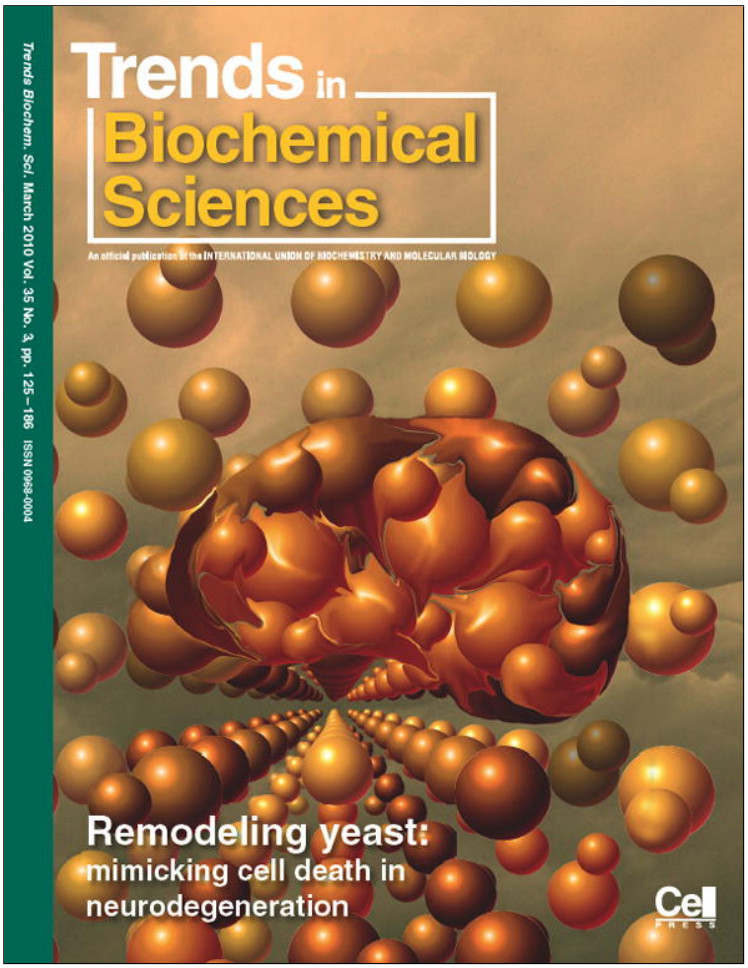

Review

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The gates of ion channels and enzymes

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Protein dynamics are essential for virtually all protein functions, certainly for gating mechanisms of ion channels and regulation of enzyme catalysis. Ion channels usually feature a gate in the channel pore that prevents ion permeation in the closed state. Some bifunctional enzymes with two distant active sites use a tunnel to transport intermediate products; a gate can help prevent premature leakage. Enzymes with a buried active site also require a tunnel for substrate entrance; a gate along the tunnel can contribute to selectivity. The gates in these different contexts show distinct characteristics in sequence, structure and dynamics, but they also have common features. In particular, aromatic residues often appear to serve as gates, probably because of their ability, through side chain rotation, to effect large changes in cross section.

Gates in different contexts

Ion channels can be viewed as the gateways into cells. A typical ion channel shuttles between an open state and a closed state; the conformational transition between open and closed states is referred to as gating. Channel opening is triggered by a specific stimulus such as the voltage or pH difference across the cell membrane or the binding of a ligand [1–5]; upon opening the channel is selective in the type(s) of ions that can pass through its pore. In the closed state the channel must be tightly shut; ion permeation is prevented by energy barriers along the pore, which arise from physical occlusion or the hydrophobic nature of residues lining the pore. The barrier region is appropriately referred to as the gate. Notably, amino acid substitutions in this region can lead to abnormal channel kinetics [6].

A different kind of channel exists in bifunctional enzymes, in which the product in one active site is the substrate of a distant, second active site. Although the intermediate product can, in principle, diffuse through the bulk solution to the final destination, a tunnel through the interior provides direct passage in a large number of enzymes [7,8]. In these enzymes, a gate might serve to prevent the passage of unintended substrates. Upon forming the intermediate product, the gate might open to allow its passage.

The active sites are buried in some monofunctional enzymes [9–12]. A tunnel then provides an entrance for the substrate and an exit for the product. A gate along the tunnel could contribute to substrate selectivity [13].

The gates in these different contexts form the focus of the present review. The main aim of the review is to expose ion channel scientists to the knowledge of gates in enzymes and vice versa. Although gates and gating mechanisms of different ion channels show distinct characteristics, they share certain common features. Some of these features also appear to be shared by enzymes that contain intramolecular tunnels.

Ion channels

Potassium (K^+) channels are among the best studied ion channels. Structure determination and functional studies of mutant proteins have contributed significantly to the identification of channel gates and the motions leading to channel opening. The pore of a K^+ channel, formed at the center of four identical subunits, is lined by the selectivity filter on the extracellular half and the inner helix on the intracellular half (Figure 1). It is generally accepted that the selectivity filter moves little during channel gating [14,15]. The gate therefore resides in the intracellular half of the inner helix.

At least three modes of gating motions have been proposed. The first consists of rigid-body motions of the pore-lining helices. For example, it was initially proposed [16] that cyclic nucleotide-gated channel 1 (CNG1) opens as the intracellular end of each inner helix rotates around the pore axis while the extracellular end remains fixed (Figure 2a). The rotation opens the channel by widening the pore at the crossing of the inner helices, which occurs within the intracellular half of the inner helix. The inner-helix crossing thus forms the gate here. Similarly, the gating motions of the mechanosensitive channel of large conductance (MscL) are now thought to involve a rigid-body tilt of the pore-lining M1 helices away from the pore axis [17–19].

In the second mode (Figure 2b), the inner helices are, upon activation, assumed to kink at a hinge around the middle; again the inner-helix crossing in the closed state forms the gate. By comparing the structures of the closed KcsA K^+ channel and the open MthK K^+ channel (Figure 1a,b), Jiang *et al.* [14] identified a kink around a Gly residue in the open state. This Gly is highly conserved (Figure 1d), suggesting that helix kinking is a common gating motion among many K^+ and related channels. In particular, substitution of the corresponding Gly (Gly466) into Ala renders the *Shaker* K^+ channel non-functional, whereas an additional substitution (of the next residue, Val467, into Gly) restores channel function [20]. (Another hinge point, within the Pro–Val–Pro sequence further toward the intracellular side, has also been proposed [21].) The helix-kinking mode was also prescribed to G protein-sensitive inwardly rectifying potassium (GIRK;

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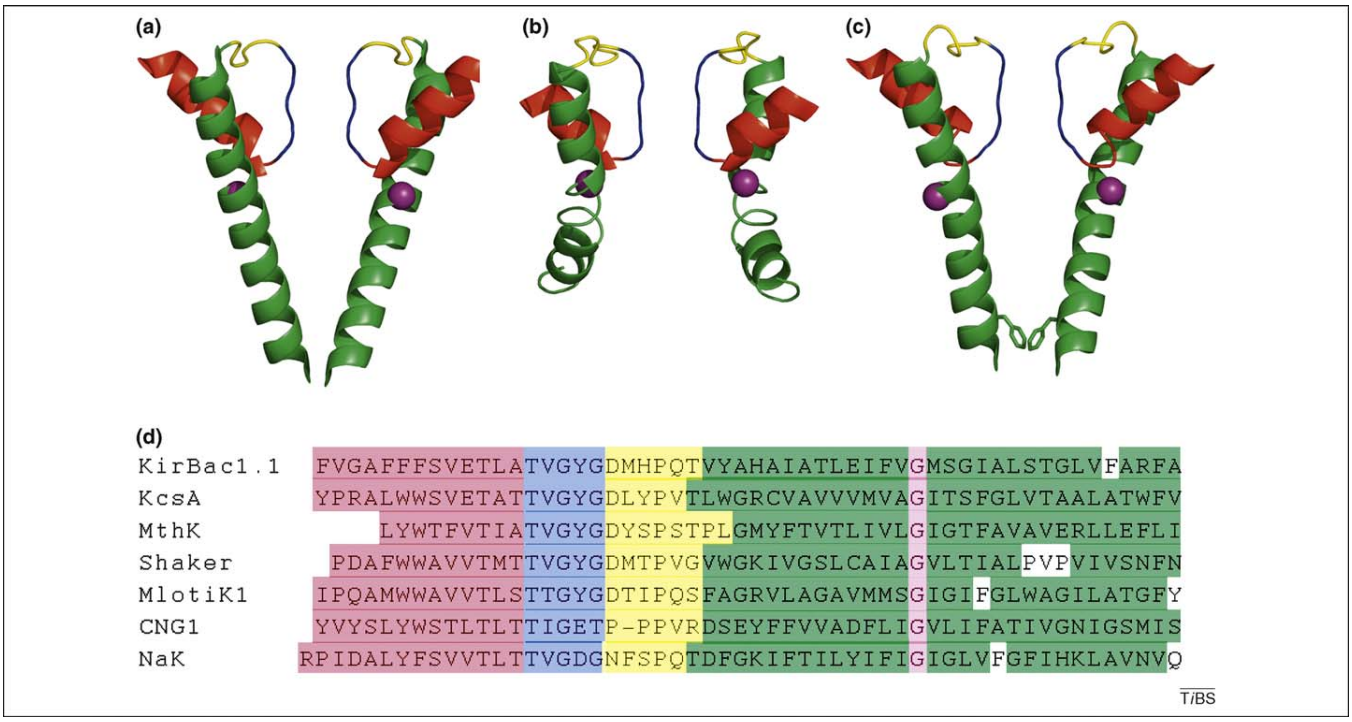

Figure 1. Structures and sequences of potassium channels. Both the structures and sequences follow the same color scheme: red, pore helix; blue, selectivity filter; green, inner helix; conserved Gly, purple. For clarity, only two of the four subunits are shown. (**a**) KcsA (PDB: 1k4c). (**b**) MthK (PDB: 1lnq). (**c**) KirBac1.1 (PDB: 1p7b). The side chain of Phe146, the putative activation gate, is displayed. (**d**) Sequence alignment of representative potassium and related channels. Several key residues noted in the text are highlighted by a white background in the sequences.

also known as Kir3) channels [22,23] and later to CNG1 [24]. The helix-kinking motion has been captured by recent X-ray structures of the open and closed forms of the non-selective cation channel NaK [15,25].

The third mode of gating motions adds a new element to the second mode: an aromatic side chain within the intracellular half of the inner helix projects into the channel pore to seal it off in the closed state. In the open state, the inner helix kinks and the aromatic side chain simultaneously retracts to widen the pore. The role of such an activation gate was assigned to Phe146 in KirBac1.1 [1] and the corresponding Tyr132 in KirBac3.1 [26]. The corresponding residue, Phe181, in a variant of mouse Kir3.1 occludes the channel pore in the closed structure (in this variant, the sequence of positions 83-177 was replaced by that of KirBac1.3) [27]. Two aromatic side chains, Phe203 and Tyr215, seem to play a similar role in the bacterial MlotiK1 channel; Ala substitutions of these residues led to significant increases in the rate of ion uptake [28]. Interestingly, whereas the residues around the inner-helix crossing, notably Gln103, form the gate in the closed state of NaK [25], the side chain of Phe92 projects into the pore to form a new constriction in the open state; Ala substitution of this residue also caused an increase in ion-conductance rate [15].

It is worth noting that the third mode of gating motions is very similar to one recently proposed for the M2 proton channel of influenza A virus [29], which is also homotetrameric and is activated by low pH on the extracellular side. Structure determination in the past decade for this protein [2,30,31] has not provided a mechanism of channel activation. Molecular dynamics simulations, however, led to the proposal that Gly34 serves as a hinge for helix kinking, and the side chain of Trp41 provides an aromatic gate [29,32]. Uptake of a proton from the extracellular side by His37 increases the propensity of helix kinking, which in turn leads to a reorientation of the Trp41 indole from being perpendicular to the pore axis to being parallel. These conformational changes poise the protonated His37 to release its proton to the intracellular side.

Recent structure determination of a bacterial pentameric ligand-gated ion channel in the open state [4,5] along with an earlier structure of a related channel in the closed state [33] has provided molecular details of the gating motions of this family of channels, which includes acetylcholine receptors. In these pentametic channels, each subunit has four transmembrane helices, and the channel pore is lined by the second (referred to as M2) of these helices. The membrane-spanning residues of the M2 helix are assigned positions –2′ to 20′, numbered from the intracellular side to the extracellular side. Relative to the closed state, the M2 helix in the open state is tilted around an axis parallel to the membrane plane. This tilt widens the extracellular end of the pore, which is constricted by three rings of hydrophobic residues (at positions 9′, 13′ and 16′) in the closed state, and slightly narrows the intracellular end of the pore. The gating motions shown by comparing the structures of the open and closed states were, in part, suggested by earlier computational studies. In particular, Yi *et al*. [34] proposed the tilt of the M2 helix as the gating motion of the α7 acetylcholine receptor. It was proposed that this motion was produced by a pull of the M1 helix by the β10 strand of the ligand-binding domain, whose propensity for an upward motion was increased by agonist

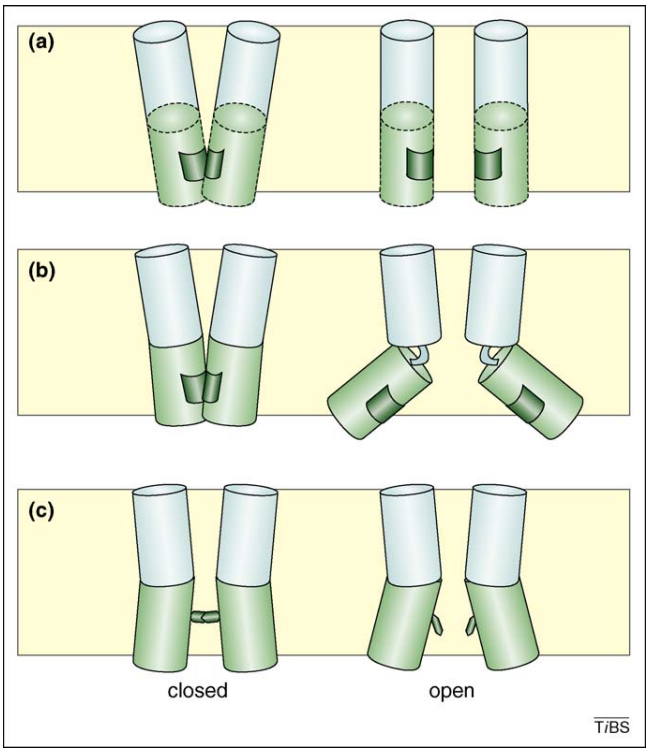

Figure 2. Modes of gating motions, as proposed in the literature for potassium and related channels. (**a**) Rotation of inner helices around the pore axis; (**b**) kinking of inner helix;(**c**) helix kinking plus aromatic gate. In each mode, the depiction on the left represents the closed state whereas that on the right represents the open state. The pale yellow background represents the membrane. The boxed regions in (**a**) and (**b**) and the aromatic rings in (**c**) constitute the gate.

binding. However, this putative role of the M1 helix does not seem to be supported by the structural data, which suggests that the M1 helix is fixed during gating. At the quaternary structure level, the five subunits of the transmembrane domain undergo a clockwise twist in the open state relative to the closed state [4]. Such a quaternary twist was predicted by normal mode analysis [35,36].

The three rings of hydrophobic residues comprise the gate [3], in the sense that they constitute the narrowest part of the closed channel pore. Several amino acid substitutions that lead to disorders in neuromuscular transmission have been found within or bordering the hydrophobic rings [6].

Bifunctional enzymes

Some enzymes catalyze two ordered reactions at two separate active sites, with a product from the first reaction becoming a substrate in the second reaction. For such bifunctional enzymes, efficient transfer of the intermediate product from one active site to another is crucial. In some bifunctional enzymes, such transfer occurs via a tunnel that connects the two active sites [7,8,37,38]. The situation then is similar to that of an ion channel: here the two active sites are similar to the two sides of the cell membrane, the intermediate product is like the permeating ion, and the tunnel is the channel pore. The tunnel might initially be closed to prevent the passage of unintended substrates. Upon completion of the first reaction, the tunnel is opened and the intermediate product is then transferred to the second active site, where the second reaction can finally proceed. In addition to its efficient transfer, sequestration within a tunnel provides a means of preventing a potentially toxic intermediate product from being released to the solvent [39].

The role of the tunnel is well illustrated by glucosamine-6-phosphate synthase, which catalyzes the conversion of D-fructose-6-phosphate (Fru6P) into D-glucosamine-6-phosphate using L-glutamine as a nitrogen source (Figure 3a) [37]. The functional unit of this enzyme is a dimer; each subunit consists of two domains (consisting of residues 1–239 and residues 249–608, respectively) (Figure 3b). The two active sites of this bifunctional enzyme are located on the N-terminal and C-terminal domains, respectively and are connected by a tunnel of 18 Å in length (Figure 3c). At the N active site, the side chain of L-glutamine is hydrolyzed to produce L-glutamate and ammonia. The ammonia produced is then transferred through the tunnel to the C active site, where it is incorporated into Fru6P to produce the final product, D-glucosamine-6-phosphate.

The first step of the two coupled enzymatic reactions is the binding of Fru6P to the C active site. The structure of the enzyme bound with Fru6P was determined by Mouilleron *et al*. [37]. They also determined a second structure, in which glucose-6-phosphate (a Fru6P analog) is bound at the C active site and 6-diazo-5-oxo-L-norleucine is bound at the N active site. The bound 6-diazo-5-oxo-L-norleucine mimics the intermediate produced at the N active site immediately after the production of ammonia. A comparison of these two structures shows how the tunnel is reorganized and opened by glutamine binding and the subsequent reaction at the N active site. The walls of the tunnel are mainly formed by Trp74 and residues 601–607 of the C-terminal tail. Upon binding 6-diazo-5-oxo-L-norleucine, the Q-loop (consisting of residues 73–81) closes the N active site. Importantly, in the Fru6P-bound structure, the Trp74 side chain seals off the tunnel, possibly explaining why this enzyme cannot use exogenous ammonia as a nitrogen source [40]. Along with the closure of the Q-loop, the χ_1 torsion angle of Trp74 changes by 75°, leading to the opening of the tunnel.

Another bifunctional enzyme, tryptophan synthase, has an $\alpha_2\beta_2$ subunit composition [38]. The α subunit catalyzes the cleavage of indole 3-glycerol phosphate into indole and D-glyceraldehyde-3-phosphate and the β subunit catalyzes the condensation of indole and L-serine into L-tryptophan (with a water molecule as a byproduct). The quaternary arrangement of tryptophan synthase shares some similarity with that of glucosamine-6-phosphate synthase (Figure 3b). Whereas the two C-terminal domains of glucosamine-6-phosphate synthase are stacked side by side, the two β subunits of tryptophan synthase are stacked back to back. The two α subunits, like the two N-terminal domains of glucosamine-6-phosphate synthase, are located at the opposite ends. The two active sites are connected by a tunnel 25 Å in length, which provides a passage way for indole, the intermediate product. Midway along the tunnel, the walls are lined in part by Tyr279 and Phe280 of the β subunit, which adopt alternative conformations, resulting in an equilibrium between the open and closed forms of the tunnel [41]. There is evidence that substrate binding to the

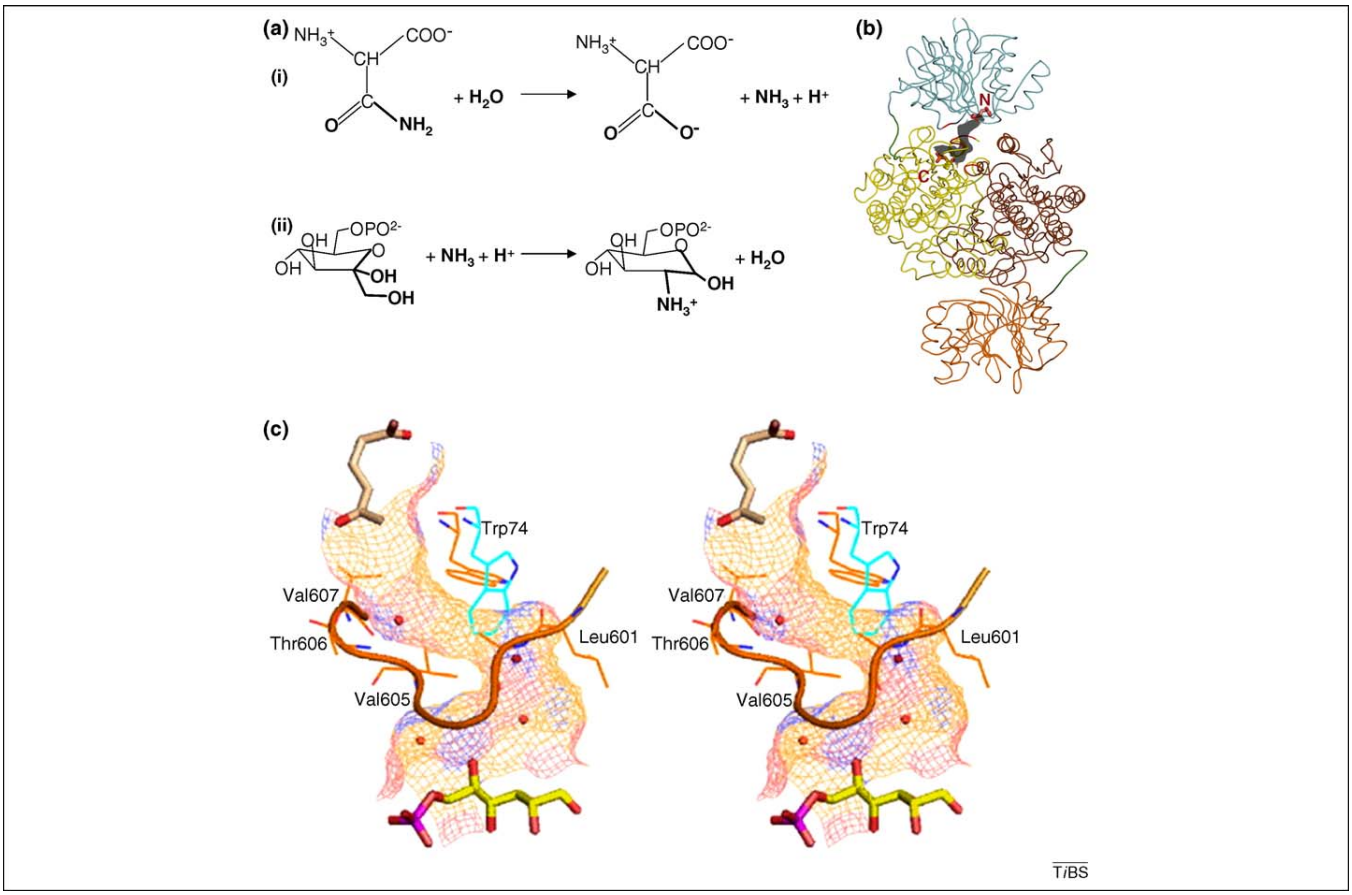

Figure 3. Enzymatic reactions and structure of glucosamine-6-phosphate synthase. (**a**) Reactions taking place at the N and C active sites. (**i**) At the N active site, the side chain of L-glutamine is hydrolyzed to produce L-glutamate and ammonia. (**ii**) At the C active site, ammonia and proton are incorporated into Fru6P to produce D-glucosamine-6-phosphate and water. (**b**) Quaternary arrangement of glucosamine-6-phosphate synthase (PDB: 2j6 h). The N- and C-terminal domains of one subunit are shown in light blue and yellow, respectively; for the other subunit, the colors are orange and brown. The ligands, 6-diazo-5-oxo-L-norleucine and glucose-6-phosphate, respectively, at the N and C active sites are shown as sticks. The tunnel between the N and C active sites is shown in gray. (**c**) The tunnel between the N and C active sites, shown as a stereo pair. The walls of the tunnel are mainly lined by the C-terminal tail (residues 601-607) and Trp74. The Trp74 side chain is shown in two conformations; in one (orange) it leaves the tunnel open whereas in the other (scyan) it blocks the tunnel. Part (**c**) is adapted from ref. [37] with permission; copyright (2006) The American Society for Biochemistry and Molecular Biology.

α subunit leads to an increase in the closed form of the tunnel by altering the conformational preferences of these residues. Upon cleavage of the substrate, the equilibrium between the open and closed forms is presumably restored, so that the transfer of indole to the β subunit can proceed.

Enzymes with buried active sites

The active sites in some monofunctional enzymes are buried; a tunnel fluctuating between alternative conformations can provide transient substrate access and product release. The burial of active sites might prevent catalyzed reactions, such as hydrolysis, of inappropriate substrates [13,42]. In addition, transfer of a substrate, typically a small molecule such as oxygen, through a tunnel could afford temporal control and regiocontrol of its reaction with a larger substrate. The monooxygenase ActVA-Orf6, which catalyzes the oxidation of a phenolic compound, 6-deoxydihydrokalafungin, at the C-6 position into the corresponding quinone, dihydrokalafungin, provides an example of regiocontrol [43]. The enzyme-bound 6-deoxydihydrokalafungin is well shielded from the solvent except for the reactive C-6 position. Oxygen can access this carbon through a narrow tunnel gated by a mobile loop consisting of residues 34–48.

Cholesterol oxidases contain tunnels, which allow for both oxygen access and hydrogen peroxide release. These enzymes are classified according to whether the cofactor flavin adenine dinucleotide (FAD) is covalently linked to the enzyme. In the type-I cholesterol oxidase of *Streptomyces* spp. SA-COO, the side chains of a large number of residues adopt two sets of alternative conformations, one of which yields an open tunnel connecting the active site to the exterior [12]. Among the residues lining the tunnel entrance is Phe359; in the F359W mutant, the larger aromatic side chain is observed only in the closed conformation (whereas other tunnel-lining residues are found in two alternative conformations) [44]. This observation shows that the equilibrium between open and closed forms of the tunnel can be easily perturbed by the size of key residues. In the type II cholesterol oxidase of *Brevibacterium sterolicum*, only three tunnel-lining residues (Ile423, Glu475 and Arg477) display alternative conformations [11]. The open conformation of Arg477 is stabilized by a salt bridge with Glu311; a Glu311 substitution seems to disrupt the open tunnel, altering oxygen binding kinetics [45].

In both types of cholesterol oxidases, cholesterol binding induces tunnel closure [11,12]. The coupled oxidation of

cholesterol and reduction of FAD (into $FADH^-$) presumably triggers the reopening of the tunnel, allowing the access of oxygen to oxidize $FADH^-$ back to FAD (the byproduct hydrogen peroxide is probably released through the same tunnel). Therefore the substrate (oxygen in the present case) reaches the active site through the tunnel at the desired time, illustrating the principle of temporal control.

Yet another potential advantage of gated access to a buried active site, which we first proposed [13], is substrate selectivity. In acetylcholinesterase (AChE), a 20-Å tunnel leading to the active site is gated by a ring of aromatic side chains [9,13,46]. The fluctuations of these side chains are such that the tunnel remains blocked for the substrate, acetylcholine (ACh), most of the time, but occasionally opens wide enough for its passage (Figure 4). Remarkably, however, the catalytic efficiency of this enzyme for ACh ($k_{cat}/K_M \sim 10^9\ M^{-1}\,s^{-1}$) is among the highest of any enzyme. This apparent conflict was resolved within a theory for the substrate binding kinetics that accounts for both the gating dynamics of the tunnel and the diffusion of the substrate toward the active site [47]. This theory predicts that when the switch between the closed and open forms of the tunnel (e.g. rotations of the aromatic side chains in the case of AChE) is sufficiently fast (relative to the diffusion of the substrate), substrate binding through the gated tunnel is effectively unhindered even when the tunnel remains closed most of the time. Such a scenario is exactly what was suggested by molecular dynamics simulations of AChE for the passage of ACh [13,46].

By contrast, for slightly larger substrates such as butyrylcholine (BCh; which harbors two additional methylene groups), the open fraction of the tunnel is substantially reduced; the effectively unhindered scenario would only occur in times scales of the gating dynamics that are too short to be physically possible. As a result, the binding rate of BCh to the AChE active site is predicted to be orders of magnitude lower than that of ACh, in agreement with the observed disparity in catalytic efficiency between these two substrates [48,49]. The dynamics of the gating aromatic side chains thus affords AChE substrate selectivity (ACh over BCh) without sacrificing the catalytic efficiency of the intended substrate, ACh. The role of the aromatic side chains in guiding substrate selectivity is further supported by the observed increase in catalytic efficiency for BCh when some of these side chains were substituted with smaller side chains [48,50]. A gated tunnel to the active site of catalase, which converts two hydrogen peroxide molecules into one oxygen and two water molecules, has also been suggested to afford similar substrate selectivity [10].

Ho recently presented an interesting discussion of the diversity of roles of intramolecular transfer of substrates and products [51]. This discussion focuses on photosystem II, the large, transmembrane multisubunit enzyme that converts water to oxygen molecules, thus providing protons for ATP production and electrons for photosynthesis. This process requires the delivery of water molecules to the oxygen-generating center, located at the heart of the enzyme, and the directional delivery of protons from this center to the appropriate side of the thylakoid membrane to maintain the proton gradient needed for ATP synthesis. The resulting oxygen molecules must also be released from the center in a controlled fashion to avoid harmful side-reactions. Each of these transfer processes is thought to involve a specific intramolecular tunnel. Particularly interesting in the present context is the suggested tunnel for water molecules, which appears to be gated in part by

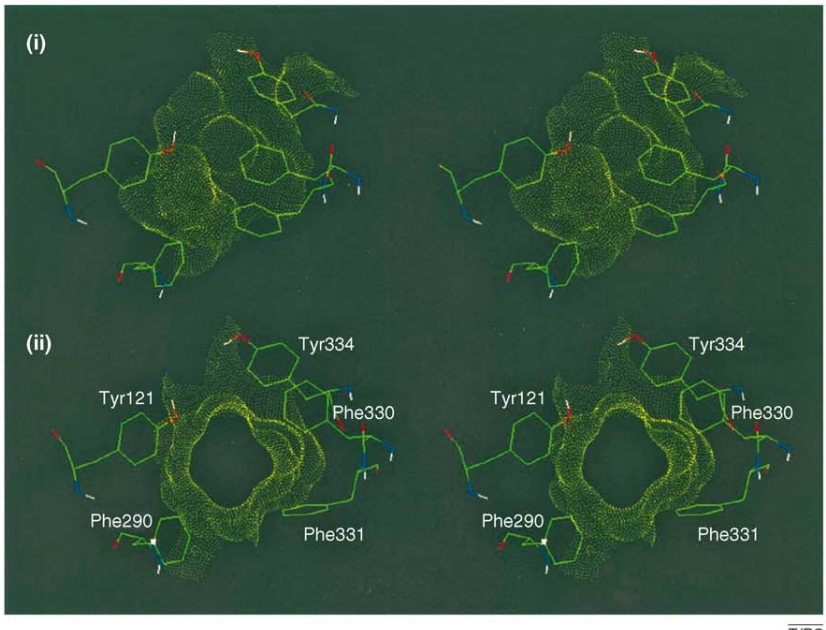

Figure 4. Opening and closing of the gate to the active site of AChE. The five aromatic residues serving as the gate are shown so that the viewer is looking down the active-site tunnel and into the active site. The surface accessible to a 1.4-Å probe is shown as dots. (**i**) Conformation at 12 ps along a 750-ps trajectory. The gate is closed to the 1.4-Å probe (but is open to a 1.2-Å probe). (**ii**) Conformation at 68 ps along the trajectory. The gate is open even to a 2.6-Å probe (but is closed for a 2.8-Å probe). Reprinted from ref. [13] with permission; copyright (1998) the National Academy of Sciences, U.S.A. A stereo pair is shown for either conformation.

aromatic residues, Tyr161, Phe186 and His190. A possible function of the gate is to moderate the flow of water molecules to the oxygen-generating center. Experimental evidence suggests that excessively rapid delivery of water molecules to this center can 'short-circuit' the catalytic process, leading to the generation of harmful species such as hydrogen peroxide.

ATP-dependent proteases are responsible for degrading the majority of unwanted proteins in cells. The proteolytic active sites of HslVU, a prokaryotic homolog of the proteasome, are sequestered in the central chamber of HslV, which is a dodecamer of two hexameric rings stacked back to back [52]. Protein substrates are unfolded and translocated to the proteolytic chamber through HslU, which forms a hexameric ring and binds to one or both ends of HslV. HslU is activated by ATP hydrolysis. Its central pore forms the translocation tunnel, which is gated by a highly conserved sequence, GVYG. Based on structures of HslU bound with different nucleotides, it was proposed that conformational changes induced by ATP hydrolysis are propagated to the gating sequence. In particular, the Tyr side chain undergoes a 180° change in direction, which is coupled to substrate unfolding and translocation. Substitutions of this residue, except with Phe, significantly impair the ability of HslVU to degrade proteins [53].

Aromatic gates

In the previous examples of transmembrane ion channels, intramolecular tunnels in bifunctional enzymes and tunnels connecting active sites to the exterior in monofunctional enzymes, aromatic residues (phenylalanine, tyrosine and tryptophan) often appear as gates. As we noted previously [13], "The aromatic rings are distinguished by their oblateness, so a small rotation leads to a large change in the width of the gate". Such rotations have been demonstrated by alternative side-chain conformations in X-ray structures and observed in numerous molecular dynamics simulations. In particular, simple libration of the χ_2 torsion angle yields a substantial change in the cross section for an aromatic side chain in a channel or tunnel. Libration of the χ_1 torsion angle or larger-scale collective motions can swing the entire ring aside. All of these types of displacements are seen in the gating dynamics of acetylcholinesterase, for example, not only in the primary tunnel to the active site, but also in secondary pathways such as the 'back door' [46,54,55]. The ability of aromatic side chains to form cation–π and π–π interactions with ionic and aromatic groups of substrates has also been noted [53].

These types of motions can be driven by dynamic processes on different temporal/spatial scales, possibly with different functional consequences. Local dynamic processes include simple, transient packing defects that can drive rapid fluctuations of the aromatic ring orientations, which could be sufficient for opening and closing gates for the displacement of small molecules such as oxygen [13,56,57]. Coupling of the side chain motions to collective dynamics of the protein is likely to be involved in the gated transport of larger ligands [52]. Such coupled local and collective motions might also provide a mechanism by which gated transport and ligand selectivity are mediated by the binding of effectors elsewhere in the protein [58], similar to that seen in ligand-gated ion channels [34].

Concluding remarks

The gates of ion channels and of tunnels in enzymes are central to activation and ligand selectivity. Their composition ranges from a small number of side chains to segments of secondary structures. An important lesson, drawn from the study of a monofunctional enzyme with a tunnel leading to a buried active site [13], but applicable to gated tunnels and channels in general, is that both the magnitudes and the time scales of the motions of gates are essential for determining the efficiency of transport. Attention to such dynamic aspects will be essential for gaining molecular insight into gating mechanisms of ion channels and regulation of enzyme catalysis.

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