Chapter 12

Role of tunnels, channels and gates in enzymatic catalysis

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1. INTRODUCTION

Enzymes, as the natural catalysts, have evolved over millions of years to perform specific reactions within living organisms. Because of their large complexity and variability, the structural basis for their efficiency and specificity is not fully understood. At the same time, there is an increasing demand to engineer enzymes for the reactions needed for production of chemicals, pharmaceuticals, food, agricultural additives and fuels[1–3].

Many of known enzymes have their active sites **buried** inside their protein core, rather than **exposed** to the bulk solvent at the protein surface[4–6]. This may be due to several reasons, such as the need of solvent absence to carry out specific chemical reaction, a means of controlling the substrate specificity, or regulating the release of products to the surrounding solvent. These buried active sites are connected to the bulk solvent through **tunnels**, which act as the exchange pathways for the substances between a bulk solvent and the active site. Hence, taking into account the very complex mixture of proteins co-localized within a living cell, the tunnels can be very important systems for accomplishing the enzyme functions. Thus, additionally to the simplistic Fischer's **lock-and-key** model[7] or the more realistic Koshland's **induced-fit** model[8], the enzymes bearing tunnels can be described by a **lock-keyhole-key**

model[4]. This model takes into account that the **key** (substrate) needs to pass through a **keyhole** (tunnel) in order to reach the **lock** (active site). Considering this model it becomes very intuitive that access tunnels represent important structural features for regulating enzymatic functions.

The enzymes frequently possess the structural elements for controlling the transport of substances through tunnels and channels, called **gates**[9]. Protein gates are dynamic systems which can reversibly switch between open and close states through conformational changes and by this way control the passage of molecules into and out of the protein. The gates provide a privileged mode for selecting the molecules that are allowed to enter the structure, as well as the frequency with which they can pass through. Protein gates have been described and studied before[9–13], but the knowledge has been very dispersed until recently Gora et al.[14] surveyed the literature and systematized the information using a newly established classification system.

Due to their primary importance for the enzyme structure and function; tunnels, channels and gates have revealed good potential for engineering of enzyme properties. There are many examples showing how mutations in the key residues defining the tunnel geometry or gate mobility have contributed to change activity, specificity, and stability of enzymes. The grand challenge in this context is to understand the structural basis and underlying mechanisms that will allow rational engineering of fully functional access pathways in the future.

2. PROTEIN TUNNELS

2.1 Structural basis and function

Many of the known enzymes possess buried active sites, and one of the possible reasons is to regulate the substrate specificity or to create the suitable environment for their

chemical reaction. Because the terminology in the literature is diverse, herein we define protein **tunnels** as the transport pathways between the surface and the active sites which are buried inside the protein structures or connecting different active sites within the proteins or protein complexes; we define **channels** as the conduits connecting different parts of the protein surface through which the molecules may pass without transformation.[4–6,15]

Structurally, the protein tunnels often contain a **bottleneck**, which is its narrowest part and is determinant of tunnel selectivity. The bottlenecks are often controlled by the gates that open and close the narrowest part of the tunnel with certain frequencies. The existence of tunnels and channels is not restricted to a small group of enzymes, but it is rather widespread and can be found in all the six enzyme classes. There are proteins containing: (**1**) channels passing throughout the structure connecting two different parts of protein surface; (**2**) one single tunnel connecting the surface with the buried active site cavity; (**3**) more than one tunnel connecting the surface with the buried active site; and (**4**) more than one active site connected with each another and with the surface by several tunnels (**Figure 1**).

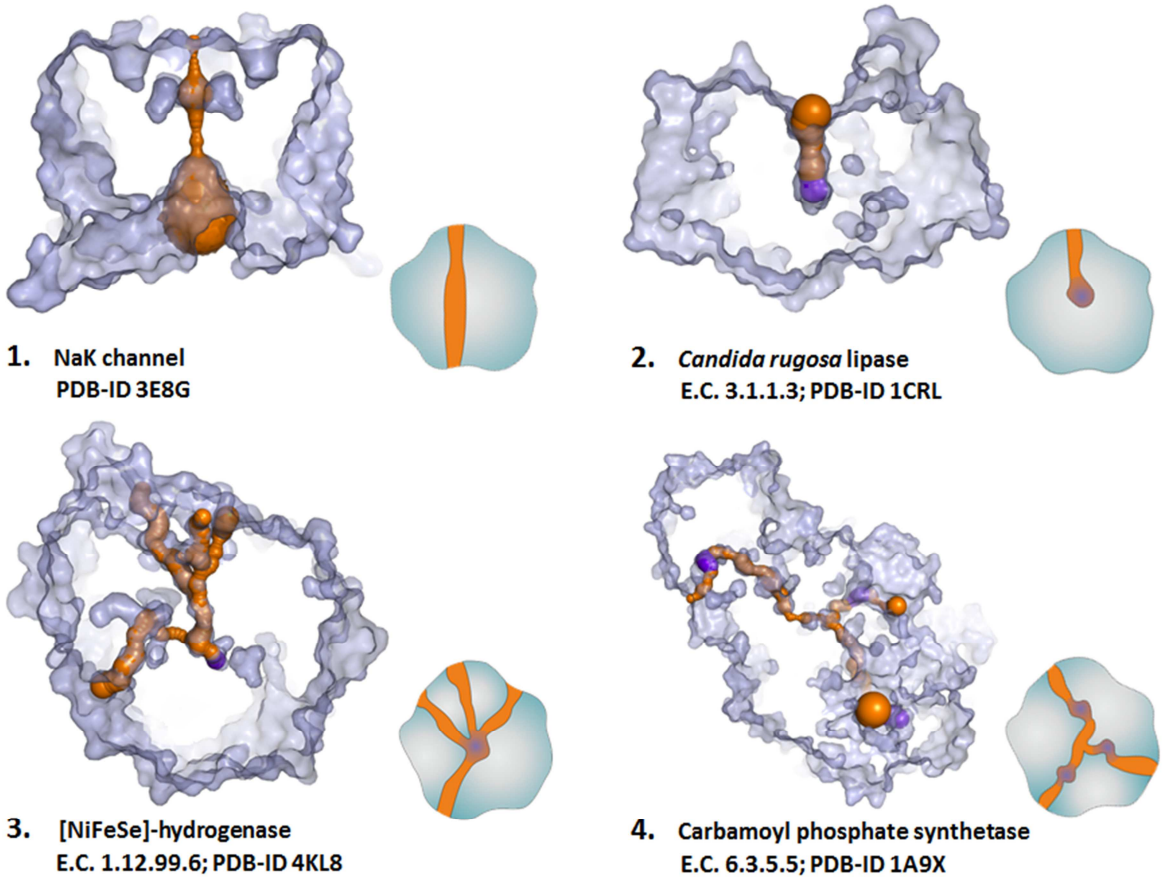


Figure 1. **Channels and tunnels in proteins.** Examples of proteins containing a channel (**1**, NaK channel), and one single tunnel (**2**, *Candida rugosa* lipase) or multiple tunnels (**3**, [NiFeSe]-hydrogenase) connecting the active site cavity with the bulk solvent, or a tunnel connecting different active sites (**4**, carbamoyl phosphate synthetase). The channels and tunnels are represented in orange colour and the active sites in purple.

In the first class (**1**), the channels serve as a pathway for the substances to cross the protein structures, for which there is usually a well regulated mechanism. We may find them, for example, in the ion channels,[16,17] which allow the crossing of specific ions through **membrane proteins** (K^+ channel, Ca^{2+} channel, etc.), in **ion pumps** (Na^+/K^+-adenosine triphosphatase[18], neurotransmitter transporter,[19] etc.), or in the **porins**[20].

In the second class (**2**), a single tunnel connecting a deeply buried active site with the surface has the role for exchange of the substrates, products, and solvent molecules throughout the catalytic cycle. Many enzymes possess one permanent tunnel as well as several transient tunnels, which can be revealed only by studying protein dynamics (**Figure 2**). Transient tunnels occur upon dynamic conformational changes or protein gating mechanisms, and their emergence may be stochastic or induced by the binding of a substrate or the presence of a ligand molecule to be transported[6]. As examples of enzymes with only one tunnel are **oxidoreductases** (e.g. cytosolic sheep liver aldehyde dehydrogenase[21], pyruvate oxidase[22], amine oxidase[23], 4-hydroxybenzoate hydroxylase[24]), **transferases** (glutathione S-transferase[25], lipoate-protein ligase A[26]), **hydrolases** (*Candida antarctica* lipase A[27], *Candida rugosa* lipase[28], *Agrobacterium radiobacter* epoxide hydrolase[29,30], neurolysin[31]), **lyases** (β-hydroxydecanoyl thiol ester dehydrase[32]), and **isomerases** (glutamate racemase[33,34]).

In the third class (**3**), several tunnels are connecting the buried active site with the surface, and they may or not serve the equivalent purpose in the catalytic process. In some cases their roles are distinct. One such an example is the cytochrome P450, which has a main 22 Å-long hydrophobic tunnel with the role in substrate access and product egress, while 12 other secondary tunnels allow the exchange of oxygen and solvent molecules and also provide alternative pathways for the product release. Similarly, the haloalkane dehalogenases possess a main tunnel, used for the halogenated substrate, alcohol and halide products exchange, and several secondary tunnels are used for the alcohol release and water solvent exchange[35]. Multiple tunnels have been identified in the structures of **oxidoreductases** (cytochromes[36], catalase[37], Ni-Fe hydrogenase[38], lipoxygenase 12/15[24], L-amino acid oxidase[39]), **hydrolases** (haloalkane dehalogenases DhaA[35,40,41] and LinB[40,42,43], acetylcholinesterase[44]), and **isomerases** (Δ5-3-ketosteroid isomerase[45]).

Finally, in the fourth class (**4**), we find multifunctional enzymes and multienzyme complexes, which contain separate active sites interconnected by the tunnels. These enzymes are able to carry out sequential reactions, in which an internal pathway conducts the intermediate products from one catalytic site to another. This mechanism may be necessary to increase the enzyme's efficiency to: (i) prevent potentially toxic intermediates to be released into the medium, (ii) avoid labile intermediates to be released into the medium and undergo side reactions, or (iii) reduce the transfer time between different catalytic sites. Such transfer process is always tightly regulated, often through molecular gating mechanism. In this type of enzymes we find **oxidoreductases** (glutamate synthase[6,46]), **transferases** (glucosamine 6-phosphate synthase[6,47], glutamine phosphoribosylpyrophosphate amidotranferase[6,48], and acetyl-CoA synthase[49]), imidazole glycerol phosphate synthase[6,50]), **lyases** (tryptophan synthase[6,51,52], and **ligases** (carbamoyl phosphate synthetase[6,53–55], asparagine synthetase[6,56]).

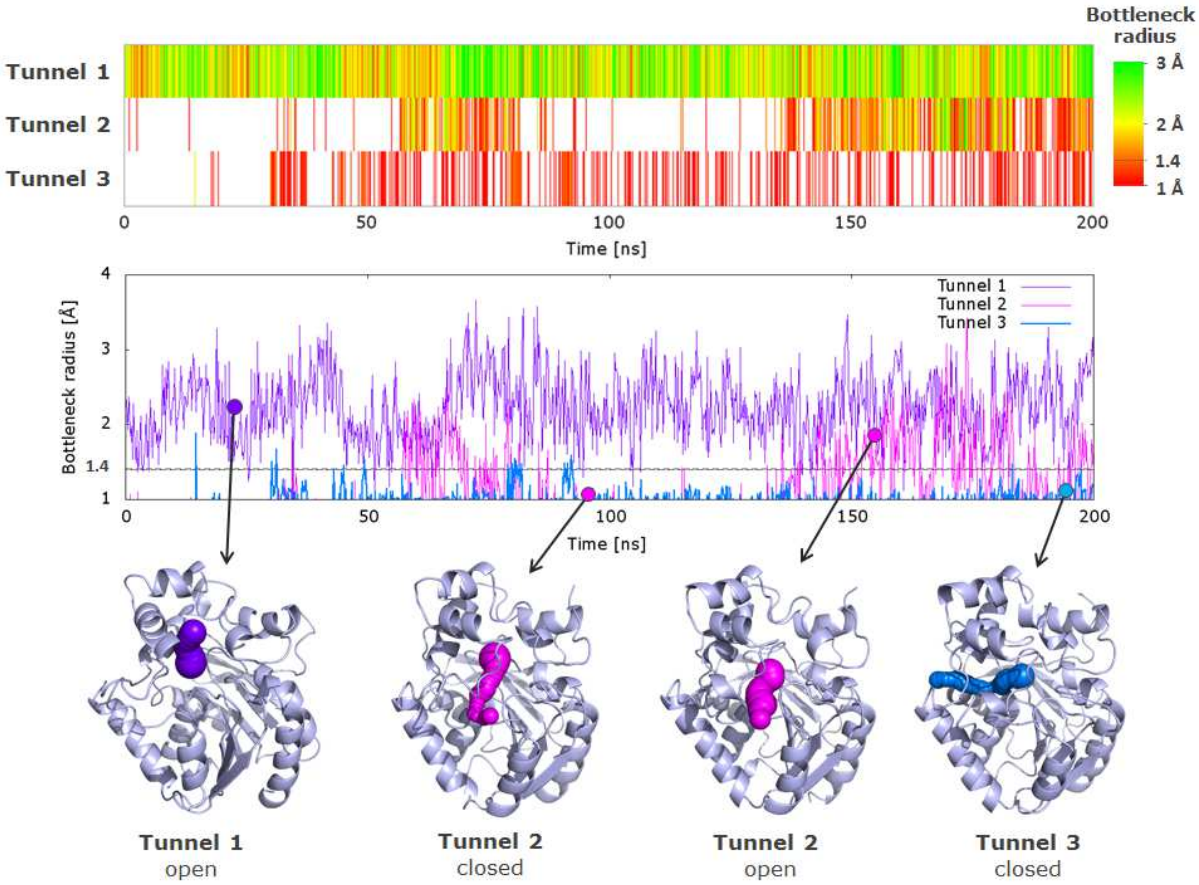


Figure 2. **Permanent and transient tunnels in proteins.** Example of a protein containing permanent and transient tunnels, shown by the dynamics of their bottleneck radii: tunnel 1 is permanently open (bottleneck radius > 1.4 Å almost all the time); tunnel 2 has closed and open periods, and hence it is considered a gated tunnel; tunnel 3 is permanently closed (bottleneck radius < 1.4 Å most of the time). Although tunnels 2 and 3 were both closed in the beginning of the simulation, they revealed different importance and behaviours with the MD. Only tunnels wider than 1 Å radius are displayed here.

The single most important function of protein tunnels is to control the ligands entry to the active site. The selection of the ligands which may pass through the tunnels prevents the formation of non-productive complexes in the binding site, which would reduce the enzyme efficiency. It may also avoid the poisoning of the active centre by certain compounds and by

this way completely inactivating the catalyst, like transition metal ions-dependent metalloenzymes. Tunnels connecting multiple active sites may also prevent the release of toxic intermediate products or metabolites into the cell. The same class of tunnels provide an excellent way for synchronizing reactions which require the contact of multiple substrates or cofactors; control the order of multistep catalytic reactions and provide the environment for carrying out reactions which require the absence of water. These functions are particularly important considering the thousands of proteins and ligands that are simultaneously co-localized within a living cell.

2.2. Identification methods

Two primary experimental methods that allow direct identification of the tunnels and channels within the protein structures to the atomic resolution are **X-ray crystallography** and **nuclear magnetic resonance** (NMR) spectroscopy**.** These experimental techniques are often followed by theoretical analyses using **molecular dynamics** (MD) simulations.

In the last few decades, advances in biological chemistry were boosted by the determination of high-resolution three-dimensional structures of proteins by **X-ray diffraction**, which allowed a deeper understanding of the underlying catalytic mechanism of enzymes at the atomic level[6]. Likewise, also the tunnels of some enzymes started being described into greater detail and their functions being better understood. The higher is the number of crystal structures solved, the deeper is the knowledge attained about that enzyme by sampling different conformational states. However, crystallography only supplies static structures and cannot show metastable conformations, and hence the insight given about the transient tunnels can be limited.

NMR spectroscopy can also provide the 3D structures of proteins, either from solution or solid state studies. But rather than a single structure, the NMR analysis supplies

results in the form of ensembles. Modern protein NMR spectroscopy has largely developed in the last 10-15 years, and currently it is possible to observe the dynamics of proteins at the atomic level by using specific methods, in the timescales ranging from picoseconds to seconds[57–59]. Therefore, NMR spectroscopy has the potential to supply relevant information regarding not only the permanent tunnels, but also the transient ones. In some cases, the transient states of tunnels and channels have indirectly been investigated by using particular methods of NMR. For example, water **magnetic relaxation dispersion** (MRD) has been used, in combination with molecular dynamics simulations, to track the internal water molecules buried inside myoglobin[60] and the bovine pancreatic trypsin inhibitor[61]; and **solid-state NMR spectroscopy** (ssNMR) has been used to track the buried water molecules within a K^+ channel in different gating modes[62].

MD simulation is the method par excellence for detecting and studying the transient tunnels in proteins. MD simulates the behaviour of a molecule under certain conditions of pressure and temperature, preferably in the presence of explicit solvent molecules. AMBER,[63] CHARMM,[64] GROMACS[65] and NAMD[66] are among the most used software packages to perform such calculations. However, this important and widespread method still has its limitations. One of them is the timescale which is possible to survey. Long timescales are very demanding in terms of calculation time and computational resources. Unless one has access to very expensive resources, such as the ANTON supercomputer[67], it is currently possible to reach only the hundreds of nanoseconds' or few microseconds' timescales, by using graphics processing units[68]. Several techniques have recently emerged to overcome such limitation and sample a broader conformation space: **accelerated molecular dynamics**[69,70], **conformational flooding**[71,72], **hyperdynamics**[73,74], and **metadynamics**[75,76], which allow sampling of a conformational space comparable to the millisecond timescale.

Tunnel dynamics can also effectively be studied by simulating protein-ligand complexes. Ligands may induce conformational changes in the protein and by this way affect the shape of the tunnel and/or frequency of gating. This can be studied by MD simulations that make use of extra forces to let the ligand move through the tunnels, either randomly in the **random acceleration molecular dynamics**, RAMD[77,78], or with directed forces in the **steered molecular dynamics**, SMD[79–81]. These procedures can also elucidate the energy profile along the ligand pathway, the preference of a certain ligand for one or another tunnel, or the tunnel-specificity towards particular ligands.

Because of the high complexity of most systems, visual inspection is unlikely to be sufficient for identifying the voids in protein structures, such as clefts, pockets, pores, tunnels, and channels. Several specialized software tools are currently available to accurately calculate those voids. The most commonly used programs for calculation of tunnels and channels in proteins are **CAVER**,[15] **MOLAXIS**,[82] and **MOLE**[83]. These programs mainly differ in the model used to describe the protein and the boundary between the surface and the bulk solvent; the algorithms used to calculate the tunnels and the cost of individual tunnels; the way of treating multiple tunnels and the ability to analyse multiple structures. All of them can identify the static tunnels in single structures, but only the first two can handle ensembles of structures to calculate dynamic tunnels. Detailed description and comparison of these tools is provided in the recent review by Brezovsky et al.[84]

2.3. Molecular engineering

It has been observed in many cases that the mutation of residues far from the active site have led to important enhancements in enzymatic properties such as activity, specificity, enantioselectivity, stability, etc. Whereas for most enzymes with solvent-exposed catalytic sites the mutations in or near the substrate-binding residues have been more successful[85], for

the enzymes with buried active sites the situation can be different. It may be difficult to find the residues susceptible to mutagenesis without disrupting the active site architecture. On the other hand, mutations targeting the residues far away from the active site are more likely to be accepted without loss of function. Considering the important roles of the access pathways, their modification appears to be an attractive possibility for generating functional variants with rationally tuned properties.

For the enzymes with buried active sites and rate-limitation at the substrate entry or product release, the **catalytic activity** can be effectively engineered by pathway modification. The most promising residues to perform positive mutations are those forming the bottleneck. There are a number of reports on activity improvements by modification of tunnel residues. Among these we may find **oxidoreductases** (cholesterol oxidase[86,87], pyruvate dehydrogenase[88], ferredoxin glutamate synthase[89], carbon-monoxide dehydrogenase[90], catalase[91–93], toluene-*o*-xylene-monooxygenase[94,95], toluene 4-monooxygenase[96–98], 4-hydroxybenzoate hydroxylase[24], cytochrom P450[99–104], **transferases** (glucosamine-6-phosphate synthase[105], β-ketoacyl-acyl-carrier-protein synthase[106], undecaprenyl pyrophosphate synthase[107], RNA-dependent RNA polymerase[108]), **hydrolases** (lipase[109,110], acetylcholinesterase[111,112], epoxide hydrolase[113], haloalkane dehalogenases[114,115]), **lyases** (tryptophan synthase[116,117], 3-hydroxydecanoyl-acyl carrier protein dehydratase[118], halohydrin dehalogenase[119]), and **isomerases** (squalene-hopene cyclase[120]), asparagine synthetase[121], carbamoyl phosphate synthetase[54,122]). In our laboratory, Pavlova et al.[115] performed saturated mutagenesis in the tunnel residues of the haloalkane dehalogenase DhaA, aiming at increasing its ability to degrade the toxic anthropogenic compound 1,2,3-trichloropropane. These efforts resulted in the discovery of a variant DhaA31 containing five mutations, four of them located in the access tunnels. DhaA31 showed 32-fold enhancement in the overall catalytic activity due to an increase in rate of the carbon-halogen bond cleavage rate and a shift of the rate-

limiting step to the product release. Modelling studies revealed that the origin of the enhanced activity is the lower number of water molecules in the active site, which would otherwise hinder the formation of the activated complex.[115]

Mutations in the tunnel residues may also modulate the **enzyme specificity** or the **enantioselectivity**. This is rationalised considering that the access tunnels are the first sieves prior to the molecules access the active site. Hence, by changing their physicochemical properties or stereochemistry, one may tune the type of substrates or stereoisomers that are able to pass through and enter the active site. Examples can be found among **oxidoreductases** (aminoaldehyde dehydrogenase[123], amine oxidase[124], toluene 4-monooxygenase[98], 4-hydroxybenzoate hydroxylase[24], cytochrom P450[99], alkane hydroxylase[125]), **transferases** (chalcone synthase[126,127], polyketide synthases[128], cellobiose phosphorylase[129], octaprenyl pyrophosphate synthase[130], undecaprenyl pyrophosphate synthase[107]), **hydrolases** (arylesterase[131], lipase[110,132,133], epoxide hydrolase[113,134], haloalkane dehalogenase[114,135]), **lyases** (hydroxynitrile lyase[136,137]), **isomerases** (squalene-hopene cyclase[120]). Chaloupkova et al.[114] from our lab designed and constructed a complete set of single-point mutants of the haloalkane dehalogenase LinB at the position L177, which is the residue located near the entrance to the main tunnel. Fifteen active variants showed activities and specificities towards the halogenated substrates very different from the wild type, and the activities correlated with the size and the hydrophobicity of the amino acid introduced.

Improvements of **protein stability** may also be achieved through tunnel engineering. It may occur in case the permeability of the tunnels to the water or organic solvent is changed in such a way that the hydrophobic packing of the protein is enhanced. In this case the protein becomes less affected by the presence of an organic cosolvent or temperature rise, thus increasing its general stability. This has been the case of the haloalkane dehalogenase variant DhaA85, which carried four mutations in the tunnel-lining residues[138]. Compared to the wild

type enzyme, this variant revealed an increase of its melting temperature by 19 °C in aqueous buffer, and a half-life rise from minutes to weeks in 40% dimethyl sulfoxide.

One last example of how significantly the enzyme catalytic properties can be affected by modifications on the access tunnel is the change of the **mechanism** of the catalytic cycle. Biedermannova et al.[139] have observed a change in the kinetics mechanism for the conversion of 1,2-dibromoethane by the LinB L177W variant, associated with a dramatic change of substrate specificity. The substitution of the tunnel-lining leucine at position 177 for a bulkier tryptophan changed the bromide ion binding kinetics from a one-step to a two-step mechanism, and significant drop in the bromide release rate (from >500 s^{-1} to 0.8 s^{-1}).

3. PROTEIN GATES

3.1 Structural basis and function

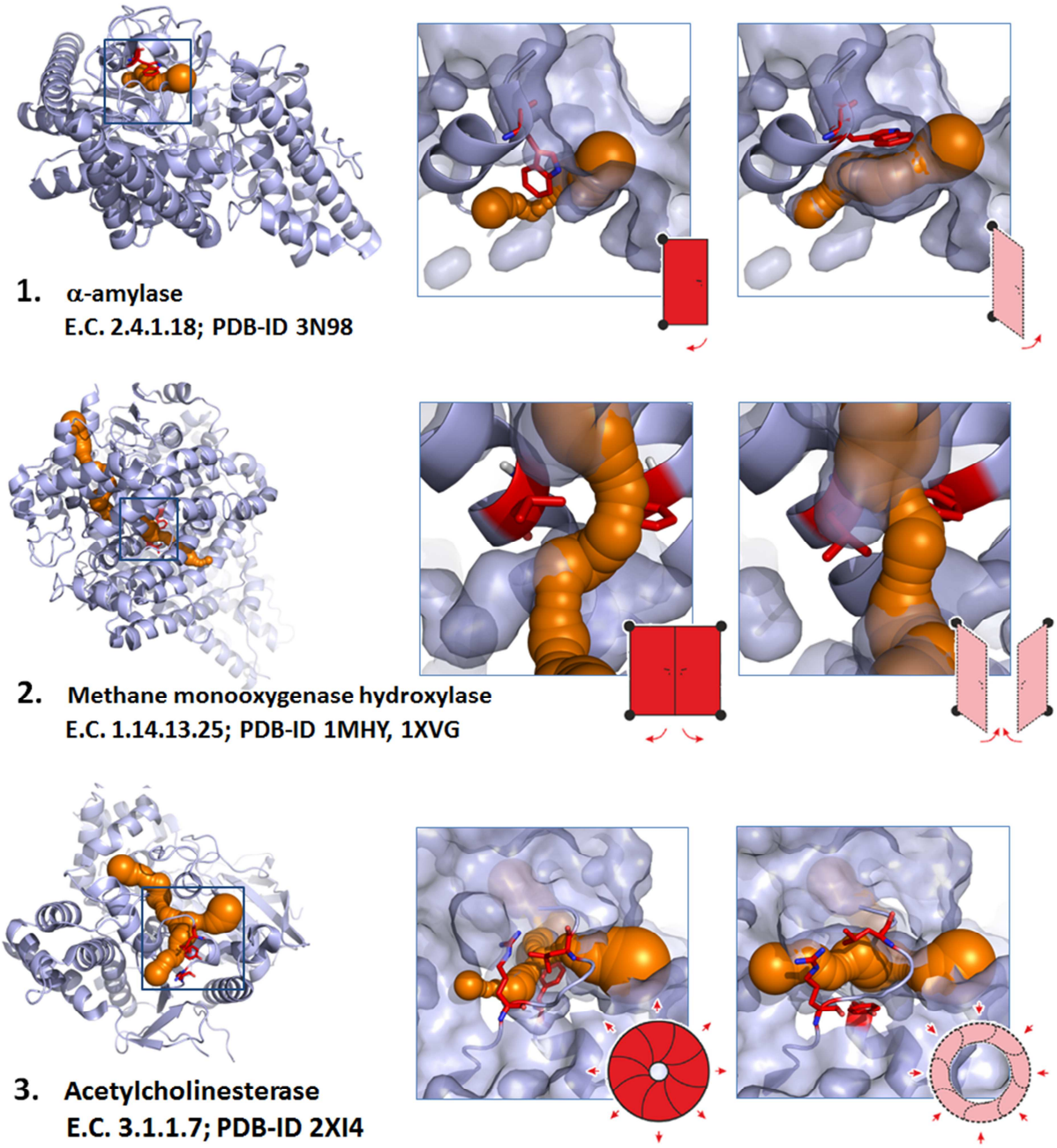
Many enzymes possessing tunnels or channels also contain some type of gate, since the traffic of ligands, ions and solvent in those pathways is susceptible to regulation. However, the gates are not limited to the enzymes containing tunnels. Molecular gates can be found in a wide variety of biological systems, such as enzymes, ion channels, protein-protein complexes, and protein-nucleic acid complexes. The gates present in enzymes may have three major roles: (**1**) control the access of the substrate to the active site; (**2**) control the access of the solvent to the active site; and (**3**) synchronization of molecular events occurring at different locations of the protein.

Considering the **substrate access** (**1**), the gates may account for the substrate specificity of the enzyme. Based on physico-chemical (polarity, lipophilicity, charge, polarizability, etc.) and geometric (bulkiness, length, stereochemistry, etc.) properties, gates can act as filters that control which compounds can pass through and which cannot. Examples of such case is the NiFe hydrogenase, which blocks the access of oxygen over carbon

monoxide[140,141], the catalase which is more permeable to the entry of hydrogen peroxide than water[142,143], cytochromes P450[36,144], epoxide hydrolases[113], and undecaprenyl-pyrophosphate synthases[107], cellobiohydrolase I[145].

Concerning the **solvent access** (**2**), in some cases the catalytic reaction requires reduced number or absence of water molecules in the active site. In those cases it is fundamental to control the access of solvent to the catalytic site by a gate. The mechanisms regulating solvent accessibility can permit entry of the solute alone, allow entry of only a limited number of water molecules, or even to restrict the access of water molecules to some parts of the active site cavity, e.g., cytochromes P450[36], carbamoyl phosphate synthetase[122], imidazole glycerol phosphate synthase[146] and glutamine amidotransferase[105,147]. In other cases, water molecules are not allowed to enter the active site, unless the substrate or cofactor is present, such as in rabbit 20α-hydroxysteroid dehydrogenase[148].

Synchronization of reactions (**3**) may occur in the enzymes with more than one active site, interconnected by the tunnels. In this case only the proper intermediate from the first reaction is allowed to cross the gate and access the second site. This may be necessary in cases of instability or toxicity of intermediate, or to avoid their unfavourable hydration. Examples of enzymes with gates involved in synchronization of reactions are the carbamoyl phosphate synthetase[122], asparagine synthetase[121], glucosamine 6-phosphate synthase[105], and glutamate synthase[149], all of which possess tunnels for ammonia transportation, and the first one also for carbamate; tryptophan synthase for indole[117], and carbon monoxide dehydrogenase/acetyl coenzyme A synthase for carbon monoxide transportation[150].


1. α-amylase
E.C. 2.4.1.18; PDB-ID 3N98
2. Methane monooxygenase hydroxylase
E.C. 1.14.13.25; PDB-ID 1MHY, 1XVG
3. Acetylcholinesterase
E.C. 3.1.1.7; PDB-ID 2XI4

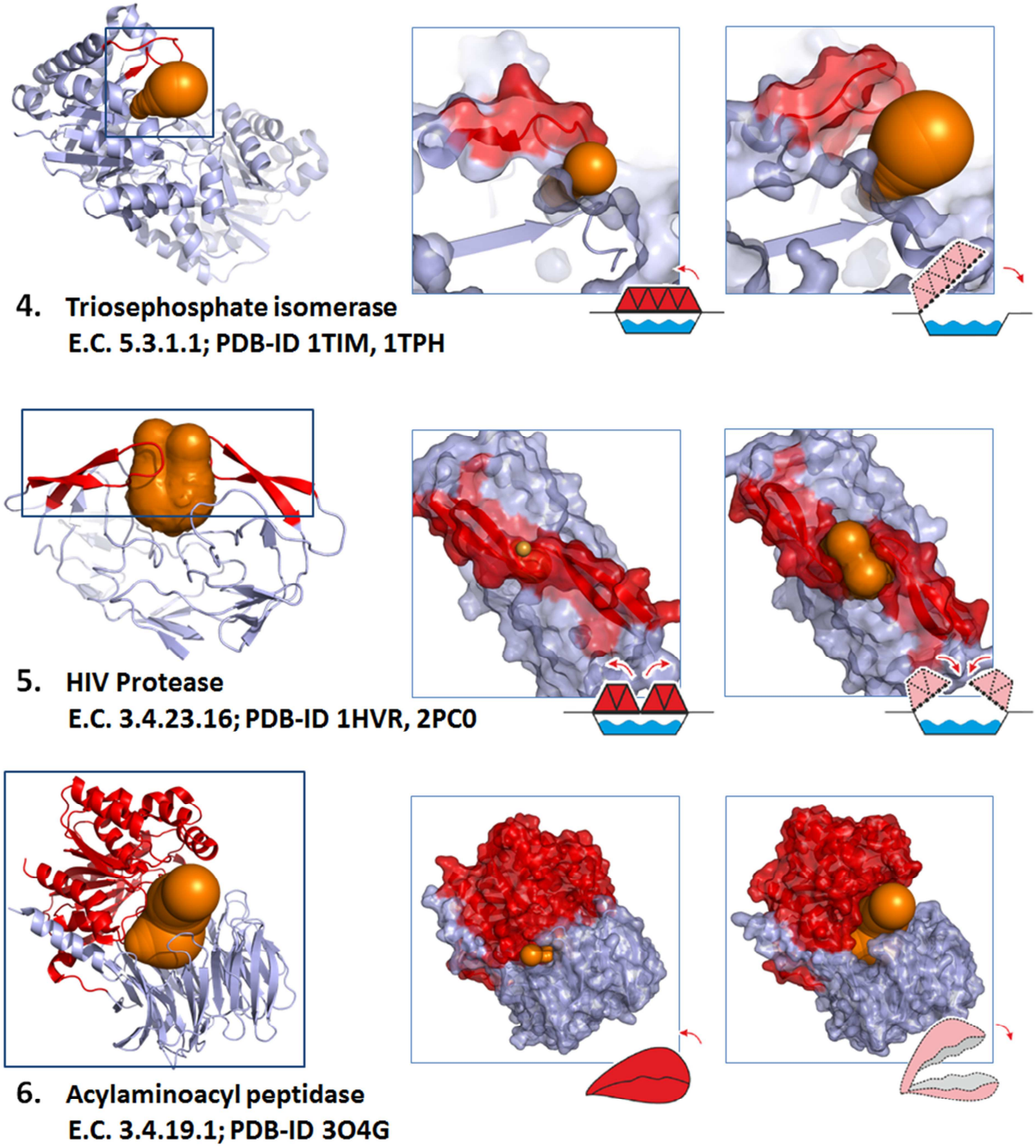


Figure 3. Classification of molecular gates. Examples and respective schematic representation: *wing* (**1**, α-amylase), *swinging door* (**2**, methane monooxygenase hydroxylase), *aperture* (**3**, acetylcholinesterase), *drawbridge* (**4**, triosephosphate isomerase), *double drawbridge* (**5**, HIV protease), and shell (**6**, acylaminoacyl peptidase). The gating elements are represented in red colour and the access tunnels in orange.

Molecular gates can operate based on a very diverse **structural basis**, involving side chain conformational changes of one or more residues, movement of the backbones of a few residues, of longer peptide chains, loops or other secondary elements, or even the motions of entire domains. Gates have been classified according to their structural-basis: (**1**) wings – single residue motion; (**2**) swinging doors – two residues motion; (**3**) apertures – backbones motion of several residues; (**4**) drawbridges and (**5**) double drawbridges – motions of loops and secondary elements; (**6**) shell – motion of a domain (Figure 3)[14].

Wing (**1**) corresponds to the side chain rotation of one single residue. It represents the simplest of all types of gating mechanisms and is also the most common[14]. The movement of wing gates cannot be large in amplitude and have quite small activation barriers. They are typically located at the bottlenecks of tunnels or channels. Interactions with certain residues, termed "anchoring residues", allow stabilization of each state. Such interactions can be H-bonds, salt bridges, π-π contact, etc. The most common amino acids involved in this type of gating are W, F and Y[14]. Examples of the enzymes containing wing gates are the imidazole glycerol phosphate synthase[146], cytidine triphosphate synthetase[151], methane monooxygenase hydroxylase[152], FabZ β-hydroxyacyl-acyl carrier protein dehydratase[118], cytochrome P450[144,153], and cellobiohydrolase I[145].

Swinging door (**2**) corresponds to two amino acids' side chains moving in a synchronized manner and represents the second most frequent type of gating. In this case, the closed state involves a close interaction between the gating residues, operated either through π-π stacking (F-F, F-Y, W-F, pairs), ionic interactions (R-E, R-D pairs), aliphatic hydrophobic contacts (F-I, F-V, F-L pairs), aliphatic interactions (L-I, L-V, R-L, pairs), or H-bonds (R-S pair). However, the most common interacting pair in the swinging-door type of gate is F-F[14]. Examples of enzymes bearing such type of gates are the acetylcholinesterase[154],

toluene-4-monooxygenase[155], and the cytochromes $P450_{3A4}$[102,156], $P450_{cam}$, $P450_{BM3}$, and $P450_{eryF}$[144,153].

Aperture (3) corresponds to another type of residue movements, this time involving the backbone atoms of two to four residues without the need for side chain movements. In this case it is common to observe several of the bottleneck residues of a tunnel performing a synchronized motion towards each other. Enzymes containing this type of gating are, for example, the carbamoyl phosphate synthetase[122], choline oxidase[157], glutamate synthases[158], extradiol dioxygenases-homoprotocatechuate 2,3-dioxygenase[159], cytochrome $P450_{eryF}$[144], and acetylcholinesterase[160].

Drawbridge (4) and **double drawbridge** (5) function by the motions of loops or secondary structure elements, involving one or two elements, respectively. These types of gates are privileged mechanisms to control the access of large ligands, which cannot be accomplished by the previously described types of gates. The loops may even be involved in the formation of the binding cavity for the substrate or cofactor. In some cases, the gating can also be part of complex machinery that controls the opening and closing of different tunnels, merges several tunnels, or even forms smaller and more selective gates. The cytochrome P450 family is a good example of such large complex gating system[36,102,161,162].

Shell (6) is represented by large motions of entire protein domains. It can be found in ion channels[12], but also in enzymes that catalyse reactions with very large substrates, such as RNA polymerase[163]. Such type of domain-displacement gating may also serve the purpose of a stricter control of the tunnel networks, in order to prevent the substrate leakage, e.g., dehydrogenase/acetyl coenzyme A synthase[90], epoxide hydrolase from *Mycobacterium tuberculosis*[164], phospholipase A2[165], and prolyl oligopeptidase[166].

Concerning the **location**, the enzyme gates can be found at: (**1**) the entrance to or even at the active site itself; (**2**) the mouth or the bottleneck of the access tunnel; (**3**) the interface between the active site and cofactor binding site (**Figure 2**).

Entrance to the active site (1) is a very suitable location for a gate which directly controls the access of the substrate to the active site. It can either prevent the entry of the substrate before the catalytic residues are properly oriented or act as a substrate sieve to control selectivity. In particular cases, the gating may even be operated by the residues which are part of the active site[167]. Examples of enzymes bearing gates at the catalytic site or its entrance are acetylcholinesterase[154], imidazole glycerol phosphate synthase[146], glutamate synthase[149], toluene-*o*-xylene monooxygenase[167], monooxygenase[168], choline oxidase[157], NiFe hydrogenases[141], carbonic anhydrases[169], formiminotransferase-cyclodeaminase[170], type III polyketide synthases[171], and FabZ β-hydroxyacyl-acyl carrier protein dehydratase[118].

Mouth or bottleneck of tunnels (2) is the most common location of a gate in enzymes[14]. Tunnels connecting buried active sites with protein surface are privileged structures to control the access of ligands and solvent. Therefore, they are naturally privileged locations for the gates. The mouth of the tunnel is the first barrier that a molecule faces before entering into a tunnel. On the other hand, the bottleneck is the narrowest part of a tunnel, and it may be regarded as one of the easiest hotspots for controlling the molecules being transferred. Figure 2 presents different dynamics of the bottleneck radius in a tunnel with and without a gate. Examples of enzymes containing gates located in tunnels are the cholesterol oxidase type I[172], toluene-4-monooxygenase[155], undecaprenyl-pyrophosphate synthase[107], homoprotocatechuate 2,3-dioxygenase[159], 4-hydroxy-2-ketovalerate aldolase/acylating acetaldehyde dehydrogenase[173], epoxide hydrolase from *Aspergillus niger* M200[113], and FabZ β-hydroxyacyl-acyl carrier protein dehydratase[118], glucosamine 6 phosphate synthase[105],

imidazole glycerol phosphate synthase[146], cytidine triphosphate synthetase[151], carbamoyl phosphate synthetase[122] and glutamate synthases[149].

Entrance to cofactor cavity (3) is also a suitable location for a gate, since the interface between the cofactor cavity and the active site often plays a critical role in the catalytic process. The gates in these locations may control the binding rate of the cofactor to the enzyme. Examples are the NADH oxidase[174], 3-hydroxybenzoate hydroxylase[175], 4-hydroxy-2-ketovalerate aldolase/acylating acetaldehyde dehydrogenase[173], and cholesterol oxidase type I[172] and type II[176]. In more specific cases, the cofactor may perform the gating function by opening the access tunnels for the substrates, such as in digeranylgeranylglycerophospholipid reductase[177].

3.2. Identification methods

Identification and description of a gating process is not simple task because of their complexity, and hence experimental and modelling techniques are usually combined with each other. The **X-ray crystallography** can provide important insights on the possible presence of gating mechanisms in some proteins. The existence of different crystal structures with amino acid residues, or even larger elements, in different conformation, may be a good indication of a gating process occurring in that system. Trough X-ray ensembles, for instance, it is possible to infer about protein flexibility, dynamics and function[178]. However, it is necessary that both open and closed states have been captured with significant representation. Examples of enzymes with gates identified by this technique are the tryptophan synthase[179], haloalkane dehalogenase LinB[43], L-amino acid oxidase[180], toluene-*o*-xylene monooxygenase[181], acetylcholinesterase[160] and phospholipase A2[165].

Similarly to the identification of the protein tunnels, the **NMR spectroscopy** may supply important evidences regarding the presence of gates in proteins. Advanced solution or

solid state NMR methods may be used to study the dynamics of protein systems and give insight to the conformational changes. These methods may survey very different timescales, ranging from picoseconds to seconds,[57–59] thus allowing to study different types of gating mechanisms. It is even possible to study the less populated conformations and the exchange rate between different conformations[62,182,183]. NMR spectroscopy has been used, for instance, to investigate the conformational changes in the gating of triosephophate isomerase[184–186], HIV-1 protease[187], and dihydrofolatereductase[188].

Fluorescence-based methods have become very popular in investigating biomolecular systems, namely in detecting and characterizing conformational changes in proteins. Hence, although not often used for that purpose, they have great potential to investigate protein gates. Fluorescence emission by fluorophore groups is dependent of the immediate surrounding environment, namely the polarity of the neighbouring molecules or residues. Hence, it can be used to detect changes in the microenvironment of fluorescent residues within the proteins, thus allowing tracking of eventual conformational changes. **Intrinsic tryptophan fluorescence emission** (ITFE), for instance, has been used to study the closed and open conformations of a dimeric phospholipase A2 homologue[165] and cytochrome c oxidase[189]. Other methods employ unnatural fluorescent probes to track the dynamics of proteins. These can be covalently bonded[190], or inserted as part of the protein by mutagenesis with unnatural fluorescent amino acids[191]. **Time-resolved fluorescence spectroscopy** can be applied with different methods and assess the dynamics of events occurring in timescales ranging from femtoseconds to nanoseconds[192]. This technique has been used, for example, to study the hydration and protein dynamics at the tunnel mouth of haloalkane dehalogenases[190,193]

Fluorescence (or Förster) resonance energy transfer (FRET) is a method that makes use two fluorophores, a donor and an acceptor, which perform nonradiative energy transfer with each other and are bound to the protein at a certain distance. The efficiency of this energy

transfer is proportional to the sixth power of the distance between the two fluorophores, and is correlated to the changes of their fluorescence spectra. Since the conformational changes in the protein will affect the distance between the two fluorophores, this method can report on the dynamics of the specific parts of protein. FRET is a versatile technique that can be used not only intra, but also intermolecularly to study the protein functions and interactions with other proteins, or even in living cells.[191,194–196] It can be applied on average ensembles or single-molecule studies, giving great insight on the dynamics and kinetics of conformational changes occurring in timescales ranging from nanoseconds to seconds or even minutes[197–199].

MD simulation is a very important theoretical technique for identifying and characterizing protein gates. This method has been described in section 2.2, and it can be used to sample the different conformational states of a protein gate, their respective energies, and interconversion frequencies. It may be difficult to survey the timescales of certain gates involving larger movements by using classical MD, namely apertures (ns–μs), drawbridges and double drawbridges (ns–μs), or shell gates (ms–s)[14]. In these cases, the enhanced-sampling techniques, i.e., **accelerated molecular dynamics, conformational flooding, hyperdynamics** and **metadynamics** must be used. In addition to these, the **Brownian dynamics** simulations have also been used to investigate gates of enzymes[157,200], while several other methods may be used to study the dynamics of ligand binding to proteins and give important insight to gating processes[201]. For the proteins bearing gates at their tunnels, the study of tunnels' dynamics is essential. For that, the use of specialized programs for performing tunnel analysis in MD trajectory is necessary to determine bottleneck residues and potential key residues involved in the gates. **CAVER** 3.0[15,84] is currently the only software tool that can handle analysis of large trajectories and supply information about various time-dependent tunnel properties.

3.3. Molecular engineering

Three main approaches of gate engineering can be followed: (**1**) Gates can be modified by mutating the residues of existing gates, the hinge or the anchoring residues. In this way it may be possible to rationally modify the gate amplitude, frequency or the affinity towards certain substrates or solvent. (**2**) Gates can be removed by mutating the gating residues in order to leave the pathway permanently open. This could lead to an increase of the ligand exchange rate, but also change the access of the solvent. (**3**) Gates can be introduced into enzyme pathways that were originally open, thus providing control over the transport of substances. It can be achieved by mutating tunnel-lining residues, preferably in the bottleneck or in the entrance.

Modification of **catalytic activity** by gate engineering can be easily rationalized considering exchange of substrates and products at gate-controlled rates, which limit the overall catalytic cycle. Gate can also control access of water molecules to the active site, making the chemical reaction more or less favoured. There are many examples wherein the enzyme activity has been changed by mutation of gate residues. Oxidation of *p*-nitrophenol in toluene-*o*-xylene monooxygenase was improved the by 15-fold due to E214G mutation[94]. Also in lipase from *Burkholderia cepacia* it was observed an overall 15-fold increase of specific activity towards (*R*,*S*)-2-chloro ethyl 2-bromophenylacetate by the double mutation L17S+L287I[110]. Several mutations on V74 and V74+L122 residues in NiFe hydrogenase attained reduced transport rates of CO and O_2 molecules through the tunnel, thereby increasing the resistance of that enzyme to the inhibition by these molecules[141]. The gate removal in tryptophan synthase with the F280C or F280S mutations led to increase the rate of indole binding[116]. In imidazole glycerol phosphate synthase, gate removal by T78A mutation increased the ammonia transfer rate and also the overall enzyme activity[146]. On the other hand, a gate disruption by R5A led to an increased access of water to the active site, which impaired

the enzyme activity. The same mutation also caused ammonia to leak through the inter-domain tunnel to the bulk solvent, resulting in 10^3-fold decrease of the cyclase reaction rate[146]. A similar ammonia leakage was observed with G359F and G359Y mutations in carbamoyl phosphate synthetase[202,203]. The gate removal in FabZ β-hydroxyacyl-acyl carrier protein dehydratase by mutation Y100A leaves the active site exposed to the bulk solvent and results in a much stronger binding of the product to the active site, reducing the enzyme activity by 50%[118]. The DhaA31 mutant with enhanced activity for 1,2,3-trichloropropane, developed in our laboratory by Pavlova et al.[35,115], contains substitutions at the main tunnel residues C176Y and V245F. Unpublished MD simulations demonstrated the existence of a gating mechanism involving these residues, which controls the access of ligands and solvent to the tunnel. This is a case of gate insertion that resulted in improvements of the enzyme activity.

Substrate-specificity of the enzymes has also been modified by gate engineering. It can be rationalized by the fact that gates control the nature and geometry of the substrates accessing the active site and change in the gate may result in shift of substrate specificity. The specificity increase of toluene-*o*-xylene monooxygenase towards the oxidation of *p*-nitrophenol was observed upon mutation of gating E214G[94]. A double mutant (L17S+L287I) of *Burkholderia cepacia* lipase resulted in 178-fold improvement of the *E*-value towards (*R*,*S*)-2-chloro ethyl 2-bromophenylacetate compared to the wild-type.[110] Q230P mutation at the hinge region of rabbit 20α-hydroxysteroid dehydrogenase decreased the flexibility of loop B, which led to narrowing its specificity compared to the wide range of substrates[148]. The gate removal from toluene-4-monooxygenase, by the D285I and D285Q mutations at the tunnel entrance, increased its ability to hydroxylate bulkier substrates as 2-phenylethanol and methyl-*p*-tolyl sulphide by 8- and 11-fold, respectively[96]. Mutations at the access tunnel of *Candida rugosa* lipase changed its substrate specificity in terms of the fatty acids' chain lengths accepted. Introduction of bulkier aromatic residues at the entrance or inside the tunnel

changed the substrate specificity profile. We speculate that these mutations are likely to have introduced some type of gating common for F and W residues, e.g., wing or swinging door gates[132].

Modulation of **product specificity** by mutation of gate residues has been reported in a few cases. *Escherichia coli* undecaprenyl-pyrophosphate synthase condenses the isopentenyl pyrophosphate with allylic pyrophosphate units to generate linear isoprenyl polymers. It was found that a gate formed by the flexible loop controls the extent of the reaction and the product release, and thus the length of the polymer formed. The L137A mutation, located at the bottom of the tunnel, led to formation of C_{70} polymer rather than the smaller C_{55} in the wild-type. On the other hand, the A69L mutant produces the smaller C_{30} polymer instead[107].

To the best of our knowledge, there have been no reports regarding **protein stability** enhancements achieved by gate modification to date. However, considering the structural basis for improving enzyme stability[204–206], it should be in principle possible to construct stable mutants by engineering enzyme gates.

4. CONCLUSIONS

Molecular tunnels, channels and gates are structural features widely represented in the protein world. Protein tunnels can be found in any enzyme containing a buried active site, in which they serve as a pathway connecting the active site with bulk solvent or connecting multiple active sites. We note that proteins containing tunnels of some type can be found in all six classes of enzymes and same is true also for enzyme gates. The gates can be very diverse in terms of localization, involved structural elements, amplitude and frequencies of motion.

Tunnels, channels and gates play important roles and in some cases are essential for enzymatic catalysis. They control the transport of small ligands and solvent molecules to and from the active site, and in this way modulate enzyme activity and substrate specificity.

Furthermore, they enable synchronization of molecular events taking place in distinct part of the protein structure and control properties of the active site environment during individual phases of a catalytic cycle.

We have surveyed existing methods suitable for the study of tunnels, channels and gates in the protein structures. The X-ray crystallography is one of the most important experimental tools for identification of permanent tunnels and channels, while it is less suitable for visualization of transient structures and the gates. The NMR spectroscopy may supply missing information on dynamical protein structures. Fluorescence spectroscopic methods may provide additional evidences for conformational changes in proteins and reveal details about the gating processes. Classical and enhanced variants of MD are currently one of the most useful tools for identifying and characterizing transient tunnels and gates. Combined with specialized void-detection tools, MD allows exploration of a large conformational space and study of the mechanisms involved in dynamical processes.

Due to their structural and functional importance, molecular tunnels and gates appear to be very attractive targets for protein engineering. The provided examples demonstrate that, by changing a specific tunnel or gate residue, it is possible to modify enzyme properties such as activity, specificity, enantioselectivity and stability. It is already possible to tune properties of a target enzyme by performing such modifications in a rational manner. With further expansion of our knowledge on enzyme tunnels and gates, it will be possible to design these fascinating structural features *de novo* in the future.

5. ACKNOWLEDGEMENTS

The authors would like to thank the Grant Agency of the Czech Republic (P503/12/0572), the Czech Ministry of Education of the Czech Republic (LO1214, LH14027 and LQ1605) for financial support. S.M. is supported by the SoMoPro II Programme (project BIOGATE, Nr. 4SGA8519), which is co-financed by the People Programme (Marie Curie action) of the Seventh Framework Programme of EU

according to the REA Grant Agreement No. 291782, and the South Moravian Region. J.B. is supported by the project REGPOT financed by the European Union (316345). Computational resources were provided by the CESNET LM2015042 and the CERIT Scientific Cloud LM2015085, provided under the programme "Projects of Large Research, Development, and Innovations Infrastructures". What was exposed here reflects only the author's view and the Union is not liable for any use that may be made of the information contained herein.

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