synthase/carbon monoxide dehydrogenase

The bifunctional enzyme ACS/CODH has been extensively studied from *Moorella thermoacetica*. ACS/CODH is utilized by anaerobic archaea and bacteria to function in the Wood/Ljungdahl autotrophic pathway for the production of acetyl CoA. Carbon dioxide is reduced to carbon monoxide and this product is coupled with coenzyme A and a methyl group from the corrinoid iron–sulfur protein (CoFeSP) to form acetyl-CoA as shown in Figure 1a [17]. The 310 kDa enzyme assembles as an $\alpha_2\beta_2$ heterotetramer and is aligned linearly with two central β subunits flanked on each side by an α subunit. Both subunits contain [Fe_4S_4] clusters [13,14]. A ribbon representation of the enzyme and the intramolecular tunnel for the passage of CO is presented in Figure 2. The catalytic

Figure 1

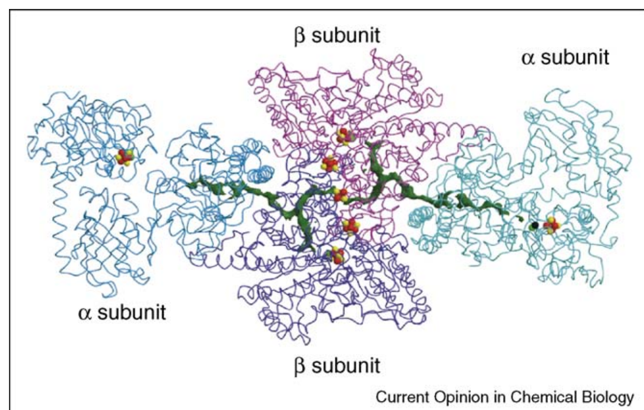


The reactions catalyzed by **(a)** acetyl-CoA synthetase/carbon monoxide dehydrogenase (ACS/CODH), **(b)** tRNA-dependent amidotransferase (GatDE), and **(c)** cytidine synthetase (CTS).

reduction of CO_2 to CO takes place within the β subunit and, which harbors the novel [Ni Fe][Fe_3S_4] metal center termed the C-cluster. There is an additional iron–sulfur cluster that bridges the two β-subunits and serves as a central electron shuttle between external redox agents and the two C-clusters via another [Fe_4S_4] cluster in each β subunit [18]. The α subunit contains the active site for the synthesis of acetyl-CoA and it contains the A-cluster. This cluster contains a [Ni_pNi_d] dimer bridged to a [Fe_4S_4] cluster. The CO is delivered to the α subunit through the intramolecular tunnel and the methyl group is delivered by a corrinoid-containing iron sulfur protein [13,14]. It has been shown that copper is inhibitory and that a two nickel complex is required for catalytic activity [19].

The molecular tunnel in ACS/CODH is a hydrophobic cavity of 138 Å that runs through the middle of the $\alpha_2\beta_2$ complex between the two A-clusters with branches to each C-cluster. CO is toxic to the cell and thus there is a practical requirement for the sequestration of this intermediate that is also coupled to direct delivery to the catalytic site. Maynard and Lindahl demonstrated that during catalysis CO does not leak from the molecular tunnel into the bulk solution [20]. The structure of ACS/CODH revealed two conformations for the α subunit: an *open* and *closed* form [13]. The proposed reaction mechanism utilizes these two conformations to orchestrate the timing of the entrance of CO and the methyl group to the active site. Functional support for the migration of CO through the intermolecular tunnel has been obtained through the mutation of specific residues that line the tunnel interior [21••]. The mutant protein A222L apparently causes a complete blockage in the tunnel between the A- and C-clusters because acetyl-CoA cannot be produced with carbon dioxide as a substrate but wild type levels of activity can be obtained using CO as the substrate. The A265M mutant enzyme has a reduced rate of acetyl-CoA synthesis with CO_2 as a substrate relative to CO, and thus the molecular tunnel is partially blocked and some CO can apparently migrate through the tunnel. Because these mutants are fully active with CO as a substrate, the results demonstrate that CO can bind to

Figure 2



Ribbon representation of ACS/CODH showing the tunnel (as a green tube) for the translocation of CO from the central β-subunits to the terminal α-subunits. The [Fe_4S_4], [Ni Fe][Fe_3S_4] and [Ni Ni][Fe_4S_4] clusters are shown in space-filling representation. One of the α-subunits (left) is in the *open* conformation whereas the other (right) is in the *closed* conformation. The coordinates were taken from PDB code 1OAO.

the α subunit without having to migrate from the β subunit through the tunnel.

tRNA-dependent amidotransferase

An indirect route of charging tRNA is found in archaea and some bacteria, involving the misacylation of the tRNA for glutamine with glutamate [22]. A subsequent chemical transformation involves a specific enzyme to catalyze the amidation of the misacylated glutamate to form the correctly charged tRNA with glutamine [10•]. Two classes of this amidotransferase have been identified. The first of these enzymes will amidate glutamate and aspartate misacylated to $tRNA^{Gln}$ and $tRNA^{Asn}$, respectively, and is found in both bacteria and archaea. The second amidotransferase only works on glutamate and is found specifically in archaea. This unusual process may have evolved because the glutamyl-tRNA synthetase recognizes both $tRNA^{Gln}$ and $tRNA^{Glu}$ [9•]. Because of the pivotal nature of this step in proper translation of the genetic code, this enzyme might prove to be an effective drug target.

The tRNA-dependent amidotransferase from the second class of enzymes was crystallized from *Pyrococcus abyssi* [10•]. The protein consists of two subunits (GatD and GatE) that form a functional $\alpha_2\beta_2$ heterotetramer. The two GatD subunits form a homodimeric complex, and are flanked by the two GatE subunits that only interact with their respective GatD. The substrate for the GatDE complex is the misacylated Glu-$tRNA^{Gln}$ formed by the glutamyl-tRNA synthetase. The GatD subunit provides ammonia through the hydrolysis of either glutamine or asparagine. GatE utilizes ATP to activate the side-chain carboxylate from the misacylated glutamate of Glu-$tRNA^{Gln}$ via phosphorylation, and then catalyzes the transfer of ammonia to give the final product, Gln-$tRNA^{Gln}$, as shown in Figure 1b [22].

The GatD subunit has an asparaginase-like core attached to a barrel domain by an 18 amino acid linker [10•]. There is a highly conserved threonine residue that plays a crucial role in amide bond hydrolysis through the formation of an acyl ester intermediate [22]. GatE has an overall bent structure that is shaped like a cradle. There are two regions of GatD involved in docking with GatE, a specific N-terminal domain and the entrance to the asparaginase catalytic site. There is a channel between the asparaginase catalytic site of GatD and the active site in GatE and it is lined with highly conserved residues [10•]. The phosphorylation of the misacylated Glu-$tRNA^{Gln}$ in the active site of GatE by ATP triggers the movement of a β hairpin loop in GatD to orientate the catalytic threonine residue in a position for hydrolysis via nucleophilic attack [22]. This conformational change also closes the active site off from access to solvent. The reorientation of this loop and the conserved residues between the two active sites suggest that the ammonia is tunneled from the site of production on one subunit to the site of utilization on the other subunit directly through the enzyme [10•]. The protein structure cannot accommodate the positioning of an activated Glu-$tRNA^{Gln}$ close to the active site of GatD, thus confirming the diffusion of ammonia through the tunnel to reach the active site of GatE [10•].

Cytidine triphosphate synthetase

CTP synthetase (CTS) catalyzes the formation of CTP from UTP, ATP and glutamine as shown in Figure 1c. CTP is a key component in the biosynthesis of DNA, RNA and phospholipids [11]. CTS is another example of a protein that has evolved a mechanism for the tunneling of ammonia between two active sites. This bifunctional enzyme has a class 1 glutamine hydrolase domain at the C-terminal end of the protein and an amidotransferase domain at the N-terminal end [11]. This enzyme plays a key regulatory role in defining the intracellular CTP levels by feedback inhibition of the product CTP and cooperative induction from the cofactor ATP and substrate UTP [11]. The significance of CTP production makes this enzyme an attractive drug target for the treatment of leukemia and parasitic infections [11]. The monomeric units of CTP synthetase dimerize via nonpolar interactions between the N-terminal ends, whereas the formation of the functional tetrameric structure is enhanced by the binding of the substrate, UTP.

An intramolecular tunnel of 25 Å traverses between the glutaminase and amidotransferase active sites [11]. CTP synthetase is unusual because there is a putative secondary tunnel entrance to the amidotransferase active site in addition to the entrance from the active site of the glutaminase domain [11]. Described as the ‘vestibule’,

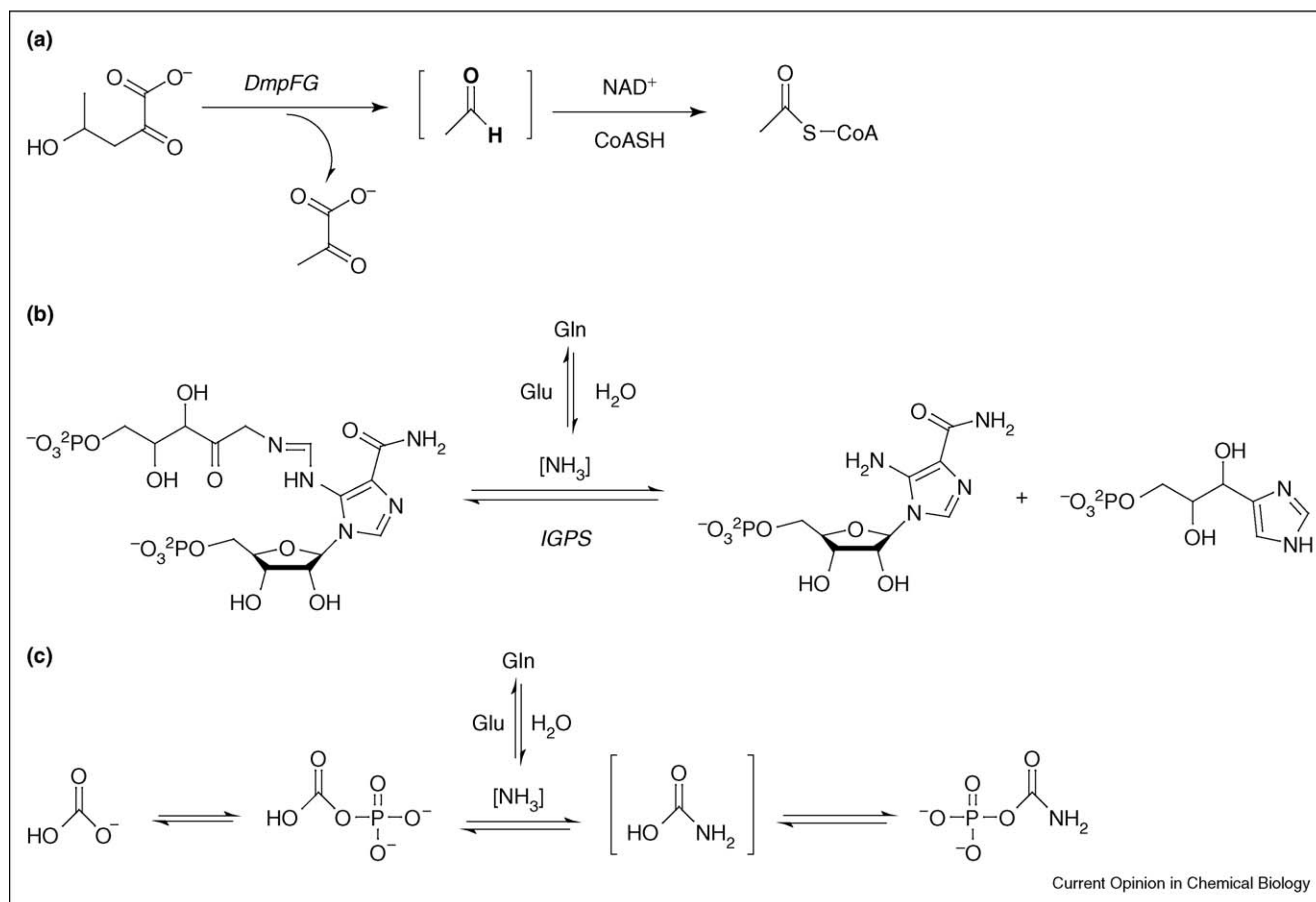
a 3.0 Å solvent-accessible gap is positioned at the base of a cleft next to the site of binding for the allosteric effector GTP. This gap provides an entry point for the alternative binding of free ammonia from bulk solvent [11]. The binding of GTP and a covalent inhibitory analogue of glutamine apparently block both entrance routes for the binding of ammonia thus increasing the utilization of glutamine when GTP is present [23]. A gating mechanism was proposed for the passage of ammonia through the intramolecular tunnel that is facilitated by multiple conformations of His57, which lies between the vestibule and the channel exit [11]. The binding of the substrate UTP induces the rotation of His57 to open the channel for the passage of ammonia. This ligand-induced change is postulated to regulate the timing for the translocation of ammonia to the amidotransferase active site.

4-Hydroxy-2-ketovalerate aldolase/acylating acetaldehyde dehydrogenase

4-Hydroxy-2-ketovalerate aldolase (DmpG)/acylating acetaldehyde dehydrogenase (DmpF) is a bifunctional enzyme (DmpFG) found in bacteria. It catalyzes the final two steps in the degradation of toxic aromatic intermediates in the *meta*-cleavage pathway of catechol [15]. DmpG, the aldolase domain, converts 4-hydroxy-2-ketovalerate to acetaldehyde, the reactive intermediate, and releases pyruvate. The acetaldehyde apparently moves through a 29 Å hydrophobic tunnel to DmpF, where it reacts with CoA in the presence of NAD^+ to produce acetyl-CoA and NADH. The complete reaction sequence is shown in Figure 3a. The two subunits have been studied separately from one another to demonstrate that DmpF alone is inactive whereas DmpG alone possesses some aldolase activity [15].

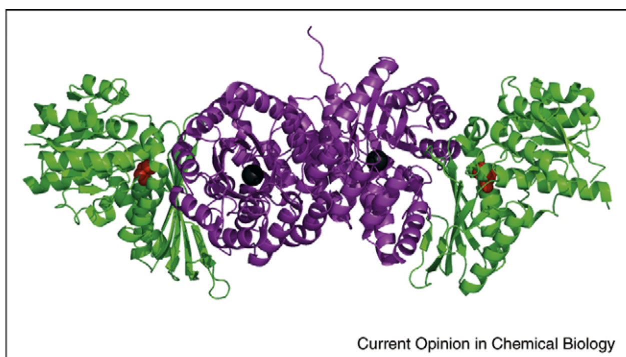
The crystal structure of DmpFG from a species of *Pseudomonas* in the presence and absence of NAD^+ has been determined to a resolution of 1.7 Å and a representation of this structure is provided in Figure 4 [15]. The bifunctional protein, DmpFG, oligomerizes to a tetramer and is composed of two heterodimeric units. The two DmpG subunits contact each other to form a central core, flanked on each end by DmpF. The DmpG subunit has two domains; an N-terminal $(\alpha\beta)_8$ TIM barrel domain and a

Figure 3



The reactions catalyzed by **(a)** 4-hydroxy-2-ketovalerate aldolase/acylating acetaldehyde dehydrogenase (DmpFG), **(b)** imidazole glycerol phosphate synthase (IGPS), and **(c)** carbamoyl phosphate synthetase (CPS).

Figure 4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Ribbon representation of the DmpFG heterodimeric complex showing the active sites for the aldolase (DmpG in purple) and dehydrogenase (DmpF in green) subunits. The active site for the aldolase subunit is marked by the location of the essential Mn^{2+} as a black sphere. The active site for the dehydrogenase subunit is marked by the location of the essential Cys132 as a red side chain. The coordinates were taken from PDB code 1NVM.

C-terminal helical communication domain [15]. The DmpF subunit also has two domains; an NAD^+ binding domain and a dimerization domain. In the crystal structure, three out of the four DmpF subunits in the asymmetric unit contain NAD^+ but the fourth subunit does not contain the cofactor. The DmpG subunit requires Mn^{2+} for catalytic activity and this metal ion was found bound to Asp18, His200 and His202 [15].

A comparison of the apo- and holo-structures has unveiled clues about the migration of the reactive acetaldehyde intermediate from one subunit to the other. In the structure with bound NAD^+, the two ends of the 29 Å tunnel are closed. Tyr291 blocks the tunnel entrance in the aldolase subunit while Ile172, Ile196 and Met198 block the tunnel exit in the dehydrogenase subunit. Entry and egress of acetaldehyde is apparently gated at both ends of the tunnel. Vrielink and colleagues have proposed that Tyr291 and His21 participate in the abstraction of the proton from the 4-hydroxyl group of the substrate as a prelude to carbon–carbon bond cleavage. The proton transfer from the substrate to His21 induces a conformational reorientation of Tyr291 that enables the intermediate acetaldehyde to enter the tunnel leading to the dehydrogenase subunit. At the other end of the tunnel, three residues, Ile172, Ile196 and Met198 exhibit multiple conformations of the side chains. In the apo-enzyme, all of the possible conformations close off exit from the tunnel. However, in the structure with bound NAD^+, the tunnel is open in one of the four conformational states. This conformation involves a unique orientation of Ile172 and an interaction of Asn172 with NAD^+. These two molecular gates apparently function to regulate the delivery of the aldehyde and keep it from becoming trapped in the tunnel. The structural observations are consistent with kinetic measurements that determined that the aldolase activity is stimulated substantially when NAD^+ is bound to the dehydrogenase subunit.

Imidazole glycerol phosphate synthase

Imidazole glycerol phosphate synthase (IGPS), also known as HisF, is an amidotransferase that utilizes an ammonia intermediate via the hydrolysis of glutamine [7,24]. This enzyme catalyzes the formation of imidazole glycerol phosphate (IGP) and 5′-5-aminoimidazole ribonucleotide (AICAR) from *N*-(5′-phosphoribosyl)-formimino-5-aminoimidazole-4-carboxamide (PRFAR) as shown in Figure 3b. The site of glutamine hydrolysis is 30 Å away from the binding site for PRFAR. Nevertheless, the catalytic activities of the two separate active sites are coupled to one another. Thus, the binding of PRFAR stimulates the hydrolysis of glutamine by 4900-fold. Site-directed mutagenesis and the structure of the substrate-bound form of IGPS have led to the proposal that the binding of PRFAR induces the reorientation of Lys258 through a conformational switch at the base of the $(\beta/\alpha)_8$ barrel that allows ammonia to pass through the interior of the barrel [25].

The tunnel of IGPS follows a well characterized TIM barrel motif, with the ammonia tunnel running the entire length of the barrel [7,24]. Conformational changes and key hydrogen bond formation have been identified in structures of the apo- and substrate-bound forms of IGPS [24]. Steered molecular dynamics (SDM) simulations have been done to probe the tunnel dynamics and energetics [26•]. These studies and other bioinformatic data suggest that two conserved residues from the *Thermotoga maritima* enzyme, Lys99 and Arg5, form salt bridges with two conserved aspartate residues, and thus open the gate for the entry of ammonia. These studies also suggest the presence of a single water molecule in the barrel that provides a hydrogen bonding partner for the ammonia [26•].

Carbamoyl phosphate synthetase

The function of the molecular tunnel of carbamoyl phosphate synthetase is perhaps the most well characterized system for the channeling of ammonia. CPS has the most complex tunnel in that three active sites are connected by two tunnel segments that traverse a distance of nearly 100 Å. Hydrolysis of glutamine occurs in the small subunit (CarA). The product ammonia travels through the first half of the tunnel to the site of binding for bicarbonate and MgATP in the N-terminal half of the large subunit (CarB) [3]. The bicarbonate is phosphorylated by ATP, and nucleophilic attack by ammonia produces carbamate. This travels through the second half of the tunnel in the C-terminal portion of the large subunit, where it is phosphorylated by the second ATP to yield the ultimate product carbamoyl-phosphate [3]. A representation of the two tunnels in CPS is shown in Figure 5. The overall reaction is shown in Figure 3c.

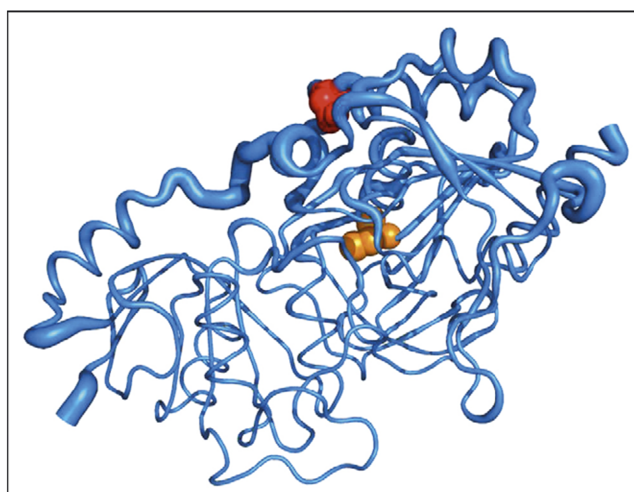
Figure 5

Ribbon representation of CPS from *E. coli* showing the small subunit in blue, the N-terminal half of the large subunit in green, and the C-terminal half of the large subunit in purple. The tunnel for the migration of ammonia and carbamate are depicted as a grey mesh. The coordinates were taken from PDB code 1BXR.

The phosphorylation of bicarbonate greatly stimulates the hydrolysis of glutamine within the small subunit [27]. Thus far, the activation of glutamine hydrolysis cannot be mimicked by the binding of competitive inhibitors to the large subunit of CPS. However, the conformational changes that are transmitted from the large subunit to the small subunit have been probed by the observation that Cys248 on the small subunit can only be labeled with the reagent *N*-ethyl maleimide (NEM) when ATP and bicarbonate are also present [28]. The NEM-labeled protein has an activated glutaminase activity, but the reactions in the large and small subunits have become uncoupled from one another. The catalytic properties of the NEM-labeled protein can be mimicked by the C248D mutant [28]. This residue is 50 Å from the MgATP/bicarbonate binding site in the large subunit. It was anticipated that the crystal structure of this mutant would reveal conformational changes at the active site of the glutaminase site near Cys269. However, although there were significant changes in the conformation of the loop near the site of the mutation, there were relatively few changes near the site for the hydrolysis of glutamine [29]. The differences in the structure of the small subunit between the wild type enzyme and the C248D mutant are shown in (Figure 6). These results indicate that relatively small conformational changes result in rather large changes in catalytic activity.

Two additional studies were conducted to probe the functional significance of the tunnels for the passage of ammonia and carbamate. Attempts were made to provide a molecular blockage in the ammonia tunnel. Gly359 was mutated to progressively larger residues and the catalytic properties of the mutants were consistent with a complete blockage of the ammonia tunnel when this residue was mutated to phenylalanine [30]. However, the crystal structure of the G359F mutant demonstrated that, instead of a blockage within the ammonia tunnel, the loop containing this residue adopted a new conformation and that perforation of the tunnel wall occurred. This loop reorientation resulted in an escape route for ammonia directly in the external solvent [31]. These results suggest that the evolution of new tunnels in existing protein frameworks is likely to involve conformational changes in the protein backbones rather than the gradual mutation of single amino acids with smaller side chains that would result in an ever longer molecular tunnel. The migration of

Figure 6



Conformational differences between the wild type CPS and the C249D mutant protein. The size of the ribbon represents the magnitude of the conformational change between the two structures. The location of C249D is shown in red and the location of the active site cysteine, C269, is shown in orange. The image was drawn using the program Pymol from Delano Scientific. The coordinates of the wild type and C249D proteins were taken from PDB codes 1BXR and 11T36, respectively.

ammonia through the tunnel leading to the large subunit of CPS has also been derailed by the mutation of residues at the interface of the large and small subunits [32•]. The functional significance of the carbamate tunnel has been probed via characterization of site-directed mutants [33]. The tunnel floor of the carbamate tunnel in CPS contains five highly conserved glutamate residues. These residues are in turn ion-paired with two highly conserved arginine residues that occupy the extreme ends of the carbamate tunnel and also interact with the bound nucleotides. It was proposed that these residues function in concert with one another to control entry of carbamate into the tunnel before phosphorylation to carbamoyl phosphate.

Conclusions

Intramolecular tunnels have been identified in a small number of multifunctional enzymes for the passage of the product of one active site for utilization as a substrate of an adjacent active site. These molecular tunnels help to sequester reactive intermediates from the hostile external environment and diminish the transit time for the dissociation from one active site and association to another active site. These systems are relevant model systems for the channeling of reaction products between successive enzymes in a biosynthetic pathway that form transient molecular complexes.

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