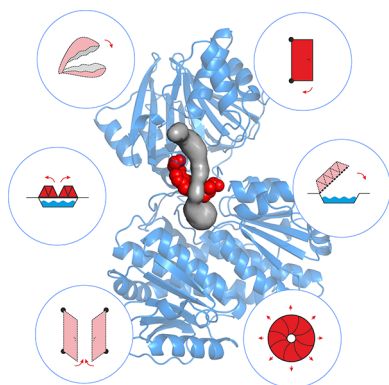


Gates of Enzymes

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1. INTRODUCTION

Enzymes are very efficient catalysts that are essential for the functioning of living organisms. The low efficiency of biocatalysts produced *de novo* relative to those that have

evolved naturally demonstrates that our understanding of enzymatic catalysis is still incomplete.^{1–4} The dynamic motion of enzymes during catalytic events is one of the many aspects of protein chemistry that are currently insufficiently well understood.^{5–9} On one hand, proteins need to have well-defined and organized structures in order to maintain stable functionality in the intracellular environment. On the other hand, some degree of flexibility is often required for catalytic activity. Molecular dynamics simulations have provided key insights into the importance of protein dynamics in catalysis, such as the observation of substrate access and product exit pathways that cannot be identified by inspecting crystal structures.¹⁰ Csermely et al. recently reported that mutations in regions that affect protein dynamics, such as hinge regions that are important in substrate binding, can have dramatic effects on catalytic activity.¹¹ In this review, we highlight the role of protein gates as another class of highly dynamic structures that play key roles in protein function.

Given the importance of gates for enzymatic catalysis, the number of studies that have examined them systematically is surprisingly small. Conformational gating in proteins was first described by McCammon and co-workers in 1981, but there have been relatively few systematic studies in this area since then.^{12–14} Moreover, much of the available data on gates in macromolecular systems is hidden or otherwise dispersed within the scientific literature, partly because there is currently no consensus regarding what defines a gate. Some authors describe all residues that affect the ligand's access to a target area as gating residues, whereas others apply the term exclusively to structural features that undergo large movements during the gating event. In this review, we define a gate as a dynamic system consisting of individual residues, loops, secondary structure elements, or domains that can reversibly switch between open and closed conformations and thereby control the passage of small molecules—substrates, products, ions, and solvent molecules—into and out of the protein structure. Under this definition, the anchoring residues that stabilize the open or closed conformations of a gate are not themselves gating residues. However, because of their various interactions with the gating residues, they can control the size and properties of the ligands that pass through the gate as well as the frequency of the exchange events.

Gates can be found in various systems, including enzymes, ion channels, protein–protein complexes, and protein–nucleic acid complexes.^{14,15} In this work, we focus specifically on gates in enzymes. We attempt to answer three basic questions—why, how, where—by describing the molecular function, structural

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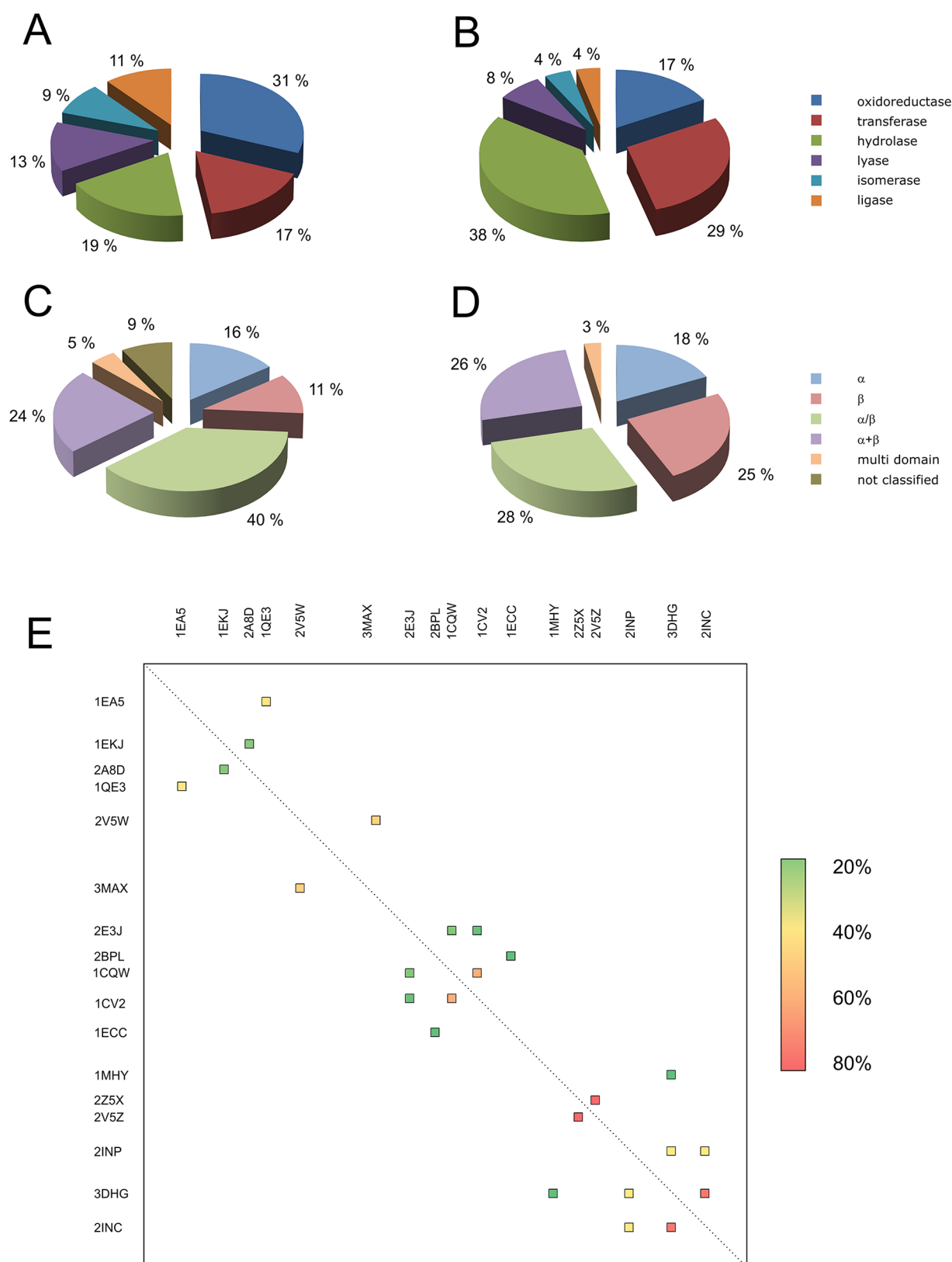


Figure 1. Distribution of (A) reviewed proteins and (B) proteins from the PDB database according to EC classes, (C) reviewed proteins and (D) proteins from the PDB database according to SCOP classification, and (E) identity matrix of reviewed enzymes (only the enzymes with sequence identities above 20% are shown for clarity).

basis, and location of gates within protein structures. We discuss 71 illustrative examples of enzymes that together contain 129 different molecular gates and propose a system for

their classification. Reviewed enzymes were chosen based on a literature search with a set of keywords corresponding to gates and conformational changes in enzymes. A preliminary set of

protein structures was filtered out, leaving only those entries for which both open and closed conformations were described. The final set represents different classes of enzymes (Figure 1A) and different protein folds (Figure 1C) and spans structurally and functionally the entire enzyme world. Among 71 chosen enzymes, only 17 have higher than 20% sequence identity with other set members (Figure 1E). The proposed classification system provides a useful framework for comparing gates of different enzymes and drawing general conclusions about gate function, structure, and position. Moreover, the classification scheme is easily extendable to describe the new gate types that will almost certainly be revealed by structural and functional analyses of newly isolated enzymes in the future.

2. MOLECULAR FUNCTION OF GATES

Analyses of protein dynamics have identified a number of enzymes with gates, suggesting that these structures are rather common. What is the molecular function of the gates? It seems that in enzymes they facilitate precise control over processes that are directly linked to catalysis. Enzyme gates can (i) contribute to enzyme selectivity by controlling substrate access to the active site, (ii) prevent solvent access to specific regions of the protein, and (iii) synchronize processes occurring in distant parts of the protein (Figure 2). The proper function of

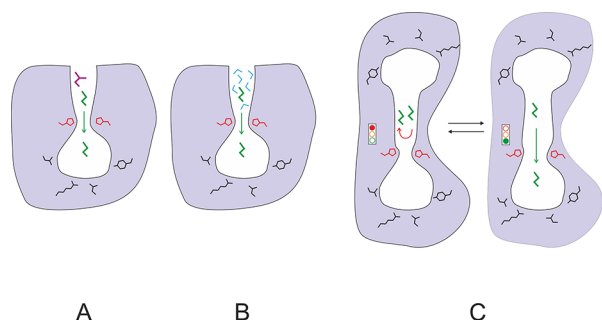


Figure 2. Schematic illustration of the molecular functions of protein gates: (A) control of substrate access, (B) control of solvent access, (C) control and synchronization of reactions. Protein is represented by the area colored in gray, active site cavity by the area in white, gating residues by red lines, substrate molecules by green or violet lines, and water molecules by blue lines.

even the simplest gates can potentially be essential for catalysis, and the gating event may even represent the rate-limiting step of the catalytic cycle. Interestingly, different gating residues within a single protein molecule may be responsible for restricting the access of specific substrates. High variability of the gating residues within an enzyme scaffold can lead to the evolution of enzyme families whose members are selective for specific substrate types. The best known example of such specialization within a single enzyme family is provided by the cytochromes P450.¹⁶

2.1. Control of Substrate Access

Enzyme selectivity has been traditionally explained by the “lock and key” model,¹⁷ which was subsequently complemented by the “induced fit” or “hand in glove”,¹⁸ “selected fit”,¹⁹ and “keyhole, lock, and key”²⁰ models. In many cases, these models provide an adequate description of enzyme selectivity based on adjustable complementarity between the active site and the cognate substrates. However, research conducted over the past decade has shown that regions located further from the active

site can also affect enzyme selectivity. Substrate access pathways, which often incorporate molecular gates, impose additional constraints on ligand binding to the active site.²⁰ The ability of ligands to traverse these access pathways can be controlled by (i) size discrimination at the narrowest point along the pathway forming a bottleneck, (ii) geometrical constraints, e.g., the curvature of the pathway, and (iii) specific molecular interactions such as hydrogen bonds, electrostatic interactions, and hydrophobic interactions with the residues comprising the access pathway. Protein gates can be regarded as molecular filters that discriminate between molecules as similar as molecular oxygen and carbon monoxide in NiFe hydrogenases^{21,22} or water and hydroxyperoxide in catalases.^{23,24} Gates act as filters in a wide range of enzymes, controlling the range of substrates that can be accepted by broad-specificity cytochromes P450,²⁵ the stereospecificity of epoxide hydrolases,²⁶ and product length in undecaprenyl-pyrophosphate synthases.²⁷

One of the first systematic descriptions of the influence of the gating process on substrate binding was reported by Szabo et al., who assumed that the switching between the open and the closed conformations of the gate was a stochastic process.^{28–30} This model was successfully used to demonstrate that despite conformation gating¹³ acetylcholinesterase can bind acetylcholine with a rate constant of $10^9 \text{ M}^{-1} \text{ s}^{-1}$ and predict the rate of formation of the enzyme–substrate complex in choline oxidase.³¹ Since gates create a barrier on the substrate access pathway, the kinetic rate constant for passage over the barrier can be obtained using Kramers’ reaction rate theory or its later modifications.^{32–34} This methodology was used to compare the results of computational and experimental studies on the passage of the tetramethylammonium cation through acetylcholinesterase³⁵ and migration of ammonia through carbamoyl phosphate synthetase.³⁶

2.2. Control of Solvent Access

Spatial localization of the hydrophobic and hydrophilic regions within the structure of a protein is important in maintaining its proper fold and can also be crucial for catalytic function. The various steps of an enzymatic reaction may require different environments. These distinct environments can be generated by having the individual steps occur in spatially separate regions of the protein, but this does not eliminate the problem of transporting the substrate between these sites. There are important problems to be addressed, including transporting polar molecules from a polar environment to a nonpolar one and separating hydrophilic compartments from hydrophobic ones within the structure of a single protein.

In some proteins, these problems are addressed by the presence of selective barriers that permit passage of solutes but not water molecules. Crystallographic and NMR data can be used to identify cavities within a protein structure accessible to water molecules. Exclusion of water from some parts of the cavity, such as the active site or a specific tunnel, is essential for functioning of numerous enzymes. In simple cases, the gates may prevent the entrance of water molecules into the cavity when a substrate or a cofactor is not present, as occurs in rabbit 20 α -hydroxysteroid dehydrogenase.³⁷ In more complex cases, the gates may permit access only to a specific part of the cavity, as occurs in carbamoyl phosphate synthetase³⁶ and imidazole glycerol phosphate synthase.³⁸ In the cytochromes P450, a “water channel” controls hydration of the substrate in the active site, which is extremely important for cytochrome activity.¹⁶

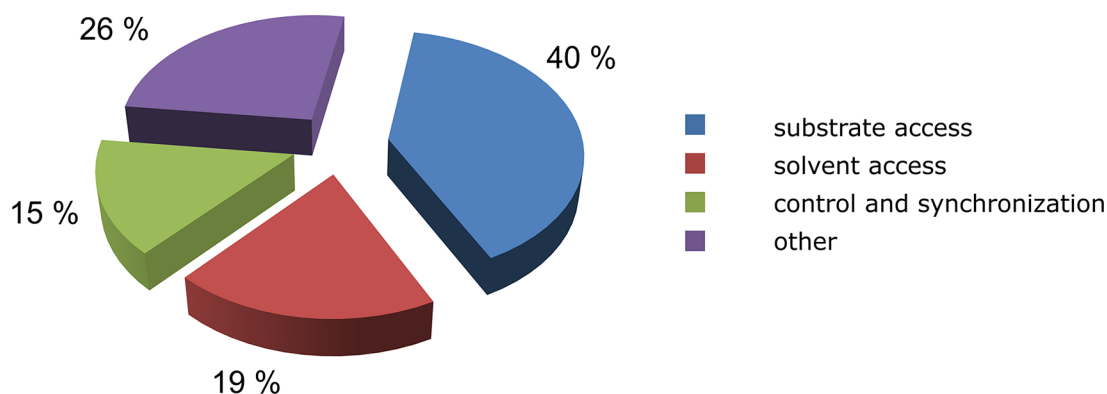


Figure 3. Distribution of gate functions; 71 proteins with 129 different gates were analyzed. Percentages shown in the figure are based on all of the identified functions of each gate; individual gate may perform multiple functions. Detailed description of the analyzed proteins is provided in Table 3.

The potential importance of gates that act as solvent barriers is further illustrated by the example of enzymes with ammonia tunnels. In these proteins, gates prevent water from entering the channel and protonating the ammonia, which is essential for maintaining its nucleophilic character.³⁹

Control of water access can be seen as a special case of the function described in section 2.1. However, when discussing water exclusion, the main emphasis is on the water permeability of the gates and their ability to distinguish water molecules from other ligands. The gate can simultaneously act as a barrier to passage of water molecules while acting as a selective filter for other molecules, allowing them to access the active site. Gates of this type resemble semiselective membranes that can distinguish between species such as water and ammonia, allowing only the second to pass. It is worth noting that passage of 'permitted' species can be facilitated by rearrangement of an individual gating residue, such as K99 in imidazole glycerol phosphate synthase. Conversely, passage of water molecules through gates of this type would often require significant conformational changes in all of the residues that comprise the gate.³⁸ This makes gates in enzymes far more sophisticated than semipermeable membranes.

2.3. Control and Synchronization of Reactions

Another function of gates becomes apparent when considering enzymes with two or more active sites. Many protein structures contain tunnels to facilitate efficient migration of intermediates and gates to synchronize chemical reactions. Such arrangements can be compared to a pair of workers on an assembly line. The second worker has to be ready before he can receive a product from the first one. Moreover, the products generated by the first worker must satisfy certain standards. The control gates located between the workers regulate the exchange of products over a well-defined period of time. Gates of this kind are common in ammonia-transferring enzymes, suggesting that they are old in evolutionary terms and functionally important.^{40–43} The need to efficiently transport ammonia within the interior of the protein may be related to its high cellular toxicity. We speculate that gates of this kind may be present in many enzymes that have multiple active sites connected by internal tunnels for the transport of intermediates. Many such enzymes have been studied in some detail, including carbamoyl phosphate synthetase, which has tunnels for ammonia and carbamate transportation;³⁶ asparagine synthetase,⁴⁴ glucosamine 6-phosphate synthase,⁴⁵ and glutamate synthase,⁴⁶ all of which have tunnels for ammonia transportation; tryptophan synthase for indole⁴⁷ and carbon

monoxide dehydrogenase/acetyl coenzyme A synthase for carbon monoxide transportation.⁴⁸

Systematic analysis of the functions of the known gates in the 71 proteins discussed in this article revealed the following distribution of gate types: 40% of the studied gates control substrate access, 19% control solvent access, 15% control and synchronize catalytic events, and 26% have other function (Figure 3).

3. STRUCTURAL BASIS OF GATES

Gates are dynamic systems that can make reversible transitions between open and closed states. They vary in size and complexity, from individual amino acid residues to loops, secondary structure elements, and even domains. The simplest gates consist of only one amino acid side chain that can close or open an access pathway by rotating. Opening and closing of more complicated systems can involve the synchronized movement of two or more residues, and the largest systems involve rearrangements of secondary elements or even entire domains (Table 1). For larger systems, movement of the gate may cause formation of a tunnel or enclosed cavity in addition to permitting or denying access to selected species.^{49,50} The following parameters can be useful for describing and discriminating between gates: (i) their constituent residues, (ii) their anchoring residues, (iii) the hinge region, i.e., the amino acids that make the structure flexible and allow it to move, (iv) the gate's position, (v) the gate's bottleneck diameter in the open and closed states, (vi) changes in the bottleneck's size over time, (vii) the energy required to switch the gate from one state to the other, and (viii) the energy required for passage of specific molecules through the gate.

3.1. Residue Motion: Wings

The energetic barriers for residue rotation are quite small, 1–16 kcal/mol.⁵¹ While generally low, such barriers can nevertheless be large enough to significantly affect the probability that a given species will be able to pass through the gate or the rate at which they do so. Depending on the particular amino acid and its surroundings, one or both states of the gate may be stabilized by interactions with anchoring residues, e.g., hydrophobic interactions, H bonds, ionic interactions, salt bridges, and π – π interactions. The strongest effect on the control of the passage is achieved when a large gating residue is located in the bottleneck of the pathway. The most common residues in this role are those whose side chains contain aromatic rings, i.e., W, F, and Y (Figure 4). Wing-type gates are common and can be found in enzymes such as imidazole

Table 1. Classifying Enzyme Gates According to Their Structural Basis




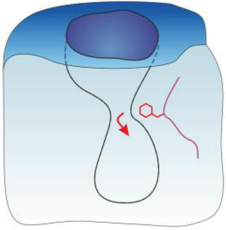
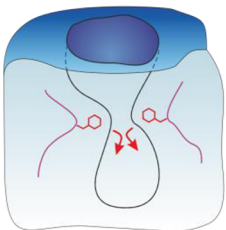
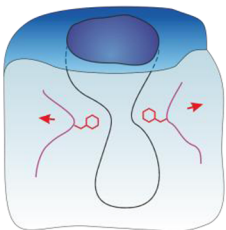

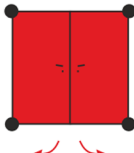
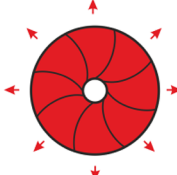
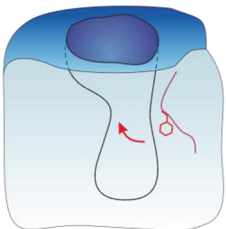
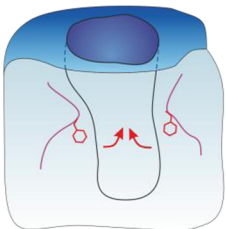
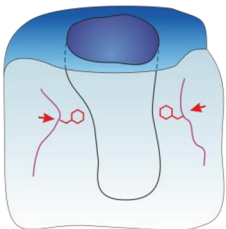

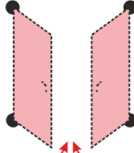
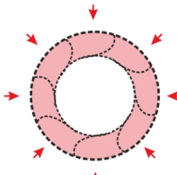

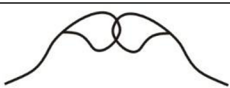

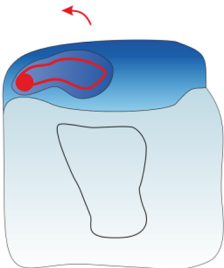
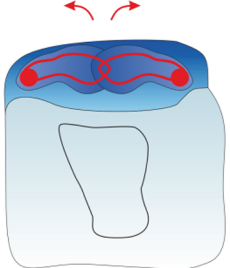
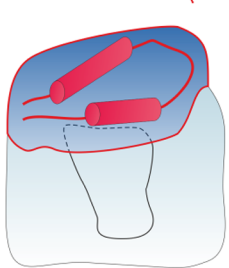
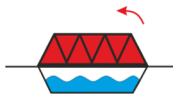


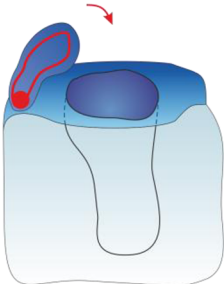
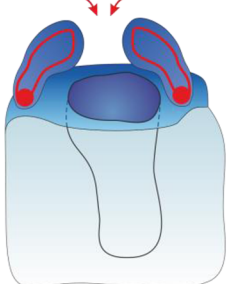
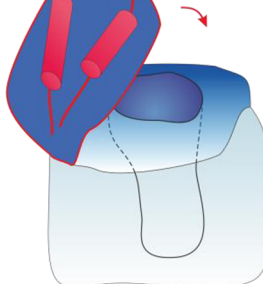
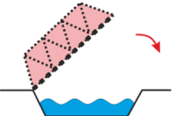
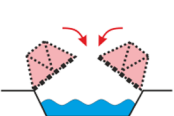

	1	2	3
Symbol			
Scheme of closed state			
Illustration of closed state			
Scheme of open state			
Illustration of open state			
Metaphor	wing	swinging door	aperture
Mechanism and moving part	side chain rotation	side chain rotation	backbone motion
Structural basis	1 residue	2 residues distant in sequence	2-4 residues distant in sequence
Amplitude of motion	< 2 Å	< 3 Å	< 3 Å
Time scale	ps – μs	ps – μs	ns – μs
Other features	anchoring residue	anchoring residues	hinge and anchoring free

Table 1. continued

	4	5	6
Symbol			
Scheme of closed state			
Illustration of closed state			
Scheme of open state			
Illustration of open state			
Metaphor	drawbridge	double drawbridge	shell
Mechanism and moving part	fragment movement	fragments movement	domain movement
Structural basis	~8-20 residues neighbour in sequence	2x ~8-20 residues neighbour in sequence	domain
Amplitude of motion	> 5 Å	> 5 Å	> 5 Å
Time scale	ns – μs	ns – μs	ms – s
Other features	hinge region, anchoring residues	hinge region, anchoring residues	hinge region, anchoring residues

glycerol phosphate synthase,³⁸ cytidine triphosphate synthetase,⁵² methane monooxygenase hydroxylase,⁵³ FabZ β -hydroxyacyl-acyl carrier protein dehydratase,⁵⁴ and cytochrome

P450.^{25,55} Even small gates of this type may require an activating agent to open. For example, the gate in the water channel of human monooxygenase CYP3A4 is created by the

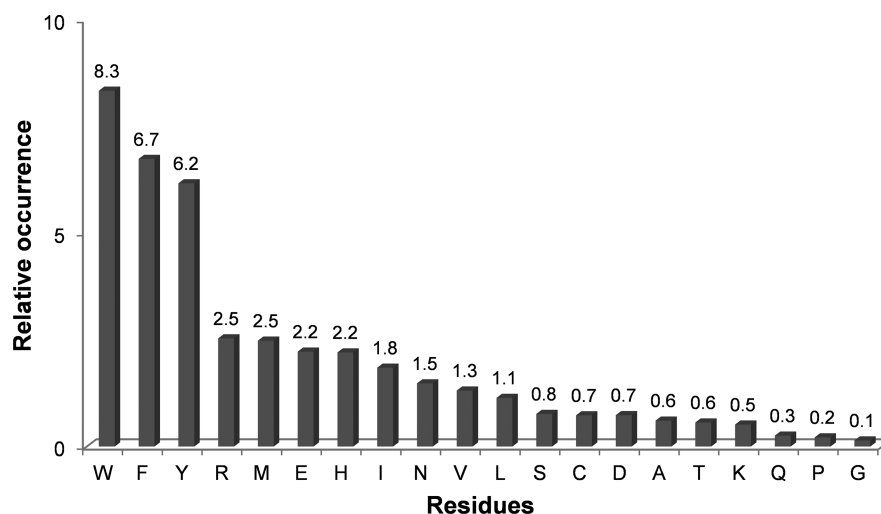


Figure 4. Relative occurrences of specific amino acid residues in wing and swinging door gates; 71 proteins with 129 gates were analyzed, and 154 residues that form wings or swinging doors were identified. Detailed description of the analyzed proteins is provided in Table 3. Values were normalized against the frequency with which each amino acid appears in all of the protein structures of the UniProtKB/Swiss-Prot database (2012_07).

interaction of the conserved residue R375 with the heme, which opens upon cytochrome P450 reductase binding to the enzyme.⁵⁶

3.2. Residue Motion: Swinging Doors

A more complex type of gate consists of two residues that can rotate but are stabilized in the closed conformation by a mutual interaction. Lario et al. introduced the phrase “swinging door” to describe gates of this type that were identified in cholesterol oxidase type I.⁵⁷ Some swinging door gates open by having both residues rotate in the same direction, while in others the two residues rotate in opposite directions. Common stabilizing interactions in swinging door gates include π stacking as occurs in the F–F pair of cytochrome P450_{3A4}^{58,59} and acetylcholinesterase,⁶⁰ ionic interactions as in toluene-4-monooxygenase⁶¹ and cytochrome P450_{cam}, P450_{BM3}, and P450_{eryF},^{25,55} aliphatic hydrophobic interactions such as those between the F–I, the F–V, and the F–L pairs of cytochrome P450_{3A4},^{58,59} aliphatic interactions such as those between the R–L and the L–I pairs of cytochrome P450_{3A4},^{58,59} and hydrogen bonds such as that between the R–S pair in cytochrome P450_{3A4}.⁵⁸ The open conformations of one or both of the gate residues may also be anchored, depending on the amino acids surrounding the gate. In comparison to wing gates, gates consisting of two residues can control wider tunnels and channels. It is worth mentioning that the individual residues that comprise a swinging door gate may simultaneously be components of another gate, as occurs in cytochrome P450_{3A4}.⁵⁸ Literature data indicate that most gates of this type consist of F–F pairs, and one way to screen for potential gates is to search for phenylalanine sandwiches.

3.3. Residue Motion: Apertures

Proteins undergo low-frequency breathing motions that may involve synchronized movements of bottleneck residues. In contrast to the previously described gates, the residues that form aperture type gates do not need to rotate and can maintain a rigid conformation. Their movements occur as a result of the synchronized relocation of the enzyme backbone during its breathing motions. The ability of a given species to pass through gates of this type depends on the length of time the gate remains in the open state, which is determined

by the enzyme’s rigidity (especially in terms of the compartments housing the gating amino acids) and the strength of the interactions between the gating residues. Gates of this kind can therefore switch between states at different frequencies, which can be adjusted by mutating the gating residues. Aperture-type gates have been identified in several enzymes including carbamoyl phosphate synthetase,³⁶ choline oxidase,³¹ glutamate synthases,⁶² extradiol dioxygenases-homoprotocatechuate 2,3-dioxygenase,⁶³ cytochrome P450_{eryF},²⁵ and acetylcholinesterase.⁶⁴

3.4. Motions of Loops and Secondary Structure Elements: Drawbridges and Double Drawbridges

The movements of loops and secondary structure elements can provide an energetically favorable method of controlling access for larger ligands. The gates described above consist of individual residues and would not provide sufficient control for enzymes that have large substrates and correspondingly large active site cavities. In many cases, the loops involved in access control also contribute to formation of substrate/cofactor binding cavities. Alternatively, in enzymes with complex systems of internal tunnels such as the members of the cytochrome P450 family, the dynamic motion of the protein structure, especially the flexible B–C and F–G loops in the cytochromes P450, plays a vital role in the opening and closing of the tunnels.¹⁶ Protein motions of this type can also merge different tunnels, creating a wider opening. Here, gating elements control the access of large substrates by merging and dividing the space shared by the tunnels.¹⁶ However, in such cases the movements of the loops can cause formation of smaller and more selective gates such as the swinging doors described in the preceding sections.^{56,58}

Movements of loops and secondary structure elements can change the solvation of a cavity or the gate itself. The equilibrium between the open and the closed conformations depends on the anchoring residues and the flexibility of the hinge region. All of these elements play important roles in the movements of large gates. The conserved GxG motif found in most cytochrome P450 family members provides a good example.⁶⁵ Depending on cytochrome isoform, the motif flanks either one or both ends of the B–C loop. It increases the

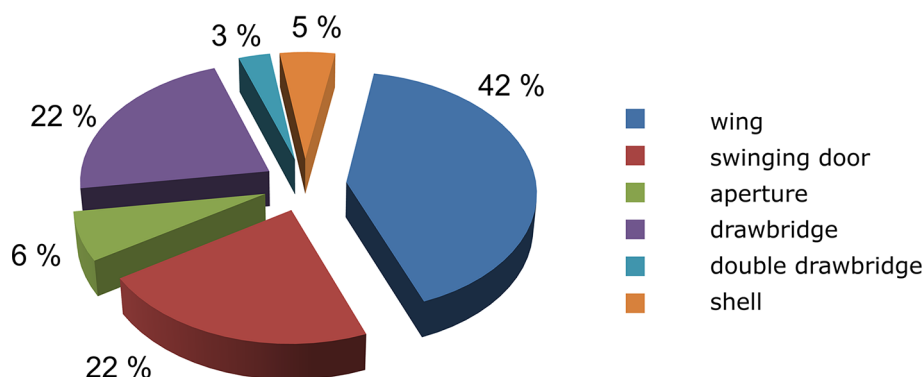


Figure 5. Frequencies of different gate types based on analysis of 71 proteins with 129 gates. Detailed description of the analyzed proteins is provided in Table 3.

flexibility of the loops, lowers the energy required for their motion, and facilitates tunnel opening and closing. The loops' variable lengths and levels of flexibility mean that each member of the P450 family has a gate with unique properties.¹⁶

3.5. Domain Motion: Shell

Large domain motions in enzymes are not generally regarded as gating systems. "Gates" of this scale are common in ion channels, which are beyond the scope of this review.¹⁴ However, one might expect that such large gates could be present in enzymes that catalyze reactions of very large substrates. Indeed, a gate of this kind has been observed in RNA polymerase, whose clamp domain opens to permit entry of promoter DNA during initiation, closes to establish a tight grip on the DNA during elongation, and then opens again to release the DNA during termination.⁶⁶ Interestingly, movement of large domains may protect enzymes from small molecule leakage and control their transport through long tunnel networks. This has been observed in carbon monoxide dehydrogenase/acetyl coenzyme A synthase, which operates as a tetrameric complex of distinct subunits.⁶⁷ The cap domain movement of epoxide hydrolase from *Mycobacterium tuberculosis* is another enzyme with a domain-scale gate that controls substrate access to the active site cavity.⁶⁸ Monomers of phospholipase A2 control access to their interface and the active site by adopting a different conformation during dimer aggregation.⁶⁹ Large domain movements often require an additional source of energy. For example, in the ATP-dependent protease HsIVU,⁷⁰ ATP hydrolysis is required to initiate conformational changes and propagate them to the residues that form the gate.

Structures of the gates found in 71 different proteins were analyzed systematically, yielding the results presented in Tables 2 and 3. The most common gate types are wings, swinging doors, and drawbridges, while apertures, double drawbridges, and shells are less common (Figure 5). However, these numbers may be distorted by the difficulty of identifying different gate classes by experimental methods or molecular dynamics simulations. Movements of only a few residues are more easily captured than those of secondary structure elements or domains due to the different time scales involved and the sizes of the moving structures. Moreover, gates may be controlled by overlapping processes that occur on different time scales and affect different structural regions.⁵⁰

4. LOCATIONS OF GATES






The roles of gates in the enzymatic catalysis discussed above suggest that these structures are natural hot spots for modifying enzyme properties. Identification of structural components of natural gates would therefore be very useful to protein designers. This raises a question: how and where should one look for the gates? Gates in proteins can be identified experimentally by protein crystallography and NMR spectroscopy and computationally by molecular dynamics simulations and normal-mode analysis.

Crystallographic analyses can provide information on alternative configurations of particular amino acids in a protein structure. The presence of residues that can adopt both open- and closed-type configurations along the access or release pathways of ligands, ions, and solvents may suggest the presence of a gate. However, in order for a gate to be detected by crystallography, it is necessary for both the open and the closed conformations to be sufficiently represented. Despite this restriction, crystallographic analyses have identified gates in tryptophan synthase,⁷¹ haloalkane dehalogenase LinB,⁷² L-amino acid oxidase,⁷³ and toluene-*o*-xylene monooxygenase.⁷⁴ In some cases, only one conformation will be present in the solved crystal structure, which may create a somewhat distorted picture, suggesting the absence of a gate when the open conformation is stabilized or the absence of a pathway when the closed conformation is stabilized.^{31,61,75}

The limitations of crystallographic analysis can be overcome by advanced NMR spectroscopy, which makes it possible to study multiple protein conformations simultaneously, over time scales ranging from picoseconds to milliseconds.^{7,76} Such analyses provide information on both the open and the closed states as well as the population of each state and rate of their interconversion. NMR techniques have been used to measure the rate of exchange between the open and the closed conformations of triosephosphate isomerase,^{77,78} HIV-1 protease,⁷⁹ and dihydrofolatereductase.^{80,81} Overall, the utility of NMR for studying distant effects of mutations on protein dynamics suggests that it has great potential for investigating gating mechanisms.⁹

Some of the most useful tools for identifying gates are the computer programs developed for detecting tunnels, channels, and cavities in protein structures.⁸² The outputs of CAVER,⁸³ MOLE,⁸⁴ and MOLAXIS⁸⁵ can be analyzed to detect bottleneck residues that form a potential gate or identify the best position for introduction of a new gate. Mutations at these "hot spots" can provide enzymes with new selectivities or

Table 2. List of Enzymes Possessing Gates Described in the Scientific Literature with Indication of Their Function, Structural Basis, and Location

No.	Enzyme name	Function				Structural basis						Location			
		Substrate accessibility	Solvent accessibility	Synchronization of reactions	Other							Active site entrance and active site	Tunnel entrance and tunnel bottleneck	Cofactor cavity	Other
01	2-Amino-2-Desoxyisochorismate Synthase PhzE	+		+		+									+
02	3-Hydroxybenzoate Hydroxylase MHBH		+		+	+								+	
03	4-Hydroxy-2-Ketovalerate Aldolase DmpG / Acylating Acetaldehyde Dehydrogenase DmpF	+		+	+	+	+						+		
04	4-Hydroxybenzoate Hydroxylase PHBH		+			+					+			+	+
05	Acetylcholinesterase AChE	+	+			+	+					+			
06	Acylaminoacyl Peptidase				+						+				+
07	α -Amylase TK1436	+			+	+						+			
08	Asparagine Synthetase			+	+	+								+	
09	ATP-Dependent Proteases HslVU	+				+							+	+	
10	Carbamoyl Phosphate Synthetase CPS – type II		+	+			+	+					+		
11	Carbon Monoxide Dehydrogenase / Acetyl Coenzyme A Synthase	+		+	+						+				+
12	Carbonic Anhydrase β – type I		+				+					+			
13	Carbonic Anhydrase β – type II	+	+	+		+		+				+	+		
14	Carboxylesterase pnbCE	+			+	+				+		+	+		
15	Catalase CAT-1 and CAT-3	+	+			+		+				+	+		
16	Cellobiohydrolase CEL 7A	+			+				+						+
17	Cellobiose Phosphorylase	+							+						+
18	Chalcone Synthase	+			+		+						+		
19	Chloramphenicol Halogenase CmlS	+							+				+		
20	Cholesterol Oxidase – type I SCHOX	+	+	+	+	+	+						+	+	
21	Cholesterol Oxidase – type II BsChOx	+			+	+									
22	Choline Oxidase	+						+	+			+	+		
23	Chondroitin AC Lyase		+		+					+		+			+
24	Copper-Containing Amine Oxidase		+			+							+		
25	Cytidine Triphosphate Synthetase CTPS		+	+		+		+			+	+		+	
26	Cytochrome P450 CYP3A4	+	+			+					+	+			
27	Dihydrofolatereductase				+			+					+		
28	Digeranylgeranylglycerophospholipid Reductase DGGR	+		+				+		+			+	+	
29	Epoxide Hydrolase H37Rv	+								+				+	
30	Epoxide Hydrolase M200	+		+	+	+						+			
31	FabZ β -Hydroxyacyl-Acyl Carrier Protein Dehydratase	+		+	+							+			
32	Formiminotransferase-Cyclodeaminase FTCD			+				+						+	
33	Glucosamine 6-Phosphate Synthase GlmS		+	+	+	+		+			+	+			
34	Glutamate Synthases GltS	+	+	+	+	+		+			+	+			
35	Glutamine Phosphoribosylpyrophosphate Amidotransferase	+	+	+	+	+		+			+	+			
36	Haloalkane Dehalogenase DhaA	+	+		+	+						+			
37	Haloalkane Dehalogenase LinB	+			+							+			
38	Histone Deacetylase HDAC1 and HDAC2	+			+	+						+			
39	Histone Deacetylase HDAC8		+	+	+							+			
40	HIV-1 Protease	+								+				+	
41	Homoprotocatechuate 2,3-Dioxygenase	+				+	+					+			
42	Hydrogenase FeFe	+					+							+	
43	Hydrogenase NiFe			+			+				+				
44	Imidazole Glycerol Phosphate Synthase IGPS		+	+	+	+						+			
45	Inosine 5'- Monophosphate Dehydrogenase				+			+				+			
46	Ketoacyl Synthase KS	+	+		+						+	+			
47	L-Amino Acid Oxidase	+			+	+						+			
48	Lipase B	+			+	+								+	
49	Lon Protease	+						+				+			
50	Mannitol 2-Dehydrogenase			+	+							+			
51	Methane Monooxygenase Hydroxylase MMOH	+		+	+	+					+	+			
52	Monoamine Oxidase A	+						+			+				
53	Monoamine Oxidase B	+			+		+				+			+	
54	Monooxygenase ActVAOrf6	+	+		+	+		+			+	+		+	
55	NADH Oxidase			+	+	+					+				
56	O-Acetylserine Sulfhydrylase Cysteine Synthase	+			+			+			+				
57	Oxidosqualene Cyclase SceOSC	+			+							+			
58	Phenol Hydroxylase PHHY	+			+	+	+					+	+		+
59	Phospholipase A2	+								+					+
60	Phosphatidylinositol-Specific Phospholipase C	+							+			+			
61	Quercetin 2,3-Dioxygenase	+				+	+						+		

62	Rabbit 20a-Hydroxysteroid Dehydrogenase		+		+		+		+		+
63	Raucaffricine O-β-Dglucosidaseglucosidase	+				+			+		
64	RNA-Dependent RNA Polymerase				+			+			+
65	RNA Polymerase	+						+		+	
66	Toluene-4-Monooxygenase T4MO	+	+		+	+	+		+	+	
67	Toluene-Ortho-Xylene Monooxygenase ToMO	+			+				+	+	
68	Triosephosphate Isomerase		+				+		+		
69	tRNA-Dependent Amidotransferase GatDE and GatCAB		+	+	+		+		+	+	
70	Tryptophan Synthase		+	+	+	+	+		+	+	+
71	Undecaprenyl-Pyrophosphate Synthase				+		+			+	

Wing Swinging door Aperture Drawbridge Double drawbridge Shell

Table 3. Detailed Description of Enzymes Possessing Gates Presented in the Scientific Literature

No.	Enzyme name		EC number
Gate function <i>Information about gate function</i>		Enzyme function <i>Information about catalysed reaction</i>	
Gate location <i>Information about gate location</i>		<i>Small picture of whole enzyme with detected tunnels and gates</i> <i>protein – light blue surface</i> <i>tunnel – dark blue wire</i> <i>gates residues – red ball and stick</i> <i>Large picture – close-up on gate residues</i> <i>protein – light blue cartoon</i> <i>tunnel profile – grey spheres</i> <i>active sites residues (if shown) – green ball and stick</i> <i>gates residues (elements) – ball and stick representation colour correspond to colour of gate name</i> <i>cofactor – yellow ball and stick</i> <i>substrate – yellow ball and stick</i>	
Gate structural basis			
<i>Schematic drawing of gate class</i> <i>? in front of a picture indicates a lack of data – classification has been made based on residues localisation</i>	GATE 1 - font colour corresponds to the colour of residues on the picture <i>Information about residues, open and closed conformation and mechanism of the changes of gate states</i> ? GATE1 - ? indicates a hypothetical gate, some data missing to fulfil all requirements, e.g., lack of information about open and close conformation * GATE1 - * indicates important residues used for gates engineering		
Gate engineering <i>Information about mutants that change gate state or create new gates, including information about mutants closing and opening tunnels</i>		<i>All pictures present results of CAVER 3.0 calculations of tunnels visualized by PyMOL. Tunnels corresponding to those reported in literature have been visualised. Reader should be aware that calculations were performed using available static structures from the PDB database, therefore calculated tunnels can differ from the tunnels described on the base of results from MD simulations.</i>	
		Tunnel	<i>Name or part of the tunnel</i>
		Length	<i>Tunnel length in [Å]</i>
		Bottleneck	<i>Bottleneck diameter in [Å]; data taken from the literature</i>
PDB: <i>Wild-type (WT) or mutants available in Protein Data Bank database</i>		Role	<i>Information about transported molecules</i>
References: <i>All references used for table preparation</i>			

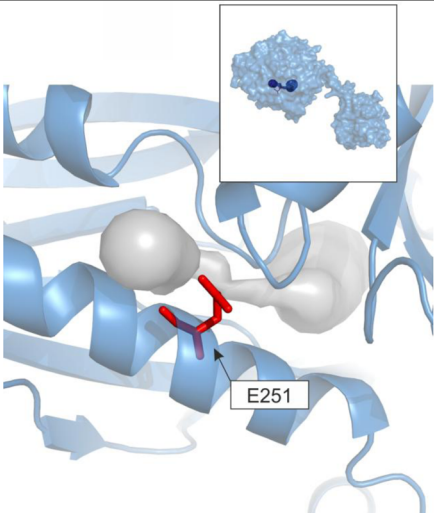

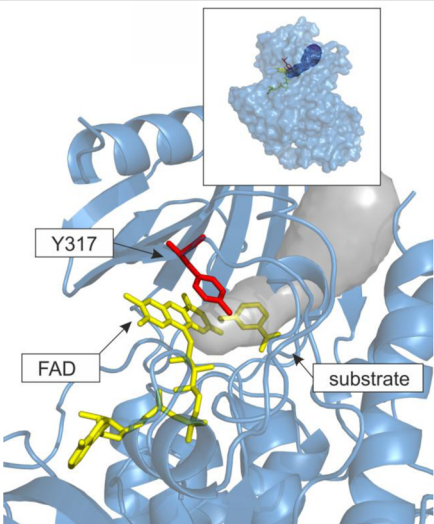

01	2-Amino-2-Desoxyisochorismate Synthase PhzE		4.1.3.27
Gate function GATE1 – Controls access of ammonia, synchronizes active sites		Enzyme function Utilizes chorismate and glutamine to synthesize 2-amino-2-desoxyisochorismate in the first step of phenazine biosynthesis	
Gate location GATE1 – Between the MST (menaquinone, siderophore, tryptophan) domain and GATaseI active site			
Gate structural basis			
	GATE1 E251 forms two hydrogen bonds with N149 in closed conformation interacts with K254 in open conformation		
Gate engineering		PDB ID: 3R74 Tunnel U-shaped Length 25 Bottleneck Role Ammonia transport	
PDB: <i>WT – 3R74 (open), 3R75 (closed)</i>			
References: ¹¹¹			

Table 3. continued

02	3-Hydroxybenzoate Hydroxylase MHBH		1.14.13.23		
Gate function GATE 1 – Controls the contact of NADPH with the isoalloxazine ring, protects FAD from the solvent			Enzyme function Conversion of 3-hydroxybenzoate to 3,4-dihydroxybenzoate		
Gate location GATE 1 – Between substrate tunnel and the FAD binding pocket					
Gate structural basis					
	GATE 1 Y317 The residue creates parallel π - π stacking interaction with FAD Opening – reorientation of both FAD and Y317				
Gate engineering					
PDB: <i>WT</i> – 2DKH, 2DKI			PDB ID: 2DKH		
References: ⁹⁸			Tunnel	E1	E2 (not shown)
			Length	20-22	32
			Bottleneck	4.5	4
			Role	O ₂ , substrate	Product

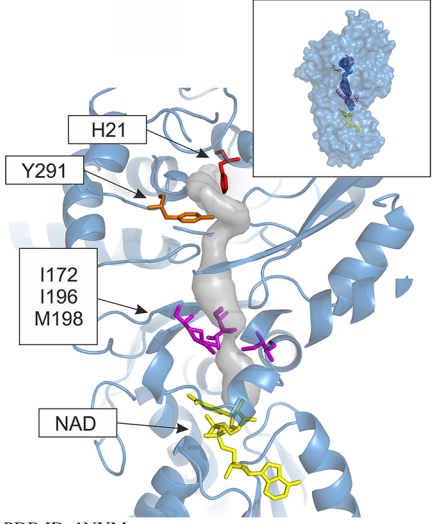




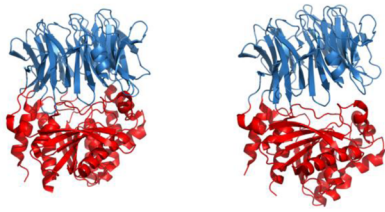

03	4-Hydroxy-2-Ketovalerate Aldolase DmpG / Acylating Acetaldehyde Dehydrogenase DmpF		4.1.3.39 / 1.2.1.10	
Gate function GATE1 – Controls access of the substrate to the DmpG active site GATE2 – Proton transfer, synchronizing two active sites by controlling the passage of the acetaldehyde GATE3 – Controls intermediate entry to the DmpF active site; facilitates interaction between N171 and NAD			Enzyme function Catalyses final two steps in degradation of toxic aromatic intermediates in the meta-cleavage pathway of catechol	
Gate location GATE1 – Tunnel entrance in aldolase subunit GATE2 – Tunnel entrance in aldolase subunit GATE3 – Tunnel exit in dehydrogenase subunit				
Gate structural basis				
	GATE1 H21 Opening – reorientation of H21			
	GATE2 Y291 Opening – reorientation of Y291			
	GATE3 I172 + I196 and M198 I172, I196 and M198 block the tunnel exit in the dehydrogenase subunit Opening – in the structure with bound NAD ⁺ – an interaction of N171 with NAD induce unique orientation of I172, additionally I196 and M198 adopt open conformations			
				
Gate engineering H21A – acetaldehyde and propionaldehyde channelling reduced by more than 70% Y291F – reduced channelling efficiencies by >30% I196L, I196F – no significant changes in the channelling efficiency			PDB ID: 1NVM	
PDB: <i>WT</i> – 1NVM			Tunnel between active sites	
			Length	29
			Bottleneck	1.7
References: ^{96,112}			Role	Acetaldehyde intermediate transport

Table 3. continued

04	4-Hydroxybenzoate Hydroxylase PHBH		1.14.13.2	
Gate function GATE1 – Controls access of the solvent GATE2 – Controls access of the solvent			Enzyme function Monooxygenation of <i>p</i> -hydroxybenzoate (<i>p</i> -OHB) to 3,4-dihydroxybenzoate	
Gate location GATE1 – Close to the FAD cofactor GATE2 – One domain of the protein			<p>PDB ID: 1IUW</p>	
Gate structural basis				
	GATE1 R220 and FAD R220 modulates the dynamics of flavin movements Opening – reorientation of R220; out – solvent exposed; in – solvent excluded			
	GATE2 Large domain movement As substrate (<i>p</i> -OHB) moves forward in the tunnel and reaches its high-affinity site, the βαβ (1-180) and the sheet domains (180-270) are expected to rotate and close the active site onto the substrate			
Gate engineering R220Q – keeps enzyme in the open conformation – loss of selectivity and decrease of activity (100-fold)				
PDB: <i>WT</i> – 1IUW, <i>Mutant R220Q</i> – 1K0I, 1K0J, 1K0L			Tunnel	
References: ¹¹³			Length	
			Bottleneck	
			Role	Substrate access

05	Acetylcholinesterase AChE		3.1.1.7	
Gate function GATE1 – Controls access of the substrate to the active site GATE2 – Controls escape of the acetic acid and/or the water molecule			Enzyme function Hydrolysis of acetylcholine	
Gate location GATE1 – Main tunnel – 12 Å from the bottom of the gorge – entrance to the active site GATE2 – Back door tunnel – on the C67– C94 wall			<p>PDB ID: 1EA5 open main tunnel and closed back door</p>	
Gate structural basis				
	GATE1 F330 F330 controls the entrance of the natural substrate Second most important residue – Y121, followed by W84, F288, F290, F331, Y334 <i>Cationic substrate (Huperzine A)</i> : – movement of F330, Y121 and D72 generate an electrostatic field affecting the substrates <i>Aromatic substrate (E2020)</i> : residues grouped in 3 groups acting as “sender” and “receiver”, compose a “conveyer belt” via π-π stacking interactions with benzene ring of E2020: Group I contains W84, F330, and F331 Group II consists of F288, F290, and Y334 Group III includes Y70, Y121, and W279 <i>In mAChE</i> F338 -Y124			
	GATE2 W84, G441 and Y442 (TcAChE); W86, G448 and Y449 (mAChE) Opening - movement of the W84 indole ring, almost 90° rotation to a position where it interacts with Y442 <i>Alternative propositions</i> ?GATE2b – E82, P76, and G77 (with some small movement of D72, E73, and N85) ?GATE2c – Between V71, N85, P86, and M90 ?GATE2d – 90° rotation of the F78 and the displacement of V431 and W432 ?GATE2e – W84, V129, and G441 ?GATE2f – Ω-loop (C67 – C94) with W84 could undergo a flap-like transition ?GATE2g – Facial rearrangement of the loop between W279 and S291			
	Gate engineering V129W – 4-fold increase of K_m V431C – 2-fold increase of K_m			
PDB: <i>WT</i> – 1W75, 1ACJ, 1ACL, 1EA5, 2ACE, 1MAH, 1QTI, 1DX6, 1EVE, 1OCE, 2XI4 (open back door)			Tunnel	
References: ^{13,35,60,64,114–123}			Length	
			Bottleneck	
			Role	Acetylcholine
			back door	Small molecules

Table 3. continued

06		Acylaminoacyl Peptidase		3.4.19.1	
Gate function GATE1 – Controls enzyme activity – only closed form is active			Enzyme function Removes acylated amino acid residues from the N terminus of oligopeptides		
Gate location GATE1 – Two domains of an enzyme monomer			 <div><div>closed</div><div>open</div></div>		
Gate structural basis					
	GATE1 Two domains may move away to form an opening of about 30°, with D376 being the hinge Open - accept substrate Closed - rearrange active site				
Gate engineering					
PDB: <i>WT</i> – 3O4G			PDB ID: 3O4G		
References: ^{124,125}			Tunnel		
			Length		
			Bottleneck		
			Role		

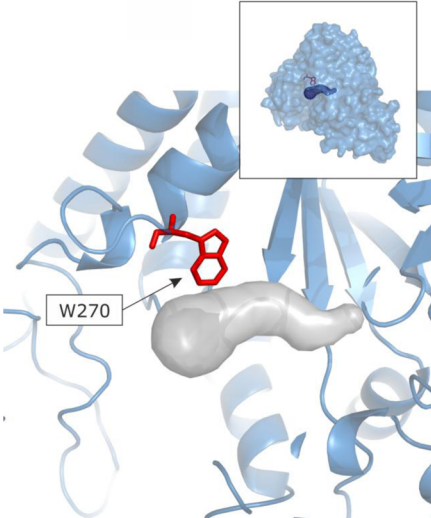

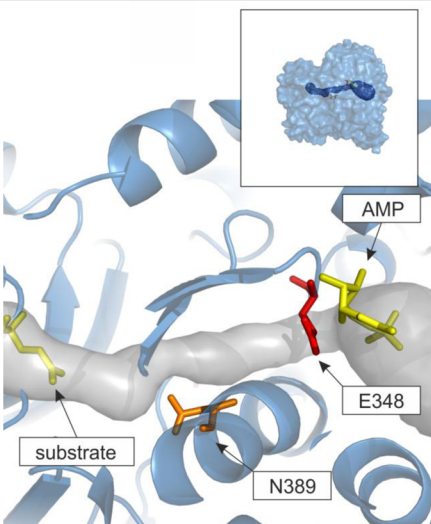


07		α -Amylase TK1436		2.4.1.18	
Gate function GATE1 – Controls access of the substrate to the active site, regulates transglycosylation			Enzyme function Formation of branch points in glycogen and amylopectin by cleavage of α -1,4 glycosidic bonds and subsequent transfer to a new α -1,6 position		
Gate location GATE1 – At the entrance of the active site			 <div><div>W270</div></div>		
Gate structural basis					
	GATE1 W270 The residue displays different conformations depending on the presence or absence of ligands in the active-site pocket Other possible gate-keepers W28, W407, W416				
Gate engineering					
PDB: <i>WT</i> – 3N8T, 3N92, 3N98			PDB ID: 3N8T		
References: ¹²⁶			Tunnel		
			Length		
			Bottleneck		
			Role		Substrate recognition and binding

Table 3. continued

08		Asparagine Synthetase		6.3.5.4	
Gate function ? GATE1 – Synchronizing active sites, establish the intramolecular tunnel for ammonia passage ? GATE2			Enzyme function ATP dependent synthesis of L-asparagine from L-aspartic acid		
Gate location ? GATE1 – C-terminal end of the ammonia tunnel linking the active site near to the ATP moiety ? GATE2 – C-terminal domain			 <p>PDB ID: 1CT9</p>		
Gate structural basis					
	? GATE1 E348				
	? GATE2 N389				
Gate engineering E348D – impairs acyl-adenylate formation, tunnel is more solvent exposed leading to loss of the ammonia because of an impaired rate of βAspAMP formation			Tunnel		
PDB: <i>WT</i> – 1CT9			Length		
References: 42,44,127,128			Bottleneck		
			Role Ammonia transport		

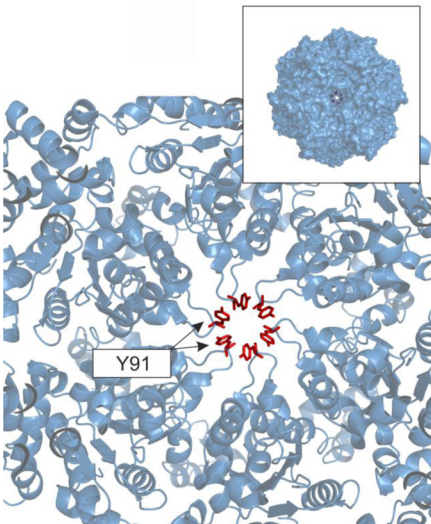

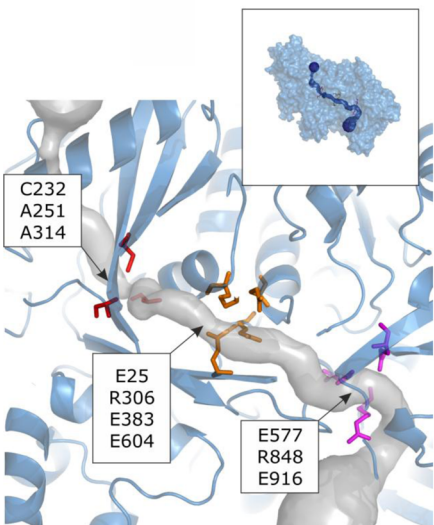



09		ATP-Dependent Protease HslVU		3.4.25.2	
Gate function GATE1 – Controls access of the substrate to the active site located inside the chamber			Enzyme function Degradation of the majority of proteins in a cell		
Gate location GATE1 – Translocation tunnel			 <p>PDB ID: 1G4A</p>		
Gate structural basis					
	GATE1 Y91 “Twist-and-open” mechanism - conformational changes induced by ATP hydrolysis are propagated to the gating sequence Y91 can move (180° rotation) from inside HslU toward HslV through the pore – closed pore has diameter 4.4Å open pore has diameter of 19.3Å HslVU works as a hexamer therefore pore diameter depends on the number of Y91 pointed toward HslV				
Gate engineering Y91F, V92I, V92A, and V92S – decrease in protein degradation activity G90P, G93P, G90A, G93P, Y91I, Y91C, Y91S, Y91A, V92F, V92C – are not capable to support the proteolytic activity of HslV					
PDB: <i>WT</i> – 1G4A, 1G4B			Tunnel		
References: 13,129,130			Length -		
			Bottleneck Closed - 4.4; open up to 19.3		
			Role -		

Table 3. continued

10	Carbamoyl Phosphate Synthetase CPS – type II	6.3.5.5																
Gate function GATE1 – Desolvation of ammonia GATE2 – Controls entry of carbamate into the tunnel prior to phosphorylation to carbamoyl phosphate GATE3 – Controls entry of carbamate into the tunnel prior to phosphorylation to carbamoyl phosphate		Enzyme function Synthesis of carbamoyl phosphate																
Gate location GATE1 – Large subunit/ammonia tunnel GATE2 – Carbamate tunnel near the carboxy phosphate binding site GATE3 – Carbamate tunnel near the site for the synthesis of carbamoyl phosphate		 <p>PDB ID: 1JDB</p> <table><tr><td>Tunnel</td><td>Whole</td><td>I part</td><td>II part</td></tr><tr><td>Length</td><td>>100</td><td>45</td><td></td></tr><tr><td>Bottleneck</td><td></td><td>3.2</td><td></td></tr><tr><td>Role</td><td>Connects all 3 active sites</td><td>Ammonia transport</td><td>Carbamate transport</td></tr></table>	Tunnel	Whole	I part	II part	Length	>100	45		Bottleneck		3.2		Role	Connects all 3 active sites	Ammonia transport	Carbamate transport
Tunnel	Whole		I part	II part														
Length	>100		45															
Bottleneck			3.2															
Role	Connects all 3 active sites	Ammonia transport	Carbamate transport															
Gate structural basis																		
 <p>GATE1 C232, A251, A314 (barrier 7.2 kcal/mol) Two more triad that may act as gate/switches for an ammonia passage are: I234-T249-L310 E217-T244-S307</p>																		
 <p>GATE2 R306, E25, ?E383, ?E604 R306 – ion pair with E25</p>																		
 <p>GATE3 R848, E577 and ?E916 R848 – ion pair with E577</p>																		
Gate engineering C232V/A251V/A314V – closing ammonia tunnel - unable to synthesize carbamoyl phosphate using glutamine as a nitrogen source G359F, G359Y – decoupling separate chemical reactions via creation an escape route for the ammonia intermediate αP360A/αH361A/βR265A – unable to utilize glutamine for the synthesis of carbamoyl phosphate via creation of an escape route for the ammonia intermediate; full catalytic activity with external ammonia source E916Q – 10-fold decrease in the rate of carbamoyl phosphate synthesis E25Q/E383Q – carbamoyl phosphate synthetase activity was diminished 50-fold E25Q/E383Q/E604Q – glutaminase activity is decreased about 5-fold, and the bicarbonate-dependent ATPase activity is diminished at least 20-fold E577Q –decrease of carbamoyl phosphate synthesis (100-fold)																		
PDB: <i>WT</i> – 1BXR, 1JDB References: 36,41,103,131–134																		

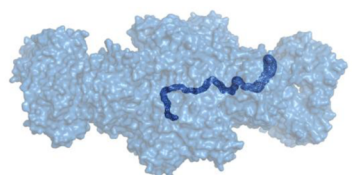


11	Carbon Monoxide Dehydrogenase / Acetyl Coenzyme A Synthase	1.2.7.4 / 1.2.99.2 / 2.3.1.169								
Gate function GATE1 – Controls the reaction, protects from CO leakage and controls CO access to Ni _p -Ni _d -[Fe ₄ -S ₄] cluster (A cluster – acetyl-CoA synthase active site)		Enzyme function α ₂ β ₂ tetramer; β subunit catalyses the reduction of CO ₂ to CO (C cluster), α subunit is responsible for the synthesis of acetyl-CoA from CO, coenzyme A (CoA) and a methyl group donated by a cobalamin-containing protein (A cluster)								
Gate location GATE1 – 20 Å from the A cluster – on the A and C cluster border		 <p>PDB ID: 1OAO Only one from two symmetrical tunnels is shown on a picture.</p> <table><tr><td>Tunnel</td><td>Length</td><td>Bottleneck</td><td>Role</td></tr><tr><td></td><td>130 between A clusters, additional two tunnels connecting C clusters 37 + additional water channel</td><td></td><td>CO transport between two active sites</td></tr></table>	Tunnel	Length	Bottleneck	Role		130 between A clusters, additional two tunnels connecting C clusters 37 + additional water channel		CO transport between two active sites
Tunnel	Length		Bottleneck	Role						
	130 between A clusters, additional two tunnels connecting C clusters 37 + additional water channel			CO transport between two active sites						
Gate structural basis										
 <p>GATE1 Large conformation change of subunit α, gating residues – residues of the α subunit N-terminal domain 143-148 Open conformation – accessible active site – closed tunnel – F512 moves to a position within 4 Å of both Ni_p and Ni_d blocking putative ligand binding to the axial coordination site of Ni_p Close conformation – closed active site – open tunnel – F229 blocks axial ligand binding to Ni_d but not to the Zn ion</p>										
Gate engineering A110C, A222L, A265M – block the tunnel between the A and C-clusters; A222L complete blocking A578C, L215F, A219F – block the tunnel between the C clusters F70W, N101Q – block a region at the ββ subunit interface that might dynamically connect the tunnel with a newly discovered water tunnel										
PDB: <i>WT</i> (<i>M. thermoacetica</i>) – 1OAO (open+closed form), <i>WT</i> (<i>C. hydrogenoformans</i>) – 1RU3 (closed form) References: 48,67,135–137										

Table 3. continued

12	Carbonic Anhydrase β – type I	4.2.1.1
Gate function ?GATE1 – Protects zinc cation site		Enzyme function Catalyses a reversible reaction to form bicarbonate from carbon dioxide and water
Gate location ?GATE1 – Vicinity of the active site		
Gate structural basis		
 ?	?GATE1 D162 donates a hydrogen bond to Q151 Acts as a gatekeeper residue by excluding anions from the zinc ligand environment that cannot donate a hydrogen bond at this position	
Gate engineering		
PDB: <i>WT</i> – 1EKJ		PDB ID: 1EKJ
References: ⁹³		Tunnel
		Length
		Bottleneck
		Role
		Acetate ion, CO ₂ transport






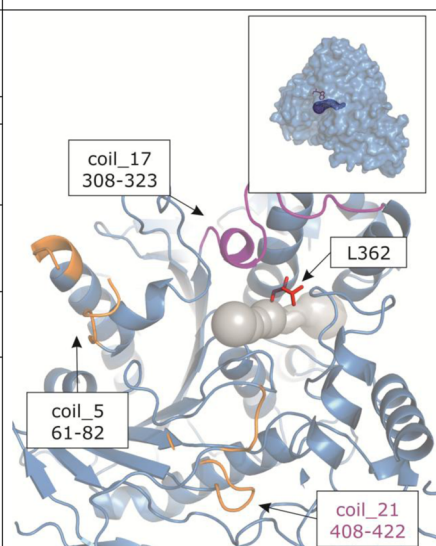
13	Carbonic Anhydrase β – type II	4.2.1.1
Gate function GATE1 – Controls access of the bicarbonate ion GATE2 – Controls access of the solvent, protects active site, controls transport of bicarbonate ion from the active site or from solution to the allosteric pocket		Enzyme function Catalyses a reversible reaction to form bicarbonate from carbon dioxide and water
Gate location GATE1 – Middle of the tunnel GATE2 – Vicinity of the active site in a narrow hydrophobic active site cleft that lies along the dimer or pseudo-dimer interface		
Gate structural basis		
 —●	GATE1 R64 – HICA Rotation of the guanidinium group allows migration of bicarbonate ion into the allosteric site	
 ?	GATE2 F61, V62?, Y83, and V87 Other anhydrases PPCA – F422, I173, Y444, Y448 Rv3588 – F70, A75, Y89, V93 Nce103 – F97, L102, G111, D59	
Gate engineering		PDB ID: 2A8D
PDB: <i>WT (HICA)</i> – 2A8D, <i>WT (PPCA)</i> – 1DDZ, <i>WT (Rv3588)</i> – 1YM3, <i>WT (Nce103)</i> – 3EYX		Tunnel
References: ^{93,138}		Length
		Bottleneck
		Role
		Acetate ion, CO ₂ transport

Table 3. continued

14	Carboxylesterase pnbCE	3.1.1
Gate function GATE1 – Controls exit of hydrolysis products by a side door GATE2 – Controls access to the active site gorge GATE3 – Controls access to the active site gorge		Enzyme function Detoxification of xenobiotics
Gate location GATE1 – Between active site and side door GATE2 – Entrance to active site gorge GATE3 – Entrance to active site gorge		
Gate structural basis		
	GATE1 L362 Rotates 180° around its C-C bond and adopts two distinct conformations	
	GATE2 Loop coil_5 (residues 61-82) and coil_21 (residues 408-422) Move away from their equilibration conformation, the active site opens and can accommodate incoming substrate	
	GATE3 Loop coil_17(residues 308-323) and coil_21 Close for substrate hydrolysis, move away to release products	
Gate engineering Δ coil_5 – k_{cat} 4.5-fold smaller Δ coil_21 – k_{cat} 3-fold smaller		 PDB ID: 1QE3
PDB: WT – 1QE3		Tunnel
References: ^{139,140}		Length
		Bottleneck
		Role



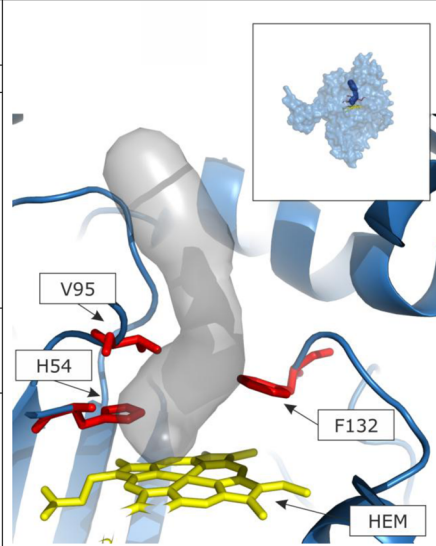
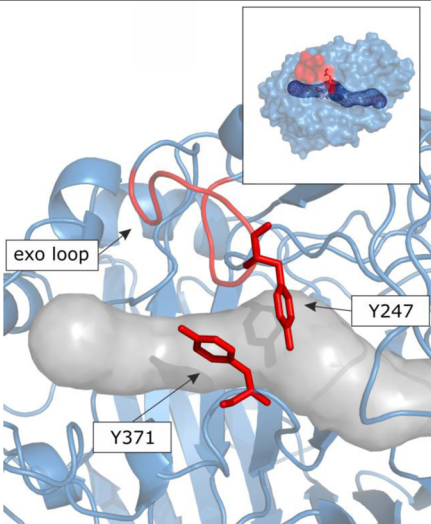

15	Catalase CAT-1 and CAT-3	1.11.1.6
Gate function GATE1 – Blocks access to the heme, controls solvation ?GATE2 – ?		Enzyme function Decomposition of hydrogen peroxide to water and O ₂
Gate location GATE1 – Entrance to the active site cavity from main tunnel ?GATE2 – Side tunnel		
Gate structural basis		
	GATE1 H54, V95, and F132 Barrier to reach the active site: H ₂ O ₂ < 4.8 kcal/mol O ₂ ~2.2 kcal/mol H ₂ O ~3.6 kcal/mol Opening of the gate may be regulated according to the H ₂ O ₂ concentration in the small cavity before the gate; H ₂ O ₂ in the cavity would change the net of hydrogen bonds and trigger opening of the gate; water molecules interacting with amino acid residues in the cavity would determine closure of the gate	
	?GATE2 (not shown on picture) CAT-1 – hydroxyl group of S198 blocks tunnel CAT-3 – hydroxyl group of T208 blocks tunnel	
Gate engineering		
PDB: WT – 1NM0		 PDB ID: 1NM0
		Tunnel
		Length
		Bottleneck
		Role

Table 3. continued

16	Cellobiohydrolase CEL7A	3.2.1.91	
Gate function GATE1 – Facilitates processing of crystalline cellulose degradation		Enzyme function Hydrolysis of amorphous and crystalline cellulose 	
Gate location GATE1 – Exo loop			
Gate structural basis			
	GATE1 Exo loop + Y247 Y247 in closed conformations interacts with Y 371 from the short loop – acting like a button – it creates tunnel, loop covers the active site during reaction time		
Gate engineering Y247F – removes hydrogen bond between Y247 and substrate - small effect on cellulose hydrolysis D241C/D249C – reduces mobility of the loop, disulphide bridge enhanced the activity on both amorphous and crystalline cellulose Deletion G245-Y252 – increases activity on amorphous cellulose, and half of the activity on crystalline cellulose			
PDB: WT – 1CEL		PDB ID: 1CEL	
References: 49,141–143		Tunnel	
		Length	50
		Bottleneck	3.5
		Role	Substrate cavity

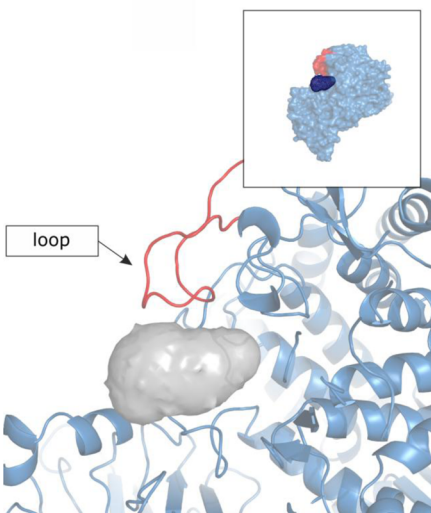

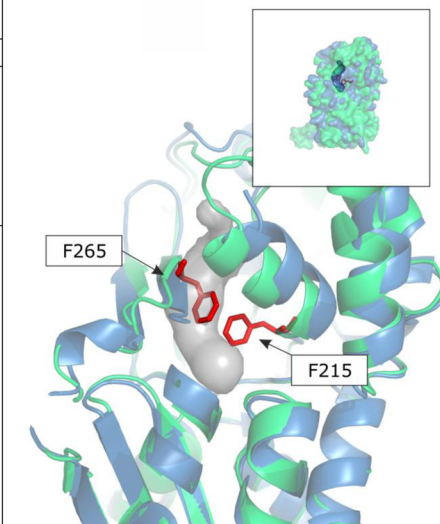

17	Cellobiose Phosphorylase	2.4.1.20	
Gate function GATE1 – Controls access of the substrate		Enzyme function Phosphorolysis of cellobiose into α -D-glucose 1-phosphate (G1P) and D-glucose 	
Gate location GATE1 – Flexible loop			
Gate structural basis			
	GATE1 Flexible loop (495–513) Loop undergoes conformational changes during substrate binding and release		
Gate engineering T508I – changes substrate specificity N156D and N163D – increase of the activity N156D/N163D/T508I/E649G/N667A – improves activity towards a whole range of β -glucosidic acceptors			
PDB: WT – 2CQT, 1V7X		PDB ID: 2CQT	
References: 144,145		Tunnel	
		Length	
		Bottleneck	
		Role	

Table 3. continued

18		Chalcone Synthase CHS		2.3.1.74	
Gate function GATE1 – Controls orientation of the substrate				Enzyme function Decarboxylative condensations of malonyl-CoA with a CoA-linked starter	
Gate location GATE1 – Between the active-site cavity and the CoA binding tunnel				 <p>PDBID: 3ALE – blue <i>Aloe arborescens</i> PCS PDBID: 1CGK – light green <i>Medicago sativa</i> CHS tunnel calculated for 3ALE structure</p>	
Gate structural basis					
	GATE1 <i>Medicago sativa</i> CHS F215 and F265 Block the lower portion of the opening between cavities and help with folding and the internal orientation of the tetraketide intermediate during the cyclization reaction; F215 facilitate the decarboxylation of malonyl-CoA by maintaining the orientations of substrates and intermediates during the sequential condensation reactions				
Gate engineering <i>M. sativa</i> CHS F215S – changes the substrate specificity via opening a space at the cavity entrance <i>A. arborescens</i> PCS *GATE2 M207G – opens a gate to two novel hidden pockets behind the active site of the enzyme - residue 207 controls the number of condensations of malonyl-CoA F80A/Y82A/M207G – provide further elongation of products					
PDB: <i>WT (M. sativa CHS)</i> – 1CGK, <i>WT (A. arborescens PCS)</i> – 3ALE				Tunnel	
References: ^{95,106,146}				Length	16
				Bottleneck	
				Role	Reaction cavity

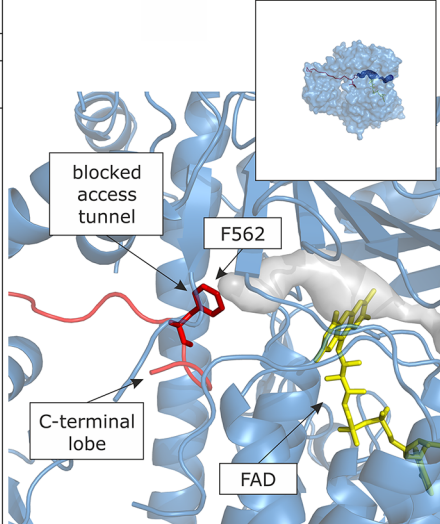

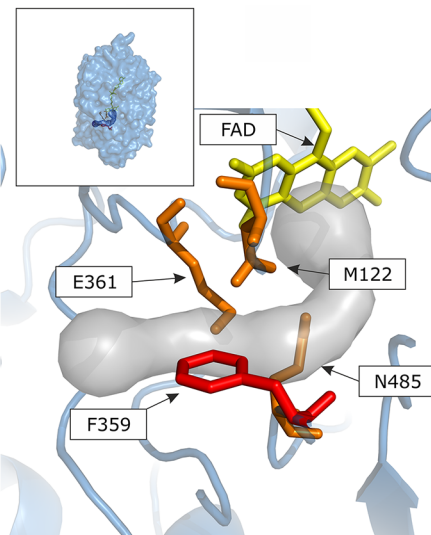


19		Chloramphenicol Halogenase CmlS		Not determined	
Gatefunction GATE1 – Blocks access to the active site				Enzyme function Formation of the dichloroacetyl group	
Gate location GATE1 – Flexible loop (the C-terminal lobe of CmlS)				 <p>PDB ID: 3I3L</p>	
Gate structural basis					
	GATE1 Flexible loop (the C-terminal lobe of CmlS) + F562 acting as a plug				
Gate engineering					
PDB: <i>WT</i> – 3I3L				Tunnel	
References: ¹⁴⁷				Length	10
				Bottleneck	
				Role	Connecting halogenation active site and FAD binding site

Table 3. continued

20		Cholesterol Oxidase – type I SCHOX	1.1.3.6	
Gate function GATE1 – Controls access of the solvent GATE2 – Controls access of O ₂ to the flavin and assures that isomerization occurs before the oxidative half of the reaction, tuning the redox state of the cofactor		Enzyme function Catalyses the first step in the pathway of cholesterol degradation		
Gate location GATE1 – Frames the entrance to the tunnel GATE2 – Gate is in the tunnel leading to the isoalloxazine ring of flavin				
Gate structural basis				
	GATE1 F359 F359 adopts two distinct alternate conformations separated by a 65° rotation of the benzene group Closed gate - maximizing hydrophobic packing interactions with V189, V124 and G347 Open conformation - the tunnel becomes solvent accessible Control of F359 gate appears to be dependent on the adopted conformation of N485			
	GATE2 N485, E361 and M122 Switch between their side-chain conformations, controls the access of O ₂ to flavin 1. Binding of steroid → rotation of the methyl group of M122 → pushes N485 sealing the tunnel and creating an ideal environment for oxidation 2. After substrate oxidation, a strong hydrogen bond forms when N485 moves to conformation near the flavin, forcing M122 to a conformation that destabilizes the binding of the oxidized product. 3. When the substrate is oxidized and the FAD cofactor is reduced, the side chain of N485 rotates toward the cofactor → tunnel opening - regulates access of oxygen to the active site			
Gate engineering F359W – rate of catalysed reaction decreases 13-fold G347N – could not be saturated with oxygen N485D – could not be saturated with oxygen				
PDB: <i>WT</i> – 1MXT, <i>Mutant F359W</i> – 3CNJ, <i>Mutant N485D</i> – 3GYI		PDB ID: 1MXT		
References: ^{57,99,148}		Tunnel		
		Length 32		
		Bottleneck 1.2		
		Role O ₂ and H ₂ O ₂ transport		

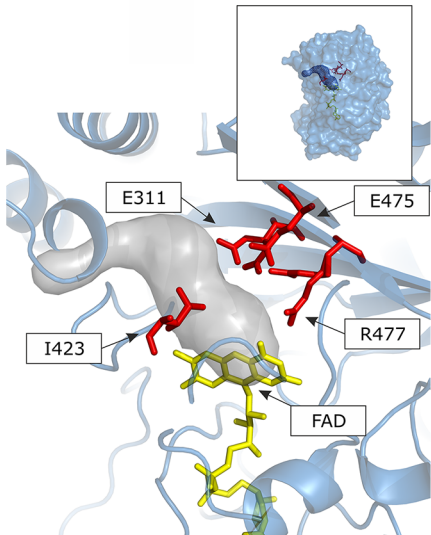


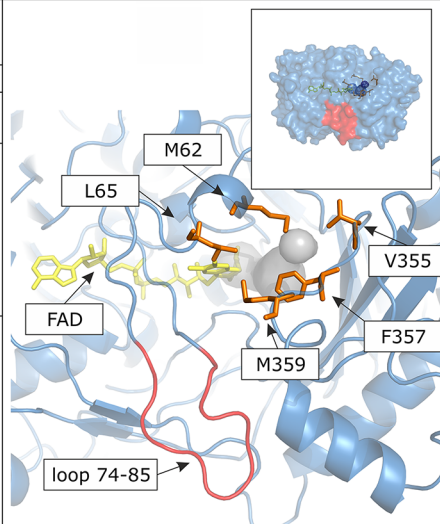


21		Cholesterol Oxidase – type II BsChOx	1.1.3.6	
Gate function GATE1 – Modulates access/reactivity of dioxygen		Enzyme function Catalyses the first step in the pathway of cholesterol degradation		
Gate location GATE1 – Bottleneck of the tunnel				
Gate structural basis				
	GATE1 E311, I423, E475 and R477 Open conformation of R477 is stabilized by a salt bridge with E311 E311 tunes the E475—R477 pair			
				
Gate engineering E311D/Q/L – cause a switch in the basic kinetic mechanism of the reoxidation with dioxygen, while BsChOx wild type and most mutants show saturation behaviour with increasing O ₂ For E311 – a linear dependence was found that would reflect a second-order process R477A – limits both oxidation and isomerization activities A204C, G309A, G309C, I423L, I423V, E475D, E475Q, R477K – no significant changes				
PDB: <i>WT</i> – 1II19		PDB ID: 1II19		
References: ^{99,149,150}		Tunnel		
		Length 23		
		Bottleneck 1.4		
		Role Oxygen transport		

Table 3. continued

22	Choline Oxidase		1.1.3.17	
Gate function ?GATE1 – Controls access of the positively charged substrate GATE2 – Controls access of the positively charged substrate			Enzyme function Oxidation of choline to glycine betaine (N,N,N-trimethylglycine) 	
Gate location ?GATE1 – Loop adjacent to the active site of choline oxidase - residues 74-85 GATE2 – Located in the tunnel above active site				
Gate structural basis				
	?GATE1 Movement of the loop can open active site			
	GATE2 M62, L65, V355, F357, and M359 Breathing motion of M62, L65, V355, F357, and M359 The weakly hydrophobic interactions between the gating residues ensure that the positively charged substrate can easily slip to the highly electronegative active site The distribution of the residues just outside the five gating residues contains a considerable amount of negatively charged amino acids, which include E63, E66, E358, and E370, which may attract and guide the positively charged choline substrate to the active site			
Gate engineering			PDB ID: 2JBV	
PDB: WT – 2JBV			Tunnel	
References: ³¹			Length	
			Bottleneck	2.5
			Role	Substrate transport

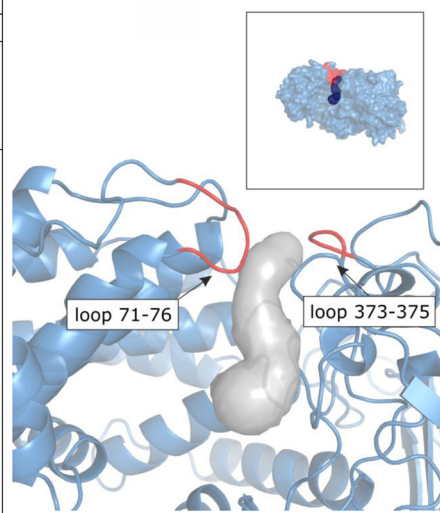

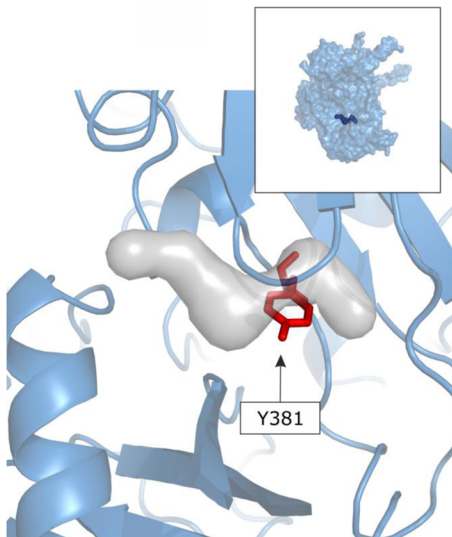

23	Chondroitin AC Lyase		4.2.2.5	
Gate function GATE1 – Creates tunnel and the active site, controls access of the solvent			Enzyme function Degradation of glycosaminoglycans 	
Gate location GATE1 – N-terminal domain and C-terminal domain				
Gate structural basis				
	GATE1 Two loops Loops are flexible and open periodically allowing the glycosaminoglycan chain to slide in; Gating involves the movement of the tips of one or two loops only, D71-W76 of the N-terminal domain and G373-K375 of the C-terminal domain			
Gate engineering				
PDB: WT – 1CB8, 1HM2, 1HMU, 1HM3, 1HMW			PDB ID: 1CB8	
References: ¹⁵¹			Tunnel	
			Length	
			Bottleneck	
			Role	Active site cavity

Table 3. continued

24	Copper – Containing Amine Oxidase		1.4.3.21
Gate function GATE1 – Blocks the back side of the active site from solvent access		Enzyme function Oxidation of primary amines to aldehydes reducing molecular oxygen to hydrogen peroxide	
Gate location GATE1 – Amine tunnel			
Gate structural basis			
	GATE1 Y381 in amine oxidase ECAO W156 in amine oxidase HCAO Y296 in amine oxidase AGAO F298 in amine oxidase PSAO Gating residues can form a π/π ring stacking interaction with the pyridine ring of the cofactor (TPQ)		
Gate engineering			
PDB: <i>WT (ECAO)</i> – 1OAC, <i>WT (HCAO)</i> – 2OQE, 2OOV, <i>WT (AGAO)</i> – 1RJO, 1RKY, <i>WT (PSAO)</i> – 1W2Z		PDB ID: 1OAC	
References: 88,152–154		Tunnel	
		Length	
		Bottleneck	
		Role	

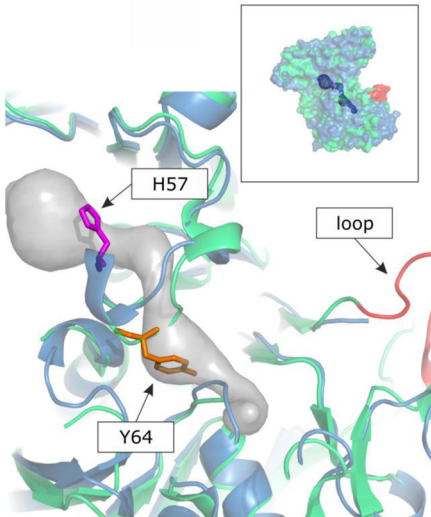



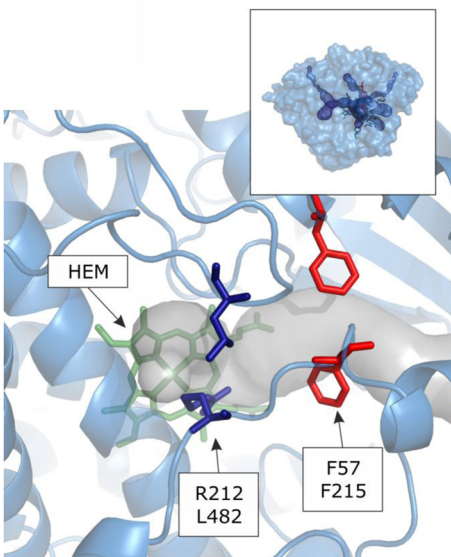

25	Cytidine Triphosphate Synthetase CTPS		6.3.4.2
Gate function GATE1 – Creates tunnel, protects tunnel from access of the solvent ?GATE2 – Controls passage of ammonia to the amidotransferase active site GATE3 – Controls passage of ammonia to the amidotransferase active site		Enzyme function Catalyses the formation of CTP from UTP, ATP and glutamine	
Gate location GATE1 – Loop of the glutaminase domain ?GATE2 – The domain interface, interacts with the amide group of the bound glutamine GATE3 – Between UTP site and the tunnel exit			
Gate structural basis			
	GATE1 (PDB ID: 1VCM) Loop The synthetase active site is exposed to the solvent and the binding pocket for the allosteric effector GTP is not properly formed After ATP and acceptor binding loop covers the entrance of the glutaminase site shielding glutamine and creates the tunnel		
	?GATE2 (PDB ID: 1VCM) Y64 Y64 might act as a door to the ammonia tunnel leading to the synthetase site		
	GATE3 (PDB ID: 2ADP) H57 1. Binding of the substrate UTP induces the rotation of H57 2. Rotation of H57 opens the tunnel for ammonia passage The ligand-induced change is postulated to regulate the timing for the translocation of ammonia		
Gate engineering		PDB ID: 1VCM – Light green PDB ID: 2AD5 – Blue Tunnel calculated for PDB ID: 2AD5 structure	
		Tunnel between active sites	
		Length	25
PDB: <i>WT</i> – 1VCM, 1VCN, 1VCO, 2AD5, 1S1M		Bottleneck	0.8-1.2
References: 52,155–157		Role	NH ₃ transport between active sites

Table 3. continued

26		Cytochrome P450 CYP3A4				1.14.13.32																											
Gate function Controls access to the active site, controls selectivity and specificity of the enzyme		Enzyme function Catalyses mono-oxygenation reactions such as hydroxylation and epoxidation, major drug metabolizing enzyme in humans																															
Gate location Depends on the P450 isoform, tunnel and the gate																																	
Gate structural basis Common gating mechanisms in cytochrome P450 family are: (i) associated with F-G-helix-loop-helix or B-C loop movement (tunnels 2a, 2ac, 2b, 2c, 2e); (ii) associated with swinging door type; (iii) associated with wing type; (iv) cofactor assisted (CPR) For details of other cytochrome P450 gates see references ^{16,59,90}																																	
<div><div><div>CYP3A4</div><div></div></div><div><div>π-stacking, aliphatic contact, H-bond, salt bridge, protein conformation changes</div><div>Tunnel</div><div>Proposed gating residues for product release</div><div>TMZ</div><div>TST-OH</div><div>2a</div><div>F57-F215</div><div>F57-F215</div><div>2b</div><div>F108-F220</div><div>F108-F220</div><div>2c</div><div>F108-F241</div><div>F108-F241</div><div>2e</div><div>F108-I120</div><div>R105-S119</div><div>3</div><div>F213-F241</div><div>F213-V240</div><div>S</div><div>R212-L482</div><div>R212-L482</div><div>W</div><div>R375 + CPR protein</div><div>Mechanism of gates opening: (i) breaking F-F π-stacking (ii) breaking aliphatic contacts between R212 and L482 - S tunnel (iii) breaking the H bond, as observed during the exit of TST-OH through tunnel 2e between R105 and S119 (iv) opening of water tunnel when the FMN domain of CPR binds to CYP3A4 via R375 rotation.</div></div></div>																																	
Gate engineering F108W, I120W – closure of tunnel 2e, switching of the midazolam entrance to another tunnel that may lead to a different active site orientation and product formation																																	
PDB: <i>WT</i> – 1TQN (CYP3A4), 1AKD (P450cam), 2D09 (CYP152A2), 1JPZ (P450-BM3), 1PQ2 (CYP2C8), 1IZO (CYP152A1), 1F4T (CYP119), 1NR6 (CYP2C5), 1OXA (P450eryF)		PDB ID: 1TQN - for clarity only gate of tunnel 2a and tunnel S are shown																															
References: ^{10,16,25,55,56,58,59,90,158,159}		<table><tr><th>Tunnel</th><th>2a</th><th>2b</th><th>2c</th><th>2e</th><th>3</th><th>S</th><th>W</th></tr><tr><td>Length</td><td>19</td><td>18</td><td>17</td><td>12</td><td>17</td><td>13</td><td></td></tr><tr><td>Bottleneck</td><td>4.1</td><td>4.0</td><td>3.9</td><td>4.2</td><td>4.1</td><td>4.5</td><td></td></tr></table>		Tunnel	2a	2b	2c	2e	3	S	W	Length	19	18	17	12	17	13		Bottleneck	4.1	4.0	3.9	4.2	4.1	4.5		<table><tr><td>Role</td><td>Different products pathways</td><td>Proton access/ water/substrate</td><td>Water</td></tr></table>		Role	Different products pathways	Proton access/ water/substrate	Water
Tunnel	2a	2b	2c	2e	3	S	W																										
Length	19	18	17	12	17	13																											
Bottleneck	4.1	4.0	3.9	4.2	4.1	4.5																											
Role	Different products pathways	Proton access/ water/substrate	Water																														

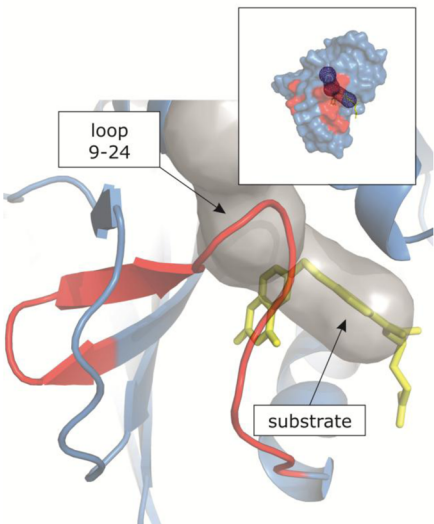

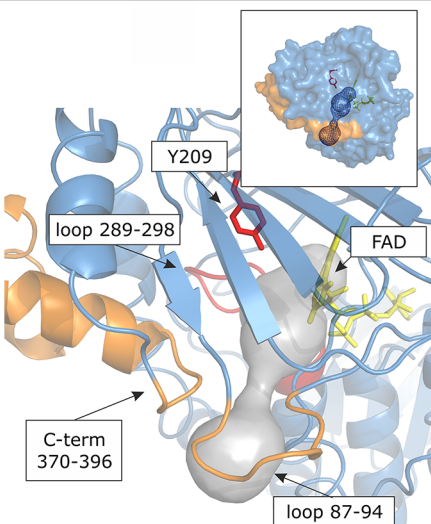

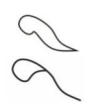
27		Dihydrofolatereductase				1.5.1.3	
Gate function GATE1 – Controls the activity		Enzyme function Catalyses the stereospecific reduction of dihydrofolate to tetrahydrofolate					
Gate location GATE1 – Entrance to the cofactor cavity							
Gate structural basis							
<div><div></div><div>GATE1 M20 loop (residues 9 to 24) In closed conformation the loop packs tightly against the nicotinamide ring of the cofactor In occluded conformation the loop projects into the active site and sterically blocks the cofactor cavity</div></div>							
Gate engineering N23PP – k_{cat} decreased 5-fold S148A – k_{cat} decreased 2-fold N23PP/S148A – k_{cat} decreased 6-fold							
PDB: <i>WT</i> – 1RX2, Mutants – 3QL0, 3QL3		PDB ID: 1RX2		Tunnel			
References: ^{80,81,160–162}		Length					
		Bottleneck					
		Role					

Table 3. continued

28		Digeranylgeranyllycerophospholipid Reductase DGGR		1.3.1	
Gate function GATE1 – Opens tunnel, controls entry of the substrate, controls reduction of FAD GATE2 – Controls substrate binding/release			Enzyme function Converts 2,3-di-O-geranylgeranylglyceryl phosphate to 2,3-di-O-phytanylglyceryl phosphate (archaeidic acid)		
Gate location GATE1 – Vicinity of the cofactor binding site (FAD) GATE2 – C-terminal helical subdomain					
Gate structural basis					
	GATE1 Glycine-rich α I– α J loop (residues 289–298) Cofactor FAD has two conformation IN (not accessible by solvent) and OUT (exposes the soalloxazine ring allowing it to be reduced by NADH (or NADPH). In IN conformation tunnel A is blocked, to open it has to turn into OUT conformation. The conformational changes in the glycine-rich α I– α J loop disrupt interaction between Y209 and T292 allowing change of the FAD position. Conserved RxxFD and LxGD motifs may play a role in FAD's IN/OUT conformational switch.				
	GATE2 Two regions, the β 6– β 7 loop (residues 87–94) and the C-terminal helices (residues 370–396) The conformation changes of the C-terminal helical subdomain may be involved in substrate binding/release				
Gate engineering			PDB ID: 3OZ2		
PDB: <i>WT</i> – 3OZ2			Tunnel	A	B
References: ¹⁰⁰			Length		
			Bottleneck		
			Role		

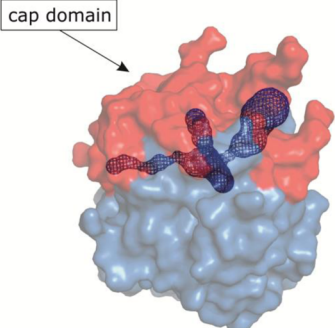

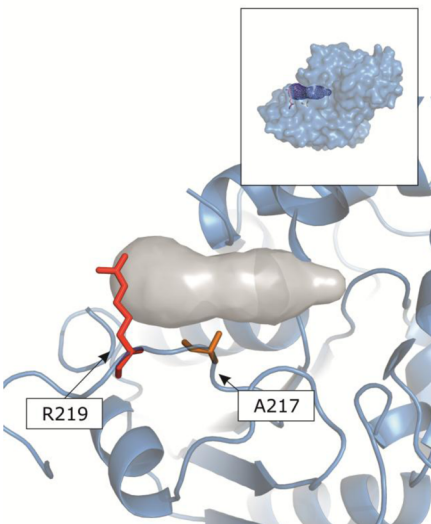


29		Epoxide Hydrolase H37Rv		3.3.2.3	
Gate function GATE1 – Regulates access to the active site			Enzyme function Hydrolysis of epoxides		
Gate location GATE1 – Cap domain					
Gate structural basis					
	GATE1 (<i>Mycobacterium tuberculosis</i> H37Rv) Movement of the cap domain regulates access to the active site				
Gate engineering					
PDB: <i>WT</i> (<i>Aspergillus niger</i> M200) – 1QO7			PDB ID: 2E3J		
References: ⁶⁸			Tunnel		
			Length		
			Bottleneck		
			Role		

Table 3. continued

30	Epoxide Hydrolase M200	3.3.2.3												
Gate function ?GATE1 – Controls enantioselectivity and activity ?GATE2 – Controls enantioselectivity and activity		Enzyme function Hydrolysis of epoxides												
Gate location ?GATE1 – Tunnel entrance ?GATE2 – Middle of the tunnel														
Gate structural basis														
 ?	?GATE1 (<i>Aspergillus niger</i> M200) R219													
 ?	?GATE2 (<i>Aspergillus niger</i> M200) A217													
Gate engineering <i>Aspergillus niger</i> M200 A217C, A217E, A217G, A217L, A217P, A217Q, A217R, A217T, A217V – at the entrance to the tunnel result in different enantioselectivity and activity; e.g.: A217G mutation results in a 33-fold decrease of activity A217V 6,6-fold increase of activity with no changes in the products enantioselectivity for the reaction with allyl glycidyl		PDB ID: 1QO7												
PDB: WT (<i>Aspergillus niger</i> M200) – 1QO7		<table><tr><td>Tunnel</td><td>C</td><td>N terminal cleft (not shown)</td></tr><tr><td>Length</td><td>25</td><td>15</td></tr><tr><td>Bottleneck</td><td></td><td></td></tr><tr><td>Role</td><td>Substrate binding cavity</td><td>sEH N terminal cleft</td></tr></table>	Tunnel	C	N terminal cleft (not shown)	Length	25	15	Bottleneck			Role	Substrate binding cavity	sEH N terminal cleft
Tunnel	C	N terminal cleft (not shown)												
Length	25	15												
Bottleneck														
Role	Substrate binding cavity	sEH N terminal cleft												
References: ^{26,163}														

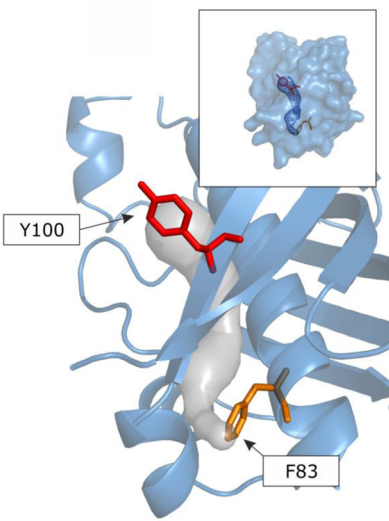


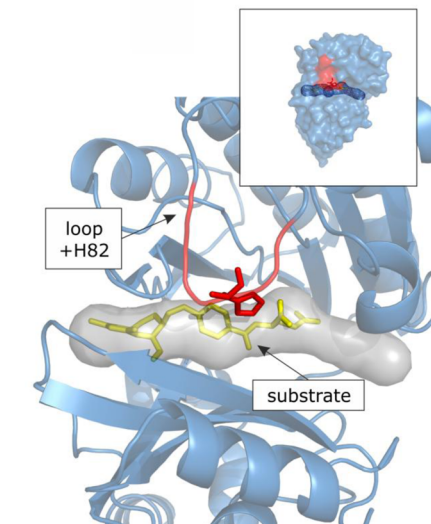

31	FabZ β -Hydroxyacyl-Acyl Carrier Protein Dehydratase	4.2.1.59								
Gate function GATE1 – Controls access of the substrate, prohibits binding of small hydrophobic molecules to the unliganded enzyme GATE2 – Controls length of the substrate		Enzyme function Elongation cycles of both saturated and unsaturated fatty acids biosyntheses in the type II fatty acid biosynthesis system (FAS II) pathway								
Gate location GATE1 – Entrance to the tunnel GATE2 – Exit (back door) of the tunnel										
Gate structural basis										
 —	GATE1 Y100 (Y88 in PaFabZ and L170 in PfFabZ) adopts either an open or closed conformation in the crystal structure In closed conformation - Y100 is stabilized by the Van der Waals interactions with M102, M154, and P112; in open one it flops ~120° and is stabilized by the Van der Waals interactions with M154, K62, and I64									
 —	GATE2 F83 Refined in two additional alternative conformations, leading the tunnel to form a L-shape or an U-shape, in closed conformation - F83 points toward I98, in open position it rotates ~120° points toward I93, exposing the exit to the bulk solvent T85 or F74 play a similar role in PaFabZ									
Gate engineering Y100A – drops the activity of the mutant to less than 50% of the enzymatic activity of the wild type, the new entrance has ~15 Å in width, completely exposes the active site to the bulk solvent – as a results ACP binds to the mutant more stronger than to the wild-type. In particular, the dissociation step of ACP from the HpFabZ mutant is extremely slow.		PDB ID: 2GLL								
PDB: WT (<i>HpFabZ</i>) – 2GLL, 2GLP, 2GLM Mutant Y100A – 2GLV, WT (<i>PfFabZ</i>) – 1ZHG, WT (<i>PcFabZ</i>) – 1U1Z		<table><tr><td>Tunnel</td><td></td></tr><tr><td>Length</td><td>20</td></tr><tr><td>Bottleneck</td><td></td></tr><tr><td>Role</td><td>Substrate binding tunnel, ACP binding groove</td></tr></table>	Tunnel		Length	20	Bottleneck		Role	Substrate binding tunnel, ACP binding groove
Tunnel										
Length	20									
Bottleneck										
Role	Substrate binding tunnel, ACP binding groove									
References: ^{54,164,165}										

Table 3. continued

32	Formiminotransferase-Cyclodeaminase FTCD		2.1.2.5												
Gate function GATE1 – Controls position of H82 in proper orientation for reaction			Enzyme function Two independent, but sequential reactions in the histidine degradation pathway 												
Gate location GATE1 – Blocks entrance from a short tunnel into active site															
Gate structural basis															
	GATE1 Loop rearrangement including a change in the histidine H82 side-chain position														
Gate engineering															
PDB: WT – 1QD1			PDB ID: 1QD1												
References: ⁹⁴			<table><tr><td>Tunnel</td><td>Main</td><td>Second</td></tr><tr><td>Length</td><td>38</td><td>9</td></tr><tr><td>Bottleneck</td><td>8</td><td>4</td></tr><tr><td>Role</td><td>Intermediate transport</td><td>Formiminoglutamate substrate entry, glutamate product exit</td></tr></table>	Tunnel	Main	Second	Length	38	9	Bottleneck	8	4	Role	Intermediate transport	Formiminoglutamate substrate entry, glutamate product exit
Tunnel	Main	Second													
Length	38	9													
Bottleneck	8	4													
Role	Intermediate transport	Formiminoglutamate substrate entry, glutamate product exit													

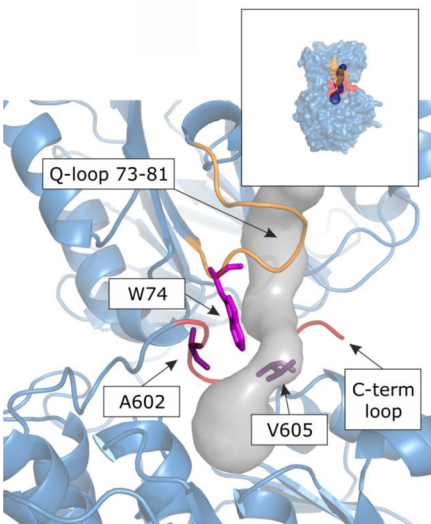



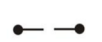
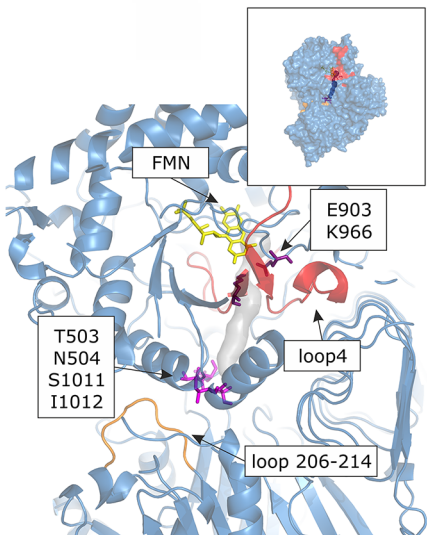




33	Glucosamine 6 Phosphate Synthase GlnS		2.6.1.16								
Gatefunction GATE1 – Protects Fru 6P site from the solvent, creates the ammonia tunnel GATE2 – Protects glutaminase active site from the solvent GATE3 – Opens ammonia tunnel, ammonia enters the sugar site, acts as a solvent barrier GATE4 – Opens ammonia tunnel			Enzyme function Catalyses glucosamine-6P synthesis from fructose-6P and glutamine 								
Gate location GATE1 – Part of the active site cavity GATE2 – Part of the active site cavity GATE3 – Tunnel between two active sites GATE4 – Bottleneck of the tunnel between active sites											
Gate structural basis											
	GATE1 C-terminal loop (residues 600–608) Open state – relaxed loop Closed state – anchored by Y28 and W74 with Q loop										
	GATE2 Q-loop (residues 73–81) Open state - anchored by R539*, Close state - after Q enters the active site anchored by Y28 and W74 with C-terminal loop										
	GATE3 W74 x1 torsion angle of W74 changes by 75°										
	GATE4 A602 and V605 A shift of the side-chain of the A602 residue is concomitant to a re-orientation of its backbone carbonyl group, which can then form a strong H-bond with the hydroxyl group of the Y28										
Gate engineering W74A, W74L, W74F – inefficient ammonia transfer A602L, V605L – efficiency of ammonia transfer decreased 2-fold			PDB ID: 2BPL								
PDB: WT – 2J6H, 1JXA, 2VF4, 2BPL			<table><tr><td>Tunnel</td><td></td></tr><tr><td>Length</td><td>18</td></tr><tr><td>Bottleneck</td><td></td></tr><tr><td>Role</td><td>Ammonia transport</td></tr></table>	Tunnel		Length	18	Bottleneck		Role	Ammonia transport
Tunnel											
Length	18										
Bottleneck											
Role	Ammonia transport										
References: ^{14,45,50,155,166,167}											

Table 3. continued

34	Glutamate Synthases GltS	1.4.7.1	
Gate function GATE1 – Controls correct conformation of active site for L-glutamine binding and hydrolysis, crucial for glutaminase activation and coupling of the glutaminase and synthase sites, creates ammonia tunnel GATE2 – Shields substrate from bulk solvent GATE3 & GATE4 – Controls transport of ammonia		Enzyme function Formation of 2 L-glutamate from L-glutamine and 2-oxoglutarate	
Gate location GATE1 – Loop protecting Fru 6P site from solvent GATE2 – Loop protecting glutaminase site from solvent GATE3 – Entrance to the interdomain tunnel GATE4 – Entrance to the synthase site (end of the tunnel)			
Gate structural basis			
	GATE1 Loop 4 (residues 933–978) Shifts to the active conformation – C-terminal residue E1013 forms a hydrogen bond with C1 and keeps correct conformation of C1 and loop 29–34 for L-glutamine binding and hydrolysis. E1013 side chain may also play a role in the precise geometry of the tunnel entry point		
	GATE2 Loop 206–214 Closing of loop 206–214 after L-glutamine binding		
	GATE3 T503, N504, S1011 and I1012 Small conformation changes of T503, N504 of the central domain, S1011 and I1012 of loop 4		
	GATE4 E903 and K966 Small conformation changes of E903 and K966 Additionally residues T507 and N508 and S976 and I977 may function as a gates for signalling between active sites	PDB ID: 1OFD – GATE3 closed	
Gate engineering E1013D – 100-fold decrease of activity, a sigmoid dependence of initial velocity on L-glutamine concentration E1013N – 1000-fold decrease of activity, exhibited hyperbolic kinetics E1013A – 1000-fold decrease of activity		Tunnel	
PDB: <i>WT</i> – 1OFD, 1OFE, 1EA0		Length	33
References: 46,62,155,168		Bottleneck	
		Role	NH ₃ transport, prevents protonation of NH ₃

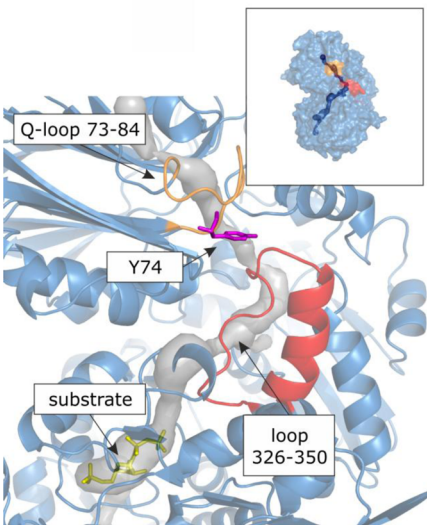



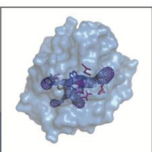





35	Glutamine Phosphoribosylpyrophosphate Amidotransferase	2.4.2.14	
Gate function GATE1 – Closes synthase site and creates a narrow, solvent-inaccessible tunnel between active sites GATE2 – Covers glutaminase site GATE3 – Protects tunnel from the solvent access and avoids wasteful release of ammonia into solution		Enzyme function Catalyses the initial reaction in <i>de novo</i> purine nucleotide biosynthesis	
Gate location GATE1 – Loop near the synthase active site GATE2 – Loop near the glutaminase active site GATE3 – On Q-loop			
Gate structural basis			
	GATE1 Loop (residues 326–350) The active site cavity is created by closure of the loop (residues 326–350) Closing of the active site creates a narrow, solvent-inaccessible tunnel between active sites I335 interacts with Y74		
	GATE2 Q-loop (residues 73–84)		
	GATE3 Y74 A key residue in coupling the glutamine and acceptor sites upon acceptor binding may play a similar gate role as W74 in GlmS		
Gate engineering		PDB ID: 1ECC	
		Tunnel between two active sites	
PDB: <i>WT</i> – 1ECF, 1ECC		Length	20
References: 155,169,170		Bottleneck	
		Role	Ammonia transport

Table 3. continued

36	Haloalkane Dehalogenase DhaA	3.8.1.5																
Gate function GATE1 , GATE2 , GATE3 , GATE4 , GATE5 – Controls access to the tunnel		Enzyme function Hydrolytic dehalogenation of various halogenated aliphatic hydrocarbons 																
Gate location GATE1 –Tunnel p1, GATE2 –Tunnel p2a, GATE3 –Tunnel p2b, GATE4 –Tunnel p2c, GATE5 –Tunnel p3																		
Gate structural basis																		
	GATE1 F144 and F149																	
	GATE2 I135																	
	GATE3 V245, W141																	
	GATE4 β-bridge interaction between P210 and A212 of the CC loop and I135 of the NC loop																	
	GATE5 W138 blocks tunnel p3																	
Gate engineering C176Y, V245F, A172F, A145F – limiting access of water to the active site		PDB ID: 1CQW																
PDB: WT (DhaA) – 1CQW, 1BN6, 1BN7		<table><tr><td>Tunnel</td><td>p1</td><td>p2a, p2b, p2c</td><td>p3</td></tr><tr><td>Length</td><td>20</td><td>22</td><td>24</td></tr><tr><td>Bottleneck</td><td>2.8</td><td>1.8</td><td>1.6</td></tr><tr><td>Role</td><td>Product</td><td>Product</td><td>Product</td></tr></table>	Tunnel	p1	p2a, p2b, p2c	p3	Length	20	22	24	Bottleneck	2.8	1.8	1.6	Role	Product	Product	Product
Tunnel	p1	p2a, p2b, p2c	p3															
Length	20	22	24															
Bottleneck	2.8	1.8	1.6															
Role	Product	Product	Product															
References: ^{109,110}																		

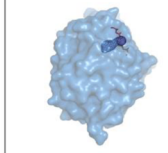


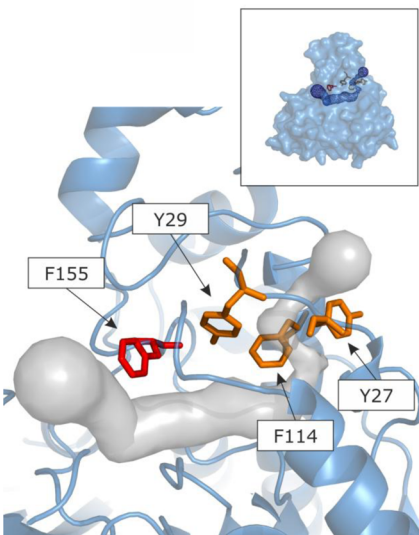



37	Haloalkane Dehalogenase LinB	3.8.1.5									
Gate function GATE1 – Controls access to the active site GATE2 – Controls selectivity		Enzyme function Hydrolytic dehalogenation of various halogenated aliphatic hydrocarbons 									
Gate location GATE1 – Tunnel bottleneck GATE2 – Tunnel bottleneck											
Gate structural basis											
	GATE1 D147 Motion of this residues is necessary for entry of large substrates										
	GATE2 L177 Partially blocks the entrance of the main tunnel										
Gate engineering L177A, L177C, L177G, L177F, L177K, L177T, L177W, L177D, L177H, L177M, L177Q, L177R, L177S, L177V, L177Y influence the substrate specificity and activity			PDB ID: 1CV2								
PDB: WT (LinB) – 1CV2			<table><tr><td>Tunnel</td><td>p1</td></tr><tr><td>Length</td><td>12</td></tr><tr><td>Bottleneck</td><td>2.6</td></tr><tr><td>Role</td><td>Product</td></tr></table>	Tunnel	p1	Length	12	Bottleneck	2.6	Role	Product
Tunnel	p1										
Length	12										
Bottleneck	2.6										
Role	Product										
References: ^{72,108,171}											

Table 3. continued

38	Histone Deacetylase HDAC1 and HDAC2		3.5.1.98																
Gate function GATE1 – Controls access to the active site through tunnel A GATE2 – Controls access to the active site through tunnel B1 GATE3 – Controls access to the active site through tunnel B2 (only HDAC1)		Enzyme function Modifies chromatin structures by removing the acetyl group from the ε-amino lysine residues																	
Gate location GATE1 – Bottleneck of tunnel A GATE2 – Bottleneck of tunnel B1 GATE3 – Bottleneck of tunnel B2 (only HDAC1)		 <p>PDB ID: 3MAX</p> <table><tr><td>Tunnel</td><td>A</td><td>B1</td><td>B2</td></tr><tr><td>Length</td><td>11</td><td>14</td><td>16</td></tr><tr><td>Bottleneck</td><td></td><td></td><td></td></tr><tr><td>Role</td><td>Access to the active site</td><td>Access to the active site</td><td>Water exchange</td></tr></table>		Tunnel	A	B1	B2	Length	11	14	16	Bottleneck				Role	Access to the active site	Access to the active site	Water exchange
Tunnel	A			B1	B2														
Length	11			14	16														
Bottleneck																			
Role	Access to the active site	Access to the active site	Water exchange																
Gate structural basis																			
	GATE1 F155 (F150) Side chain of F155 has to rotate ~ 180°																		
	GATE2 F114 + Y27 and Y29 (F109 + Y22 and Y24) F114 residue is located between two tyrosine rings in closed conformation Opening requires unzipping of the gate																		
	GATE3 Y303 Observed only in HDAC1 - Y303 residue may rotate opening access through tunnel – anchoring residue in closed position M30																		
Gate engineering																			
PDB: WT (HDAC2) – 3MAX																			
References: ¹⁷²																			

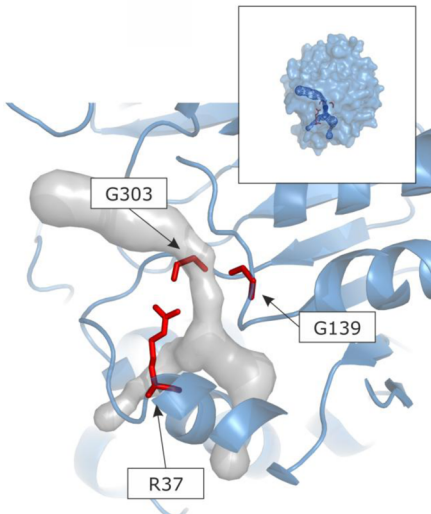

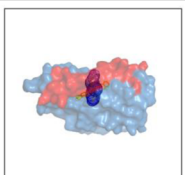
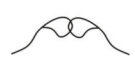
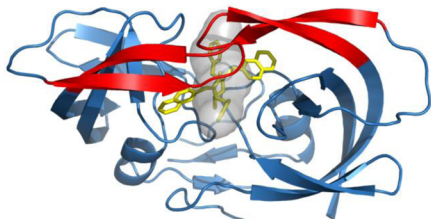
39	Histone Deacetylase HDAC8		3.5.1.98												
Gate function GATE1 – Regulates water or product (acetate) transit from the active site through the internal tunnel		Enzyme function Deacetylation of the ε-amino group of specific lysine residues within histones and other proteins													
Gate location GATE1 – Centre of the internal tunnel		 <p>PDB ID: 2V5W</p> <table><tr><td>Tunnel</td><td></td><td></td></tr><tr><td>Length</td><td>11</td><td>14</td></tr><tr><td>Bottleneck</td><td></td><td></td></tr><tr><td>Role</td><td colspan="2">Access to active site</td></tr></table>		Tunnel			Length	11	14	Bottleneck			Role	Access to active site	
Tunnel															
Length	11			14											
Bottleneck															
Role	Access to active site														
Gate structural basis															
	GATE1 R37 A structural reorientation of R37 and the loop is required for opening the access to the active site via the 14 Å ‘internal’ tunnel. R37 forms multiple hydrogen bond interactions with the backbone carbonyl oxygen atoms of conserved G303 and G305 positioned in a loop between the β8 and α10-helix Gating interaction between G139 and G303														
Gate engineering R37A – the values for k_{cat}/K_M decrease 530-fold															
PDB: WT – 2V5W															
References: ¹⁷³															

Table 3. continued

40	HIV-1 Protease	3.4.23.16
Gate function GATE1 – Controls access to the active site		Enzyme function Central role in processing HIV-1 viral polypeptide precursors 
Gate location GATE1 – β -turn flaps		
Gate structural basis		
	GATE1 β -turn flaps (residues 43–58) Movement of two β -turn flaps controls access to the active site	
Gate engineering WT – open 14% of time (Brownian dynamics simulations) G48V/V82A – open 2% of time (Brownian dynamics simulations) I84V/L90M – open 2% of time (Brownian dynamics simulations) L90M, G48V – open 14% of time (Brownian dynamics simulations) F53L – unstabilised semi open conformation due to lack of F53-I50 interaction		
PDB: <i>WT</i> – 1HHP, 1HVR		 PDB ID: 1HVR
References: ^{79,89,174–177}		Tunnel
		Length
		Bottleneck
		Role Active site gorge

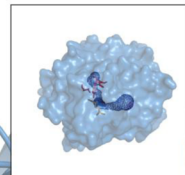
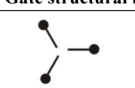
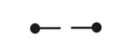
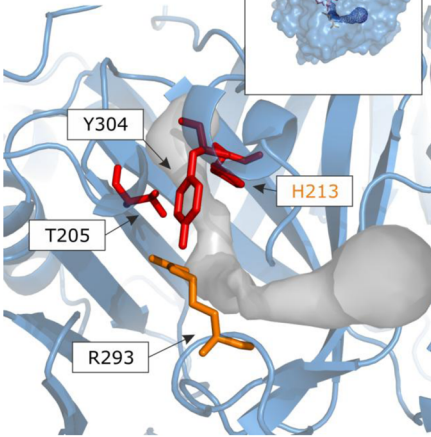

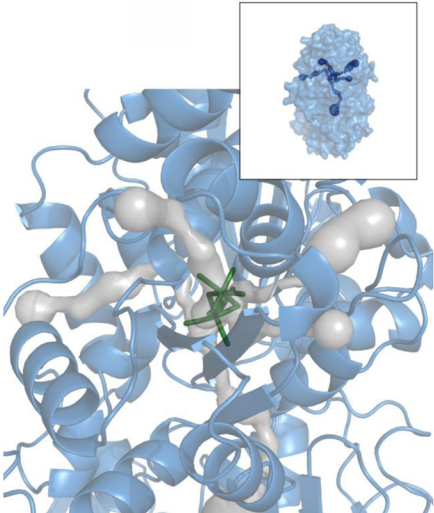
41	Homoprotocatechuate 2,3-Dioxygenase	1.13.11.15
Gate function GATE1 – Controls O ₂ diffusion pathway GATE2 – Controls alternative O ₂ diffusion pathway		Enzyme function Degradation of catechol and its derivatives by cleavage of aromatic rings 
Gate location GATE1 – Just below the protein surface GATE2 – Below the protein surface		
Gate structural basis		
	GATE1 T205, H213, and W304 Breathing motion of protein causes synchronizing movement of residues	
	GATE2 R293, H213 The fluctuation of R293 along with H213 could result in opening of the pathway	
Gate engineering		 PDB ID: 2IG9
PDB: <i>WT</i> – 2IGA, 2IG9		
References: ⁶³		
		Tunnel
		Length
		Bottleneck
		Role O ₂ transport

Table 3. continued

42	Hydrogenase FeFe	1.12.7.2								
Gate function Controls access of oxygen and hydrogen to the active site		Enzyme function Catalyses the reversible oxidation of molecular hydrogen								
Gate location Not specified, between dynamic cavities										
Gate structural basis										
	No permanent tunnel – O ₂ moves from cavity to cavity as the cavities fluctuate inside the protein									
Gate engineering										
PDB: <i>WT</i> – 1HFE, 1FEH		 <p>PDB ID: 1FEH Representative tunnels connecting internal cavities calculated with probe radius 0.6 Å</p> <table><tr><td colspan="2">Tunnel</td></tr><tr><td>Length</td><td>30 – 35</td></tr><tr><td>Bottleneck</td><td>2</td></tr><tr><td>Role</td><td>H₂ and O₂ transport</td></tr></table>	Tunnel		Length	30 – 35	Bottleneck	2	Role	H ₂ and O ₂ transport
Tunnel										
Length	30 – 35									
Bottleneck	2									
Role	H ₂ and O ₂ transport									
References: ^{88,178–180}										


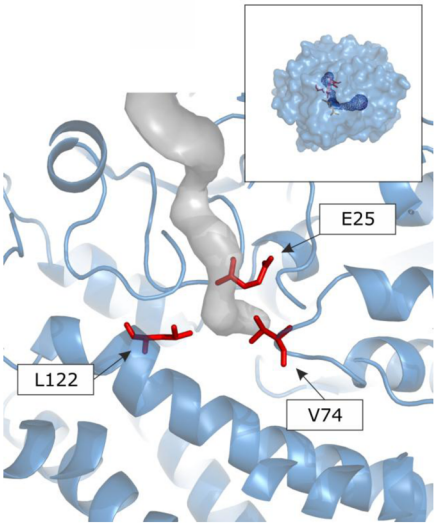
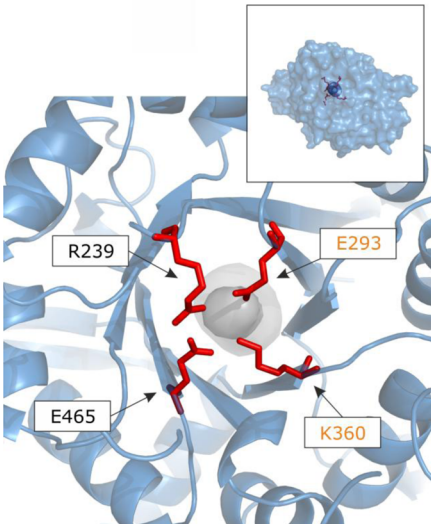


43	Hydrogenase NiFe	1.12.2.1								
Gate function ?GATE1 – Protects the active site against O ₂		Enzyme function Catalyses the reversible oxidation of molecular hydrogen								
Gate location ?GATE1 – Vicinity of NiFe cluster, bottleneck close to the active site										
Gate structural basis										
	?GATE1 E25, V74 and L122 The size of the amino acids at positions 122 and/or 74 may determine the accessibility of the active site and therefore the resistance to O ₂									
Gate engineering V74Q, V74M, V74E, V74N, V74W, V74F, V74D, L122M/V74M, L122F/V74I, L122A/V74M – two contributions, size and polarity, are independent and have different effects to H ₂ , CO, O ₂ molecules, different influences on reaction rate and inhibition effect										
PDB: <i>WT</i> – 1YQW <i>Mutants</i> – 3CUR, 3CUS, 3H3X		 <p>PDB ID: 1YQW</p> <table><tr><td colspan="2">Tunnel</td></tr><tr><td>Length</td><td>30</td></tr><tr><td>Bottleneck</td><td></td></tr><tr><td>Role</td><td>O₂ transport</td></tr></table>	Tunnel		Length	30	Bottleneck		Role	O ₂ transport
Tunnel										
Length	30									
Bottleneck										
Role	O ₂ transport									
References: ^{21,22}										

Table 3. continued

44	Imidazole Glycerol Phosphate Synthase IGPS	2.4.2
Gate function GATE1 – Prevents penetration of bulk water molecules into chambers I and II GATE2 – Controls ammonia transfer, discriminates between ammonia and water		Enzyme function Catalyses the closure of the imidazole ring within histidine biosynthesis and provides 5-aminoimidazole -4-carboxamide ribotide (AICAR) for use in the <i>de novo</i> synthesis of purines
Gate location GATE1 – Near the entrance of the (β α) ₈ barrel of hisF GATE2 – Between chamber I and chamber II		
Gate structural basis		
	GATE1 (for water) R5 (R239), E46 (E293), K99 (K360), and E167(E465) numbers from yeast or (<i>Thermotoga maritima</i>) Four strictly conserved gate residues act as the wall barrier for water molecules	
	GATE2 (for ammonia) K99 (K360) and E46 (E293) Conformational change of all residues – high barrier 25 kcal/mol for ammonia transfer K99 (K360) side chain rotation barrier 10 kcal/mol Both K99 and R5 – fully open gate barrier 2 kcal/mol Ammonia enters to chamber II through a side-opening between residues K99 and E46, the only requisite is the slight bending of the side chain of K99 (K360) - eliminates the need for an energetically costly gate-opening mechanism	
Gate engineering Y138F – experimentally no change of kinetics and stoichiometry, in simulations Y138 is not a gate but prevents bulk water from entering the interface during a reaction, keeping ammonia sequestered within the tunnel R5A (R239A) – loss of ammonia through new hole and results in a 10 ³ decrease in <i>k_{cat}</i> / <i>K_M</i> values for the cyclase reaction, in simulations possibly because the mutation allowed water molecules to access chamber II; these additional water molecules increased the energetic barrier to ammonia entry and passage through the tunnel K99A (K360A) – experimentally 3-fold decrease in the overall reaction stoichiometry, in simulations larger opening between chamber I and chamber II, and simultaneously deprives E167 and E46 of a salt-bridge partner T78A – allowed a rapid and unhindered conduction of ammonia through the tunnel T78F, P76F –block the ammonia conduction Any mutation K181 (K196), D98 (D359), and Q123 (Q387) – decouples the two reactions		PDB ID: 1JVN Gate in closed position blocking the tunnel
PDB: <i>WT</i> – 1KA9, 1JVN		Tunnel
References: 38,43,155,181–186		Length
		Bottleneck
		Role
Ammonia transport		

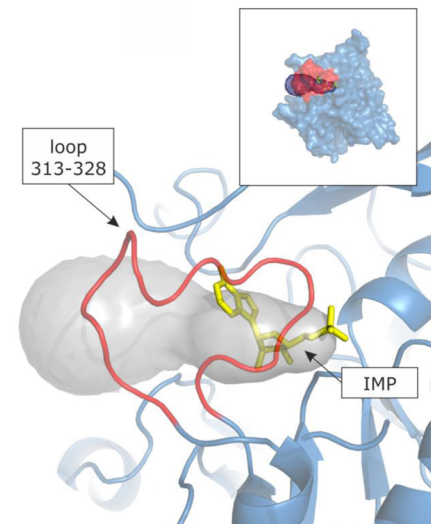

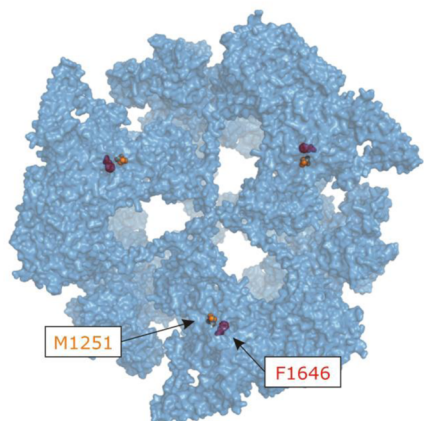


45	Inosine 5'- Monophosphate Dehydrogenase	1.1.1.205
Gate function GATE1 – Conformational change converts the enzyme from a dehydrogenase into hydrolase		Enzyme function Transformation of inosine 5'-monophosphate into xanthosine 5'-monophosphate
Gate location GATE1 – Loop covering active site cavity		
Gate structural basis		
	GATE1 C319 loop (313-328) adopts different conformations during the dehydrogenase and hydrolase reactions	
Gate engineering		
PDB: <i>WT</i> – 1ME9, 1LRT, 1ME8, 1PVN		Tunnel
References: 187		Length
		Bottleneck
		Role

Table 3. continued

46	Ketoacyl Synthase KS		2.3.1.86	
Gate function GATE1 – Shields the active site GATE2 – Controls access of the substrate			Enzyme function Elongates an ACP- or CoA-associated acyl chain by adding C2 units through a ping-pong decarboxylating condensation mechanism	
Gate location GATE1 – Entrance to the active site GATE2 – Centre of the acyl-binding tunnel				
Gate structural basis				
	GATE1 F1646 Shields the active site, flips and allows access to the nucleophilic cysteine			
	GATE2 M1251 rotates and unlocks the inner part of the fatty acid binding cavity			
Gate engineering			PDB ID: 2VKZ	
PDB: <i>WT</i> – 2VKZ			Tunnel	
			Length	
			Bottleneck	
			Role	
References: ¹⁸⁸				

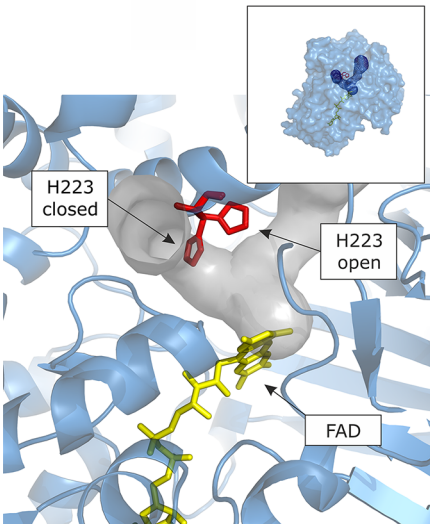


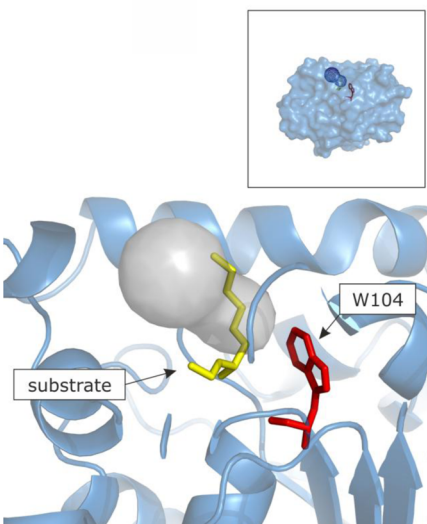
47	L-Amino Acid Oxidase		1.4.3.2		
Gate function GATE1 – Controls access to the oxygen tunnel, binds the substrate initially			Enzyme function Oxidative deamination of L-amino acid substrates		
Gate location GATE1 – 15 Å from surface in main funnel					
Gate structural basis					
	GATE1 H223 H223 has two conformations and can act as a gate and binds the substrate initially H223 conformation A closing oxygen tunnel → substrate entry and deprotonation of the zwitterion → substrate into Michaelis position → H223 turn into conformation B → opening of oxygen tunnel				
Gate engineering					
PDB: <i>WT</i> – 1F8R, 1F8S			PDB ID: 1F8S		
			Tunnel	Main funnel	Y shape tunnel
			Length	25	9
			Bottleneck	4.2	2
References: ^{73,189}			Role	Access to active site	O ₂ access and H ₂ O ₂ release

Table 3. continued

48	Lipase B	3.1.1.3
Gate function (lipase from <i>Candida antarctica</i>) GATE1 – Controls regioselectivity, controls length of the substrate		Enzyme function Hydrolyse triacylglycerols and a broad range of other substrates, important for asymmetric synthesis
Gate location GATE1 – Bottom of the substrate tunnel		
Gate structural basis		
	GATE1 W104 The stereospecificity pocket is defined by T42, S47 and W104 Pocket is buried under a surface helix and delimited by the side chain of W104 <i>R</i> enantiomer of butanoic ester fits well into the active site pocket after a small movement of the side chain of W104	
Gate engineering W104A, W104Q – change in substrate specificity		
Gate creation in other lipases Lipase from <i>Burkholderia cepacia</i> – mutation in bottleneck for increased enantioselectivity – the best double mutant L17S/L287I - 15-fold increased activity and a tenfold enhanced enantioselectivity <i>Candida rugosa</i> lipase 1 – mutation of amino acids in different position inside the tunnel – P246F, L413F, L410W, L410F/S300E, L410F/S365L – different chain length selectivity		
PDB: <i>WT</i> (<i>Candida antarctica</i>) – 1LBS, 3ICV, 3ICW, <i>WT</i> (<i>Burkholderia cepacia</i>) – 3LIP, <i>WT</i> (<i>Candida rugosa</i>) – 1LPO		
References: 86,190–192		PDB ID: 1LBS
		Tunnel
		Length
		Bottleneck
		Role
		Access to active site


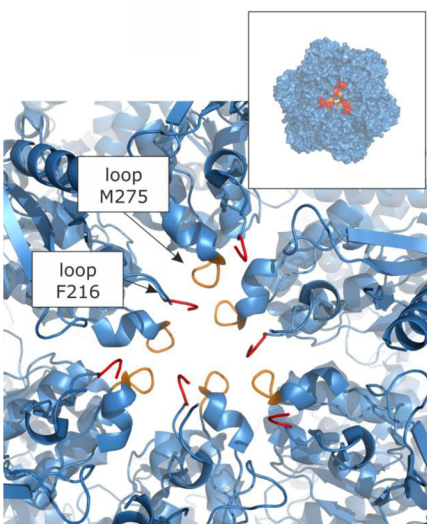

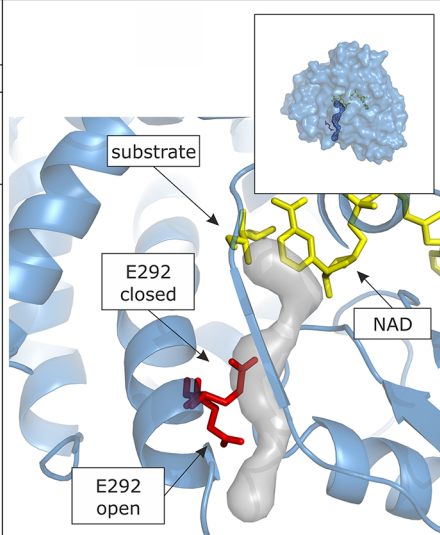

49	Lon Protease	3.4.21.53
Gate function GATE1 – Controls the access of the substrate		Enzyme function ATP-dependent proteolysis
Gate location GATE1 – At the entrance to an internal unfolding and degradation chamber		
Gate structural basis		
	GATE1 F216 loop	
	GATE2 M275 loop	
Gate engineering F216A – lost almost all of the ATP-dependent proteolytic activity against a casein and the aromatic peptide		
PDB: <i>WT</i> – 3K1J		
References: 193		PDB ID: 3K1J
		Tunnel
		Length
		Bottleneck
		Role

Table 3. continued

50		Mannitol 2-Dehydrogenase		1.1.1.67	
Gate function GATE1 – E292 functions as a gate in water chain mechanism of proton translocation			Enzyme function Dehydrogenation of mannitol		
Gate location GATE1 – Bottleneck of the tunnel connecting active site with protein surface					
Gate structural basis					
	GATE1 E292 may adopt two conformation open and closed				
Gate engineering E292A – 120-fold decrease in a rate of microscopic steps preceeding catalytic oxidation of mannitol					
PDB: WT – 1LJ8 (closed), 1M2W (open)			PDB ID: 1M2W (open), 1LJ8 (closed)		
References: ¹⁹⁴			Tunnel		
			Length		
			Bottleneck		
			Role Proton transfer		

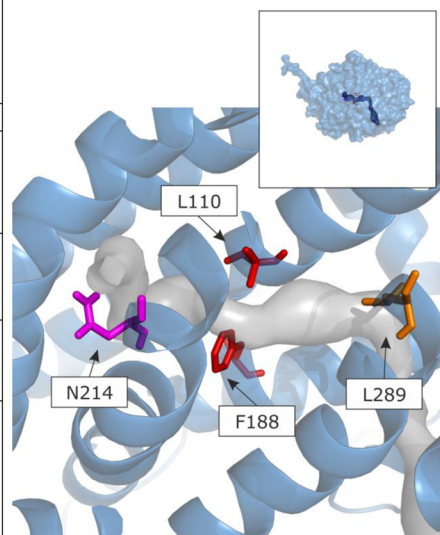



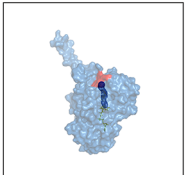

51		Methane Monooxygenase Hydroxylase MMOH		1.14.13.25	
Gate function GATE1 – Controls transport of the substrate between cavities 1 and 2 GATE2 – Allows substrate to traverse the protein cavities GATE3 – Controls entrance of small substrates such as O ₂ , H ₃ O ⁺ , CH ₄			Enzyme function Converts hydrocarbon substrates either to alcohols or epoxides		
Gate location GATE1 – Separates cavities 1 and 2 GATE2 – Separates cavities 2 and 3 GATE3 – One of the residue from active site is positioned at the interface between the cavity and the surface above the iron-coordinating residue					
Gate structural basis					
	GATE1 L110, F188 Conformation changes of L110 and F188 opens the access between the cavities 1 and 2, other residue involved T21				
	GATE2 L289 Moves to allow the substrate analogues to traverse between the cavities 2 and 3, other residues creating the bottleneck F109, V285 and Y291				
	GATE3 N214 Conformation changes – movement of N214 forms a deep crevice in the four-helix bundle		PDB ID: 1MHY		
Gate engineering			Tunnel		
			Length		
			Bottleneck		
			Role		
PDB: WT – 1MHY, 1XVG, 1XVF, 1XVE, 1XVB, 1XVC, 1XU5, 1XVD, 1XU3					
References: ^{53,74,195}					

Table 3. continued

52	Monoamine Oxidase A		1.4.3.4
Gate function GATE1 – Controls the access of the substrate		Enzyme function Oxidative deamination of biogenic amines and amine neurotransmitters (serotonin, dopamine and epinephrine) 	
Gate location GATE1 – At the entrance to the active site cavity			
Gate structural basis			
	GATE1 Loop 99-104 The loop movement is regulating access to the active site cavity and the loop flexibility is critical for opening the entry for substrates/inhibitors		
Gate engineering G110A – increase of K_m of 5-fold G110P – increase of K_m of 19-fold			
PDB: <i>WT</i> – 2Z5X, <i>Mutant G110A</i> – 2Z5Y		PDB ID: 2Z5X	
References: ^{196,197}		Tunnel	
		Length	
		Bottleneck	
		Role	

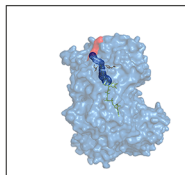


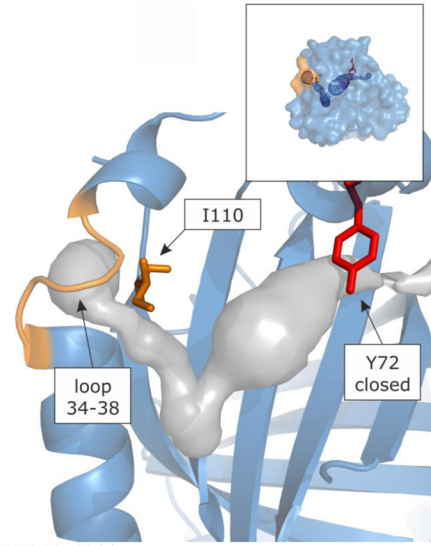


53	Monoamine Oxidase B		1.4.3.4
Gate function GATE1 – Controls the access of the substrate GATE2 – Controls the size of the active site cavity by separating or merging two smaller cavities		Enzyme function Oxidative deamination of biogenic amines and amine neurotransmitters (serotonin, dopamine and epinephrine) 	
Gate location GATE1 – At the entrance to the active site cavity GATE2 – Separates two internal cavities			
Gate structural basis			
	GATE1 Loop 99-104 F103 side chain conformation movement is synchronized with the conformation changes of I199 residues (I199 open → F103 closed)		
	GATE2 I199, Y326 Movement of side chain residues separate or merge the entrance cavity and the substrate cavity		
Gate engineering I199A/Y326A – exhibits inhibitor binding properties more similar to those of monoamine oxidase A		PDB ID: 2V5Z	
PDB: <i>WT</i> – 2V5Z, <i>Mutant I199A/Y326A</i> – 3ZYX		Tunnel	
		Length	
		Bottleneck	
		Role	

Table 3. continued

54		Monooxygenase ActVAOrf6		Not determined													
Gate function GATE1 – Controls access of the substrate, hydrogen bond donor and acceptor/proton gate GATE2 – Controls opening of the narrow tunnel, can also control exit for H ₂ O, O ₂ , H ₂ O			Enzyme function Oxidation of a phenolic compound 6-deoxydihydrokalafungin at the C-6 position into the corresponding quinone dihydrokalafungin														
Gate location GATE1 – Entrance to the active site GATE2 – Opposite side of the bound substrate			 <p>PDB ID: 1LQ9</p>														
Gate structural basis																	
	GATE1 Y72 The residue possess two possible conformation that can act as a gate and act as hydrogen bond donor and acceptor																
	GATE2 I110 + loop (residues 34-38) Conformation changes close and open the narrow tunnel; gate can also control an exit path for H ₂ O																
Gate engineering			<table><tr><td>Tunnel</td><td></td><td></td></tr><tr><td>Length</td><td></td><td></td></tr><tr><td>Bottleneck</td><td></td><td></td></tr><tr><td>Role</td><td>Proton transfer</td><td>Oxygen/water transport</td></tr></table>			Tunnel			Length			Bottleneck			Role	Proton transfer	Oxygen/water transport
Tunnel																	
Length																	
Bottleneck																	
Role	Proton transfer	Oxygen/water transport															
PDB: <i>WT</i> – 1LQ9																	
References: ^{14,92}																	

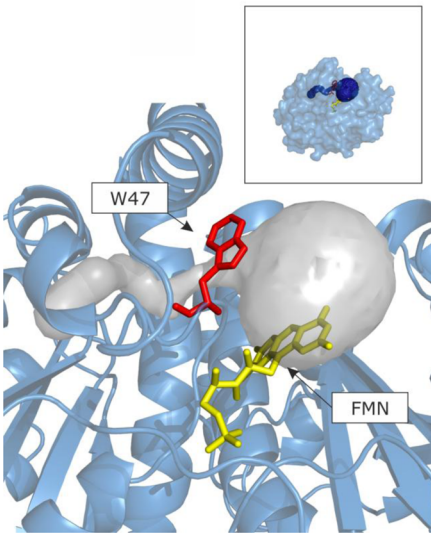

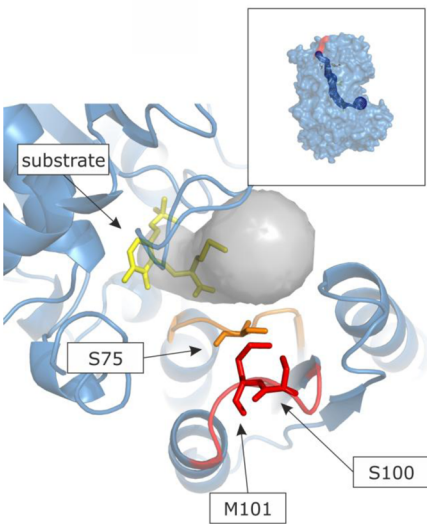


55		NADH Oxidase		1.6.99.3													
Gate function GATE1 – Controls accessibility of the flavin ring and plays a crucial role during the catalytic cycle			Enzyme function Hydride transfer from NADH to the intrinsic flavin cofactor														
Gate location GATE1 – Above the active site			 <p>PDB ID: 1NOX</p>														
Gate structural basis																	
	GATE1 W47 and cofactor FAD Cofactor-assisted gating mechanism, W47 moves from the original position toward the pyrimidine section Closed conformation is stabilized by the hydrogen bonds between cofactor and peptide backbone Stabilization of the open form may have advantages during the initial steps of the substrate binding, it may slow down the product dissociation																
Gate engineering																	
PDB: <i>WT</i> – 1NOX			<table><tr><td>Tunnel</td><td></td><td></td></tr><tr><td>Length</td><td></td><td></td></tr><tr><td>Bottleneck</td><td></td><td></td></tr><tr><td>Role</td><td></td><td></td></tr></table>			Tunnel			Length			Bottleneck			Role		
Tunnel																	
Length																	
Bottleneck																	
Role																	
References: ^{97,199}																	

Table 3. continued

56		O-Acetylserine Sulfhydrylase Cysteine Synthase	2.5.1.47	
Gate function GATE1 – Controls access to the active site GATE2 – Controls access to the active site			Enzyme function Production of cysteine from the O-acetyl-serine intermediate	
Gate location GATE1 – Bottleneck of tunnel 12–20 Å from the active site cavity GATE2 – Bottleneck of tunnel 8–12 Å from the active site cavity			 PDB ID: 1Z7Y Tunnel Length ~ 28 Bottleneck Role Access to the active site	
Gate structural basis				
	GATE1 Motion of side chains in addition to backbone movements M101 or S100 on the loop of the upper domain			
	GATE2 Motion of side chains in addition to backbone movements S75 on the Asn-loop for the inner gate			
Gate engineering				
PDB: <i>WT</i> – 1Z7Y, 1Z7W				
References: ²⁰⁰				

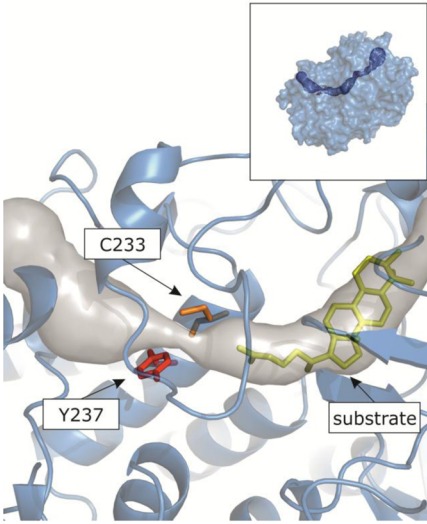



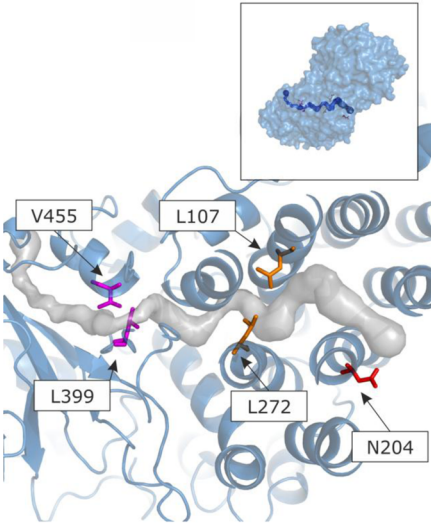



57		Oxidosqualene Cyclase SceOSC	5.4.99.7	
Gate function GATE1 – Controls access of the substrate ?GATE2 – Controls access of the substrate			Enzyme function Cyclization of the 2,3-oxidosqualene into lanosterol	
Gate location GATE1 – Bottleneck between tunnel and active site cavity ?GATE2 – Bottleneck between tunnel and active site cavity			 PDB ID: 1W6K Tunnel Length Bottleneck Role Connects active site cavity with bulk solvent	
Gate structural basis				
	GATE1 Y239 (Y237 in <i>HsaOSC</i>) A rotation of the side chain stabilized by a hydrogen bond bridge with P228 could open the channel and enable the substrate to enter the active site			
	?GATE2 T235 (C233 in <i>HsaOSC</i>)			
				
Gate engineering Y239F – 5-fold decrease in enzyme activity, mutation keeps the channel in a closed conformation Y239A – 2-fold decrease in enzyme activity, mutation keeps the channel in a open conformation				
PDB: <i>WT (HsaOSC)</i> – 1W6K				
References: ²⁰¹				

Table 3. continued

58		Phenol Hydroxylase PHH		1.14.13.7	
Gate function GATE1 – Controls access to the active site GATE2 – Closes cavity 2 GATE3 – Closes cavity 2			Enzyme function Hydroxylates aromatic compounds		
Gate location GATE1 – Entrance to the tunnel GATE2 – Between active site pocket and cavity 2 GATE3 – Between cavity 3 and cavity 2			 PDB ID: 2INP Tunnel Length Bottleneck Role Substrate transport		
Gate structural basis					
	GATE1 N204 The residue shift is redox-dependent Helix E orients N204 away from the active site and opens the tunnel N204 in closed conformation forms a hydrogen bond with S72				
	GATE2 L107 and L272				
	GATE3 L399 and V455				
Gate engineering					
PDB: <i>WT</i> – 2INP, 2INN					
References: ^{74,195}					

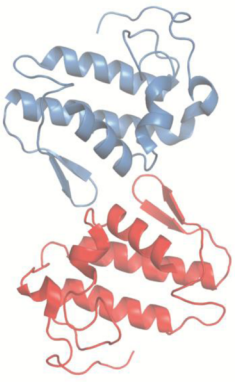

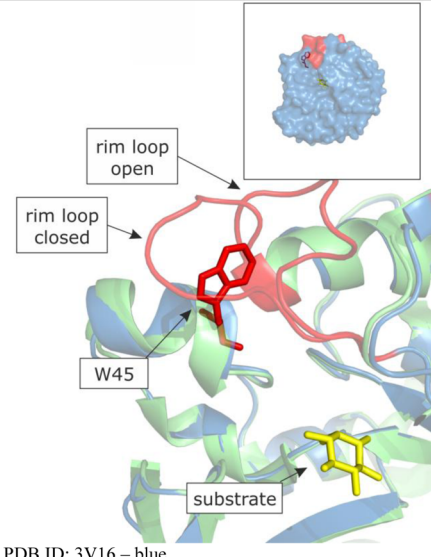

59		Phospholipase A2		3.1.1.4	
Gate function GATE1 – Controls access to the interface and active sites			Enzyme function Catalyses the hydrolysis of acyl bonds in sn-3-phospholipids		
Gate location GATE1 – Position of monomers			 PDB ID: 1CLP – open Tunnel Length Bottleneck Role		
Gate structural basis					
	GATE1 The dimer interface might act as a hinge The homodimer can be observed in open and closed conformations formed by different angle between monomers				
Gate engineering					
PDB: <i>WT</i> – 1CLP (open), 1PP2 (closed)					
References: ^{69,202,203}					

Table 3. continued

60	Phosphatidylinositol-Specific Phospholipase C	4.6.1.13
Gate function GATE1 – Controls access to the active site, controls product exit		Enzyme function Catalyses the cleavage of glycan-phosphatidylinositol anchored proteins 
Gate location GATE1 – Entrance to the active site		
Gate structural basis		
	GATE1 Rim loop (residues 241-252) and W45 Rim loop undergoes the pH-dependent movement with a maximum backbone displacement of 9.4 Å between the acidic and basic forms H258 serves as an anchoring residue holding F249 residue from a loop by π -cationic interaction W45 closes the product exit pathway when the loop is in extended position	
Gate engineering H258Y – keeps the rim mobile loop in extended conformation		
PDB: <i>WT</i> – 3V16, 3V18, <i>Mutants H258Y</i> – 3V1H		PDB ID: 3V16 – blue PDB ID: 3V18 – light green
References: ²⁰⁴		Tunnel
		Length
		Bottleneck
		Role

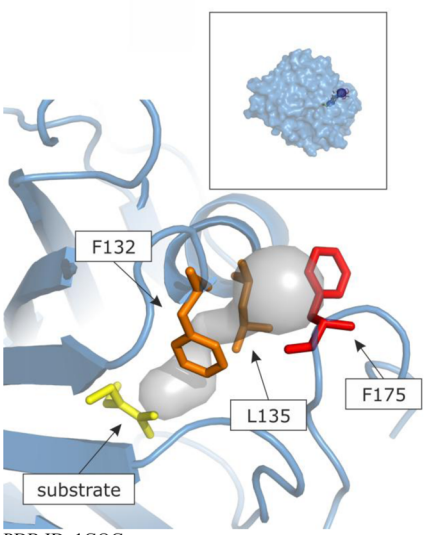


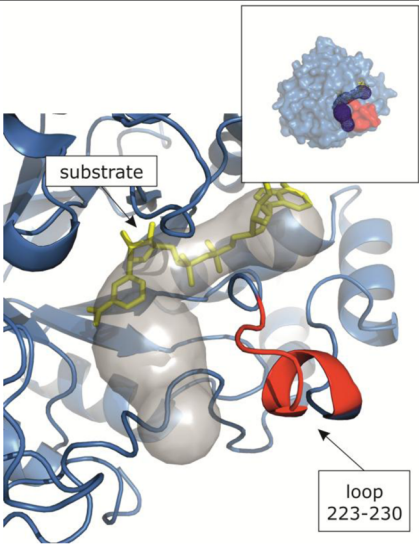

61	Quercetin 2,3-Dioxygenase	1.13.11.24
Gate function GATE1 – Controls access of small molecules into the active site GATE2 – Controls access of small molecules into the active site		Enzyme function Degradation pathway of flavonoids 
Gate location GATE1 – Tunnel connecting the bulk solvent and the active site cavity GATE2 – Tunnel connecting the bulk solvent and the active site cavity		
Gate structural basis		
	GATE1 F175 Side-chain of F175 switches from the enzyme surface to the bulk and opens access to the cavity This tunnel opening occurs as soon as the cavity is inflated by a sufficient number of water molecules (16–18)	
	GATE2 F132 and L135 Side-chains of F132 and L135 are structurally mobile enough to provide access for the entry of small molecules into the enzyme active site	
Gate engineering		PDB ID: 1GQG
PDB: <i>WT</i> – 1GQG, 1JUH, 1H1I		Tunnel
References: ⁷⁵		Length
		Bottleneck
		Role

Table 3. continued

62	Rabbit 20a-Hydroxysteroid Dehydrogenase		1.1.1.149	
Gate function GATE1 – Protects cavity from the solvent in the absence of a steroid			Enzyme function Dehydrogenation of hydroxy steroid	
Gate location GATE1 – Loops making-up the active site cavity				
Gate structural basis				
	GATE1 Flexible loop B (residues 223–230) Movement of the loop partly controlled by the nature of Q230 Loops A and C mostly contribute in the pocket creation changing depth and size of the cavity			
Gate engineering E230P – changes in the selectivity by reduced loop flexibility K274R – increases flexibility of loop by and eliminating K274–E227 contact, high k_{cat} for NADPH-dependent reduction of xylose				
PDB: <i>WT</i> – 1Q5M, 1Q13, <i>WT (AKR5H1)</i> – 2WZT 2WZM small loops			PDB ID: 1Q13	
References: ^{37,205–208}			Tunnel	
			Length	
			Bottleneck	
			Role	Substrate steroid binding and orienting towards cofactor NADP(H)

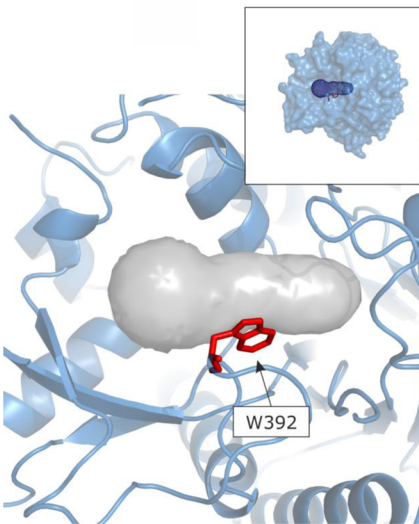


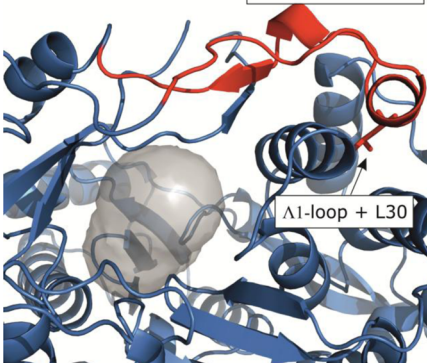
63	Raucaffricine O-β-Dglucosidaseglucosidase		3.2.1.125	
Gate function GATE1 – Ccontrols the access of the substrate			Enzyme function Hydrolyses alkaloid raucaffricine to aglycone vomilenine	
Gate location GATE1 – At the entrance to the active site cavity				
Gate structural basis				
	GATE1 W392 Conformation of W392 is controlled by S390			
Gate engineering S390G – leads to more flexible conformation of W392 F485W – results in more fixed conformation of W392				
PDB: <i>WT</i> – 4A3Y, <i>Mutants E186Q</i> – 3U5U, 3U57, 3U5Y			PDB ID: 3U5U	
References: ²⁰⁹			Tunnel	
			Length	
			Bottleneck	
			Role	

Table 3. continued

64	RNA-dependent RNA polymerase	2.7.7.48	
Gate function GATE1 – Controls enzyme activity		Enzyme function RNA replication	
Gate location GATE1 – Surface loop			
Gate structural basis			
	GATE1 Δ1-loop + L30 Δ1-loop is involved in keeping the enzyme in a closed conformation, makes interaction with L30 (closed conformation is active, open is inactive)		
Gate engineering L30R and L30S – open conformation R222E – exhibited 2-fold reduction in activity, positively charged residue inside the tunnel K151E – activity was 7–10-fold higher at 21 °C, and 2–3-fold higher at 37 °C, positively charged residue near the tunnel entrance			
PDB: <i>WT</i> – 2XWH, 2XXD, 3MWV			
References: ²¹⁰		PDB ID: 2XWH	
		Tunnel	
		Length	19
		Bottleneck	
		Role	NTP transport


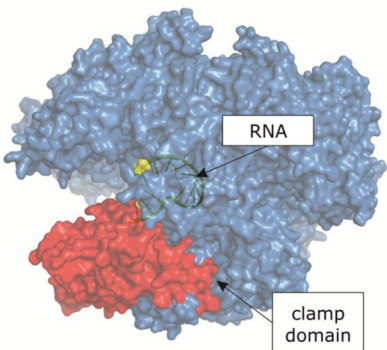
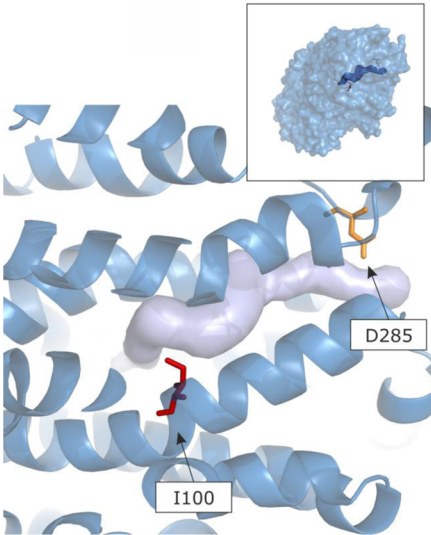



65	RNA Polymerase	2.7.7.6	
Gate function GATE1 – Controls the access to the DNA-binding tunnel		Enzyme function RNA synthesis	
Gate location GATE1 – Entrance to the DNA binding tunnel			
Gate structural basis			
	GATE1 Clamp domain may open to permit entry of promoter DNA during initiation, close to establish the tight grip on DNA during elongation and then open again to allow release of DNA during termination		
Gate engineering			
PDB: <i>WT</i> – 2O5J, 116H, 116V			
References: ^{66,211}		PDB ID: 116H	
		Tunnel	
		Length	
		Bottleneck	
		Role	

Table 3. continued

66	Toluene-4-Monooxygenase T4MO		1.14.13		
Gate function GATE1 – Controls access to the active site, controls selectivity GATE2 – Controls access to the tunnel GATE3 – Protects cavity from access of the solvent		Enzyme function Hydroxylates toluene primarily at the para position to cresol			
Gate location GATE1 – Boundary between the active site pocket and the tunnel GATE2 – Tunnel entrance GATE3 – Cavity entrance		 <p>PDB ID: 3DHG</p>			
Gate structural basis					
	GATE1 I100 Side chain rotation				
	GATE2 D285 Steric blocking by negatively charged residue D285				
	GATE3 R60, E64, E75, R69 Interactions between E64 and R60 and between E75 and R69, ionic gate for the cavity				
Gate engineering D285I, D285Q – improves oxidation of bulky substrates (11-fold) D285S – improves oxidation of styrene (1.7-fold) I100A – improves oxidation of bulky substrates (35-fold) I100G – improves oxidation of methyl-p-tolyl sulphide (11-fold)		Tunnel			
PDB: WT – 3DHG, WT (<i>T4moC</i>) – 1VM9, WT (<i>T4moD</i>) – 1G10		Length			
References: ^{61,105}		Bottleneck			
		Role	Substrate binding cavity		

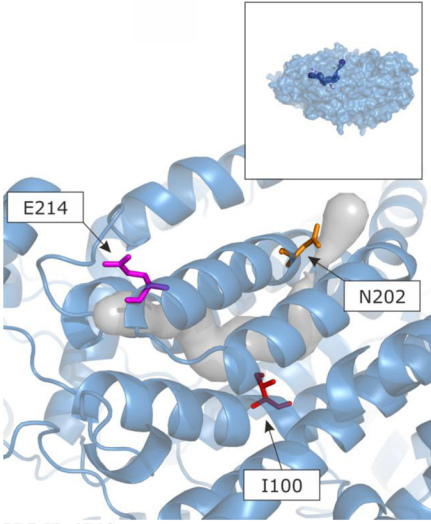


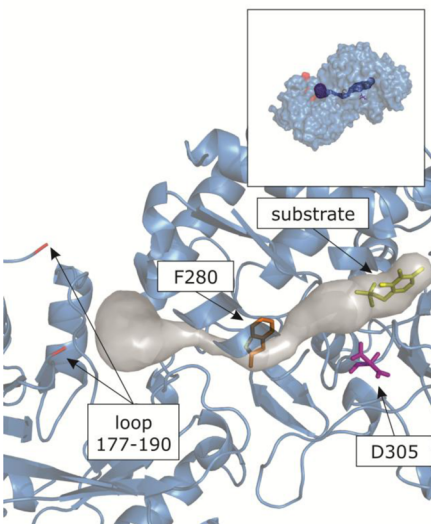



67	Toluene-Ortho-Xylene Monooxygenase ToMO		1.14		
Gate function GATE1 – Controls access to the active site, controls selectivity GATE2 – Open pore allows access to the diiron centre for substrate (maybe also dioxygen or protons)		Enzyme function Catalyses hydroxylation of aromatics, oxidize benzene to phenol, catechol and trihydroxybenzene			
Gate location GATE1 – Border of the pocket near Fe atoms – entrance to active site GATE2 – Entrance to the tunnel		 <p>PDB ID: 2INC</p>			
Gate structural basis					
	GATE1 I100 Residues H96, I100, T201, F205, and F196 border the pocket – I100 is an analogue of L110 (gate) in MMOH Ile100 contributes to defining the hypothetical para site, but it is also at the boundary between the active site pocket and the tunnel which connects the pocket to the surface of the protein				
	GATE2 N202 The conserved tunnel is gated by N202. The residue shifts in a redox-dependent manner. Its side chain is oriented away from the active site in the oxidized form and points inward in the reduced or Mn(II) reconstituted forms of the hydroxylase. This motion correlates with the carboxylate shift that occurs upon reduction of the dimetallic centre.				
Gate engineering I100A, I100W, F205L, A107V, A107I – decreased or removes activity Creation of new GATE3 E214 – hot spot for new gate creation localized on helix E at the entrance to the tunnel 23 Å from the active site, mutation of those residue regulates access to the tunnel (E214A, E214G, E214V, E214W, E214F, E214Q, E214P) E214G – 15-fold improvement for p-nitrophenol oxidation				Tunnel	
PDB: WT – 2INC, 1T0Q		Length	30-35		
References: ^{74,91,101,195,212,213}		Bottleneck			
		Role	Substrate and product transport		

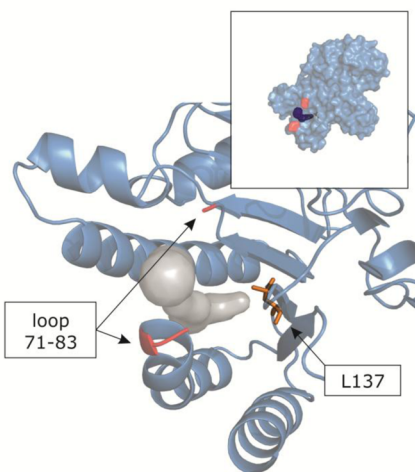

Table 3. continued

68		Triosephosphate Isomerase		5.3.1.1	
Gate function GATE1 – Controls access of the water to the active site			Enzyme function Catalyses isomerisation D-glyceraldehyde 3-phosphate to dihydroxyacetone phosphate		
Gate location GATE1 – Entrance to active site					
Gate structural basis					
	GATE1 Loop 6 (residues 166 to 176). Residues 169 – 173 move as a rigid body, which position is controlled by flexible three residues N-terminal and C-terminal hinge regions In closed conformation loop sequesters the enzyme reaction from solvent. Closed conformation is stabilised by conserved anchoring residues from loop 7				
Gate engineering P(166)VW-AIGTG-KTA to P(166)GG-AIGTG-GGG mutant – k_{cat} decreases 2500-fold Y208F – k_{cat} decreases 2400-fold Y208T – k_{cat} decreases 200-fold			Tunnel Length Bottleneck Role		
PDB: <i>WT</i> – 1TIM (open), 1TPH (closed)					
References: ^{77,78,214–216}					

69		tRNA-Dependent Amidotransferase GatDE and GatCAB		6.3.5	
Gate function GATE1 – Protects active site from access of the solvent, avoids wasteful release of ammonia into solution ?GATE2 – Controls ammonia transport			Enzyme function Conversion of Glu-tRNA ^{Gln} into Gln-tRNA ^{Gln} or Asp-tRNA ^{Asn} into Asn-tRNA ^{Asn}		
Gate location GATE1 – β hairpin loop surrounding asparaginase active site (in GatDE) ?GATE2 – Inside ammonia tunnel (in GatCAB)					
Gate structural basis					
	GATE1 GatDE β hairpin loop D β 7-D β 8, residues 100–118 in GatD. G100, the first glycine in the strictly conserved GGT motif, active conformation substrate in contact with T102				
	GATE2 GatCAB in <i>A. aeolicus</i> (<i>S. aureus</i>) E128 (E125), K90 (K88) E128 (E125) side chain can blocks the tunnel via a salt bridge with K90 (K88)		Tunnel Length Bottleneck Role		
Gate engineering			GatDE GatCAB		
PDB: <i>WT (GatDE)</i> – 1ZQ1, <i>WT (GatCAB)</i> – 3H0L, 3H0M, 3H0R					
References: ^{155,217–219}					

Table 3. continued

70	Tryptophan Synthase	4.2.1.20
Gate function GATE1 – Creates the tunnel and protects substrate binding cavity from the access of the solvent GATE2 – Controls transport of the indol GATE3 – Controls access to the subunit β and activates the β active site		Enzyme function Synthesis of L-tryptophan; subunit α cleavage of 3-indole-D-glycerol 3-phosphate; subunit β pyridoxal phosphate dependent condensation of indole with L-serine
Gate location GATE1 – Surface loop from subunit α at the border between the subunits β and α GATE2 – Tunnel wall in subunit β GATE3 – Subunit β		
Gate structural basis		
	GATE1 Loop L177 - A190 A disordered surface loop in the subunit α after substrate binding becomes ordered and clamps down over the active site, isolating this region from solvent	
	GATE2 F280, Y279 The residue F280 can adopt alternative conformations in closed interact with C170 in open one it adopts Y279 position, Y279 moves toward the subunit α and interacts with part of the flexible loop-2 residues (54-61) of the subunit α	
	GATE3 D305 In the closed conformation D305 creates the H-bonded salt bridge with R141 with the associated H-bonding network involving S197 and S199	
Conformation changes are initiated by binding IGP - the allosteric communication between the two sites that results in full coupling of the reaction at the subunits α and β		PDB ID: 1BKS
Gate engineering E49F, G51L, D60Y – decreases activity (2-fold) F280C, F280S – increases transport C170W, C170F – chemical modification of C170 (C170-NEM, C170-MMTS), obstructs the tunnel and accumulates indole intermediate		Tunnel
PDB: <i>WT</i> – closed 3CEP, open conformations: 1BKS, 1KFK, 1KFJ, 1TTP, 2CLL, 2CLM, 2CLO		Length 25 – 30
References: 47,71,102,220,221		Bottleneck
		Role Indol transport between two active sites

71	Undecaprenyl-Pyrophosphate Synthase	2.5.1.31
Gate function GATE1 – Controls length of the final product		Enzyme function Condensation reactions of isopentenyl pyrophosphate with allylic pyrophosphate to generate linear isoprenyl polymers
Gate location GATE1 – Loop closing the entrance to the tunnel		
Gate structural basis		
	GATE1 71 – 83 loop The helix $\alpha 3$ in open tunnel conformation is kinked by $\sim 30^\circ$ at E96, and the helix $\alpha 3$ in closed tunnel conformation is kinked by $\sim 45^\circ$ at A92. Reaction starts with closed conformation \rightarrow when the chain length reaches C55, the dimethyl end is stopped at the end of the tunnel \rightarrow $\alpha 3$ helix move away from the closed position into the open position (71 – 83 loop) \rightarrow fully synthesized C55 long product can exit easily through the open gate	
Gate engineering *GATE2 L137A – removes the floor of the tunnel and allows formation of a longer chain length products (normal length C55), bulky side chain of L137 serves to block further elongation of undecaprenyl-pyrophosphate A69L – results in long lived accumulation of a short chain intermediate C30 final product C55 A143V – similar to wild type, rate 3-fold lower S71A, N74A, or R77A – decreases in k_{cat} values (25–200-fold) W75A – increases in K_m for farnesyl pyrophosphate (8-fold) E81A, S71A – increases in K_m for isopentenyl pyrophosphate (22–33-fold)		
PDB: <i>WT</i> – 1JP3		
References: 27		PDB ID: 1JP3
		Tunnel
		Length 30
		Bottleneck
		Role Active site cavity

substrate binding since it is believed to slow down product dissociation.^{22,97} Other gates of this type have been reported in 3-hydroxybenzoate hydroxylase,⁹⁸ 4-hydroxy-2-ketovaleate aldolase/acylating acetaldehyde dehydrogenase,⁹⁶ and cholest-

terol oxidase type I⁵⁷ and type II.⁹⁹ Moreover, cofactors themselves can also function as gates. The FAD cofactor of digeranylgeranylgerolphospholipid reductase has two different conformations, referred to as the “in” and “out”

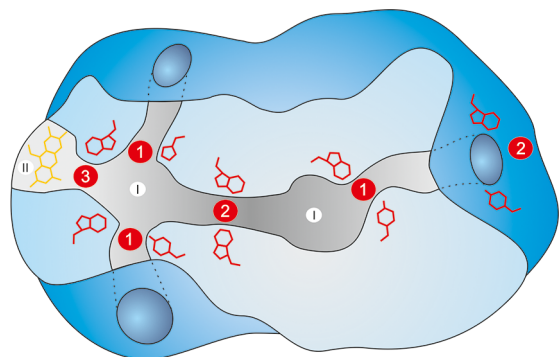


Figure 6. Locations of gates within a protein structure. Schematic representation of an enzyme with two active sites connected by a tunnel (I), a cofactor cavity (II), and multiple access tunnels. Gating residues in red may be located at the entrance to the active site (1), at the entrance or the bottleneck of the tunnel (2), and between the active site cavity and the cofactor cavity (3).

conformations. In the “in” conformation, the tunnel is blocked by FAD. To open the tunnel, FAD has to adopt the “out” conformation.¹⁰⁰

Gates are most commonly located at the tunnel entry and the tunnel bottleneck (51%). This is to be expected because the bottleneck represents the narrowest point of the tunnel, and its diameter often dictates the tunnel’s permeability. Another common location is the entrance to the active site cavity (28%). Gates at the entrance of the cofactor cavity are less common (5%), which is not surprising since not all enzymes have a cofactor cavity. In 16% of the cases studied, the gate was not located within any of these functional regions (Figure 7).

5. ENGINEERING OF GATES

The average rate of evolution of the gating residues in the cytochrome P450 family is significantly greater than that for the protein sequence as a whole.³⁹ The high rate of evolution at the gating residues suggests that gate engineering may be an attractive alternative to other rational enzyme design strategies. This idea is supported by a few observations: (i) the gates are often spatially separated from the active site, and so mutations at the gating residues should not be deleterious to protein function, (ii) the opening and closing of the access pathways can affect ligand exchange and thus enzyme activity and selectivity, and (iii) gate modification can modulate the solvent’s ability to access the active site, which in turn affects

solvation and stabilization of the transition state and also product release. In the following section, we describe selected cases in which an enzyme’s catalytic properties have been successfully altered by modifying its gates.

5.1. Gate Modification

Gates can be modified by substitution of the gating residues, hinge residues, or anchoring residues depending on the nature of the gate in question. Modification of the gating residues has been shown to change the selectivity and activity of toluene-*o*-xylene monooxygenase, with the E214G mutation improving oxidation of *p*-nitrophenol by a factor of 15.¹⁰¹ A similar improvement in overall activity was achieved in a lipase from *Burkholderia cepacia* by the mutations L17S + L287I. This double mutant also exhibited a 10-fold increase in enantioselectivity compared to the wild-type enzyme.⁸⁶ The T78F or P76F mutations in imidazole glycerol phosphate synthase override some of the control exerted by the wild-type gate and block the passage of ammonia through the tunnel.³⁸ In NiFe hydrogenases, mutations of the V74 and V74 + L122 residues changes the rates of transport for H₂, CO, and O₂, thereby modulating the overall rate of reaction.²¹

Gates that incorporate secondary structure elements are dependent on hinge and anchoring regions. It has been demonstrated that modification of the hinge region can change an enzyme’s activity and selectivity. Notably, the Q230P mutation in rabbit 20A-hydroxysteroid dehydrogenase decreases the flexibility of a key loop and thereby changes its selectivity.³⁷ Similarly, access to the active site in the HIV-1 protease is controlled by two β -turn flaps.⁸⁹ Results from Brownian dynamics simulations suggest that the G48V + V82A or I84V + L90M mutations in this enzyme reduce the likelihood that the active site will be exposed at any given point in time from 14% in the wild type to 2% in the mutants.⁸⁹ The importance of the anchoring residues in the HIV-1 protease was demonstrated by the F53L mutation, which generates a semiopen conformation due to removal of the stabilizing F53–I50 interaction.⁸⁹

5.2. Gate Removal

Removing gates typically increases substrate and product exchange rates but also allows more extensive access of water molecules to protein tunnels and cavities. The overall effect of gate removal is therefore equal to the combined effects of these two processes. Gate deletion has been shown to increase the rate of substrate binding to tryptophan synthase.¹⁰² The F280C and F280S mutations both increased the rate of indole binding

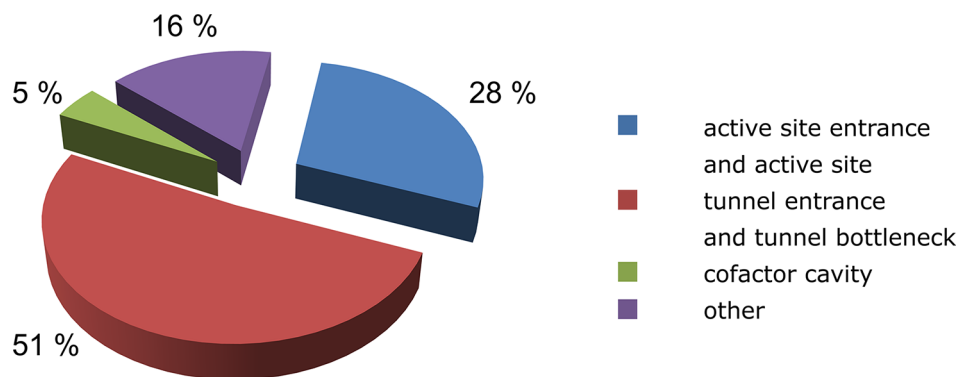


Figure 7. Distribution of gate locations within protein structures based on analysis of 71 proteins with 129 gates. Detailed description of the analyzed proteins is provided in Table 3.

by a factor of 2. Similarly, the T78A mutation allowed ammonia to rapidly pass through the tunnel in imidazole glycerol phosphate synthase.³⁸ The R239A mutation in the cyclase caused a 1000-fold decrease in the enzyme's k_{cat}/K_m value and decoupling of the reaction.³⁸ This dramatic change in enzyme catalytic efficiency was attributed to creation of a new route for ammonia release. A similar leakage of ammonia was caused by the G359F and G359Y mutations in carbamoyl phosphate synthetase.^{103,104} Negative consequences of gate removal were also observed for the FabZ- β -hydroxyacyl-acyl carrier protein dehydratase (HpFabZ),⁵⁴ in which the Y100A mutation leaves the active site completely exposed to the bulk solvent. As a result, the acyl carrier protein binds to the HpFabZ Y100A mutant much more strongly than to the wild-type HpFabZ, decreasing the mutant enzyme's activity by more than 50% due to the very slow dissociation of the acyl carrier protein.

In some cases, gate removal enables bulky substrates to access the active site cavity. Mutations D285I and D285Q in toluene-4-monooxygenase improved its ability to oxidize the large and bulky substrates 2-phenylethanol and methyl *p*-tolyl sulfide by factors of 8 and 11, respectively, while the D285S mutation improved the rate of styrene oxidation 1.7-fold.¹⁰⁵ The L137A mutation in undecaprenyl-pyrophosphate synthase removed the bottom of the tunnel in this enzyme, allowing formation of products with longer chain lengths.²⁷ A similar situation was encountered in type III polyketide synthases from *Aloe arborescens* PCS, in which the M207 residue controls the number of condensations of malonyl-CoA.¹⁰⁶ The M207G mutation opened a connection between the tunnel and two hidden pockets located behind the active site, resulting in formation of extended products. Further product elongation was achieved with the triple mutant F80A + Y82A + M207G.

5.3. Gate Insertion

To best of our knowledge, there have been no reports of an intentional introduction of a new gate into an enzyme structure. However, there have been studies in which an access tunnel was systematically modified with multiple substitutions, and it is reasonable to expect that some of these mutations might have created new gates. More research will clearly be needed to confirm this expectation. To verify successful intentional insertion of a new gate into a protein structure, it would be necessary to confirm the existence of both the open and the closed conformations at a position where previously only a single conformation could be adopted.

Site-directed mutagenesis targeting specific residues at various positions along the access tunnel of *Candida rugosa* lipase has been used to alter the acceptable substrate chain length for this enzyme. The mutants for which this was observed were P246F, L413F, L410W, L410F + S300E, and L410F + S365L.¹⁰⁷ We note that the aromatic residues F and W, which are common in wing and swinging door gates, were introduced in each of these variants.

In another study, the residue L177 that is located near the entrance to the access tunnel of the haloalkane dehalogenase LinB from *Sphingobium japonicum* UT26 was substituted with all of the natural amino acids, yielding 19 mutants with significantly altered substrate specificity and activity.¹⁰⁸ Preliminary computational analyses of these variants using molecular dynamics revealed that the two residues possessing a single aromatic ring (F and Y) exhibited large fluctuations, as might be expected for gating.

Residue A217 is located at the entrance to the tunnel in the epoxide hydrolase EH from *Aspergillus niger* M200. This residue was substituted with C, E, G, L, P, Q, R, T, and V, and the effect of each mutation on the enzyme's activity and enantioselectivity was studied.²⁶ The mutants exhibited different enantioselectivity and activity relative to the wild type. For instance, the activity of the A217G mutation toward allyl glycidyl ether was lower than that of the wild type by a factor of 33, whereas the A217V mutation increased activity toward this substrate 6.6-fold.

Residues I135, W141, C176, V245, L246, and Y273 are positioned close to the entrance to the main and side tunnels of the haloalkane dehalogenase DhaA from *Rhodococcus rhodochrous* NCIMB 13064. These residues were simultaneously permuted in an attempt to improve this enzyme's activity against 1,2,3-trichloropropane. The most successful mutant, which featured the I135F, C176Y, V245F, L246I, and Y273F substitutions, showed 26-fold greater activity toward the target substrate than did the wild type.¹⁰⁹ In this mutant, three aromatic residues were introduced in place of aliphatic ones in the vicinity of the tunnels. Computational analysis of product release from the mutant suggests that substitutions introduced a transient rather than permanent structural feature and gating residues prevented access of water to the active site.¹¹⁰ Crystallographic analysis of the mutant revealed two distinct conformations for the Y176 side chain.¹¹⁰

6. CONCLUSIONS

This review highlights the importance of gates in enzymes. Gates play vital roles in controlling the catalytic activity and selectivity of enzymes and are more common in protein structures than is generally thought. In particular, gates control substrate access to the active site and product release, prevent or restrict solvent access to specific regions of the protein, and can synchronize processes occurring in distinct parts of the enzyme. Our literature survey of 129 gates in 71 enzymes revealed a large variety of systems with sophisticated structures. We presented a rigorous definition of gates and established a new scheme for their classification. The large number of inspected cases allowed us to build a catalogue of gates assigned to six distinct classes—wings, swinging doors, apertures, drawbridges, double drawbridges, and shells—with three different functions and three distinguishable locations. We also presented summary statistics that give a preliminary overview of the propensity of specific amino acid residues to occur in particular gate classes. The proposed classification scheme can be easily extended and updated but even in its present form can provide guidance for analysis and engineering of gates in biomolecular systems.

The biochemical relevance and specific location of gates within protein structures make them attractive targets for protein engineering. Attempts to rationally redesign gates typically involve computer-assisted gate identification followed by modification using focused directed evolution. This approach is compatible with a recent trend in protein engineering that stresses construction of small and smart libraries. Gate modification and deletion have been demonstrated in numerous cases, but the intentional insertion of new gates remains a challenge. Convenient methods for identifying gates in protein structures are essential prerequisites for their engineering. In silico, this can be achieved by coupling the software tools developed for describing pathways to tools developed for study of protein dynamics. Of the available

experimental techniques, NMR spectroscopy is particularly suitable for analysis of highly dynamic protein structures and can be expected to play an indispensable role in the study of gate dynamics at the atomic level. The field would also benefit from development of new experimental techniques for monitoring the passage of ligands through the protein pathways. One day it will be possible to control the catalytic properties of enzymes by rational engineering of their gates. To achieve this goal, we have to learn how gates evolved, how they interact with the other parts of the protein structure as well as with the ligand and solvent molecules, and how they fulfill their biological functions.

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