Chapter

Structural Determinants for Ligand Accommodation in Voltage Sensors

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Abstract

After ligand binding, many ion channels undergo rearrangements at the voltage sensor domain (VSD) that often modulate their gating activity with important physiological repercussions. Since the VSD is dynamic, it is interesting to establish a correlation between the potential mobility of this element in terms of its intrinsic flexibility and its ability to accommodate several ligands by induced-fit mechanisms. We presume that these associations are not causal since the flexibility of the VSD could have an important impact on the ligand coupling event. Many significantly flexible ion channels show a general architecture and composition compatible with important conformational changes and capable of accommodating chemically diverse agonists. In this contribution, the structural bases of this subtle and probably unexpected relationship between the VSD flexibility and its influence during the dynamic coupling of the ligand are exposed. Thus, given its physiological relevance, the study of ion channel malfunction can be associated with ligand accommodation events to the VSD, which could depend on its local flexibility. This could contribute to a better understanding of the molecular bases of a variety of physiological disorders. In consequence, considering these effects during the protein/ligand interaction could be determinant to the rational design of novel drugs.

Keywords: voltage sensor, side-chain flexibility, side-chain rotamers, RMSD, induced-fit docking, conformational selection

1. Introduction

Every cell is defined by its membrane. This amphiphilic molecular matrix is highly organized and complex in terms of its lipid and protein components. Ion channels have evolved to mediate ion transport throughout membranes always in favor of electrochemical gradients from free-living bacteria to mammal neurons [1, 2]. In doing so, these membrane proteins are part of complex physiological networks which include signal transduction processes, cellular communication, or the propagation of electrical signals [3]. The voltage-gated ion channel (VGIC) superfamily comprises dozens of variations on a common theme—(i) a voltage sensor domain (VSD) and (ii) a pore domain (PD) [4]. This modular architecture has in turn evolved into activation mechanisms as diverse as the detection of changes in the potential across

the membrane, the binding of diverse chemical ligands, local membrane stretching, or subtle changes in temperature or the pH. In consequence, those physical-chemical variables are often interrelated modulating the ion channel gating but not clearly defined as exclusive stimuli for a determined protein [5]. In voltage sensing, the VSD performs important conformational rearrangements moving through the membrane-electric field and coupling this motion to the opening of the permeation pathway at the PD. To do this, four transmembrane segments (S1–S4) at the VSD respond sensing the electric field by translocating the so-called gating charges and by reorganization of the dipole moments into an aqueous crevice around the S4 segments, so that at any membrane potential, the charged side-chains of basic residues (mainly Arg and Lys) are essentially both hydrated and ionized either above or below the plane of the lipid bilayer (i.e. depolarized or hyperpolarized, respectively) [6].

In that sense, the VSD is clearly a mobile and intrinsically flexible element. A more detailed analysis of this mobility has revealed the relative flexibility of the different regions into this domain and clearly demonstrate that helices S1, S2, and the N-terminal part of S3 (S3a) are relatively more static than the so-called VSD *paddle* (S3b-S4) which loop linker show enhanced flexibility at higher temperatures in molecular simulations [7]. In those *in silico* studies, it is also evident that the segment S4 undergoes hinge bending and swiveling about its central axis, motion facilitated by the conformational instability of the S3a helix [7, 8]. The intrinsic

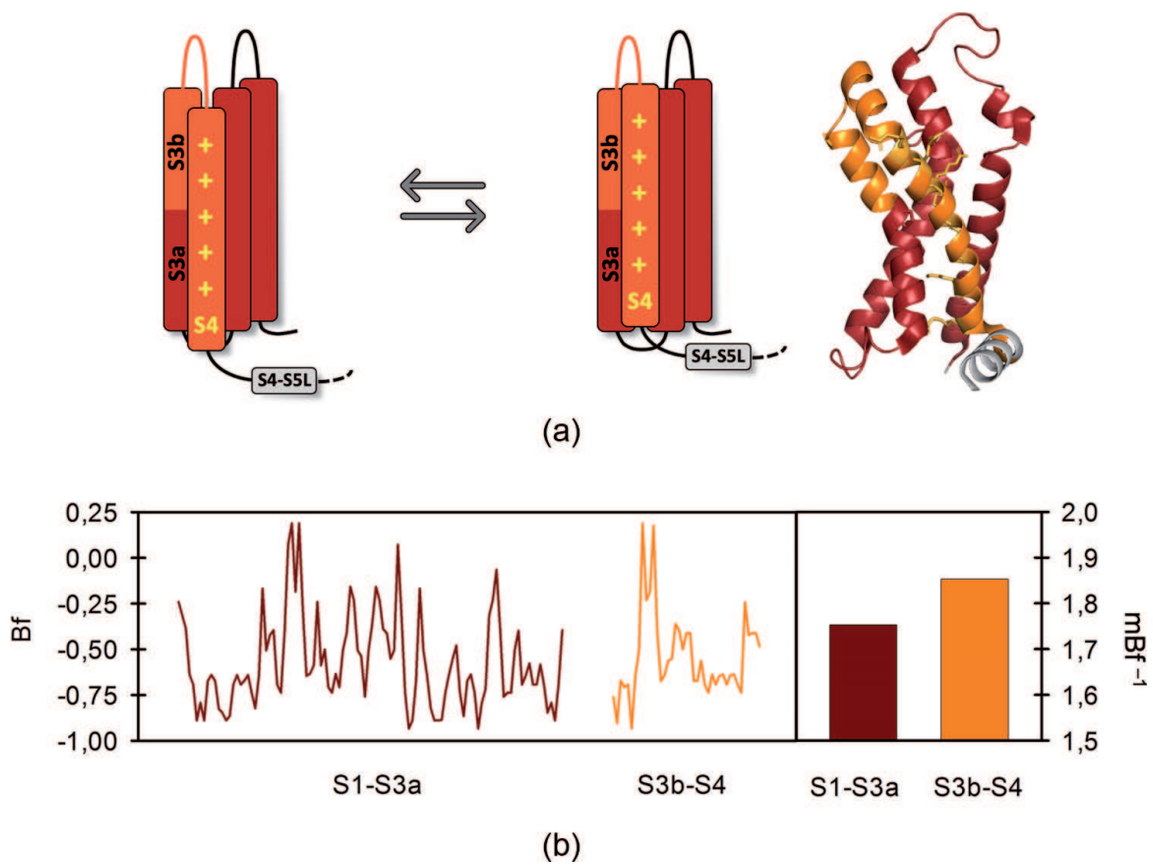

Figure 1.
Flexibility plot for the VSD of Kv1.2-Kv2.1 paddle chimera (PDB code: 2r9r) calculated using the method previously reported by us [5]. a. Cartoons showing the hyperpolarized (left) and depolarized (right) conformations of the VSD. b. Plots showing B-factors normalized to a Gumbel distribution according to our previous studies. Segment S3b-S4, involved in channel activation and the electromechanical coupling exhibits higher flexibility than the rest of the voltage sensor (S1–S3a).

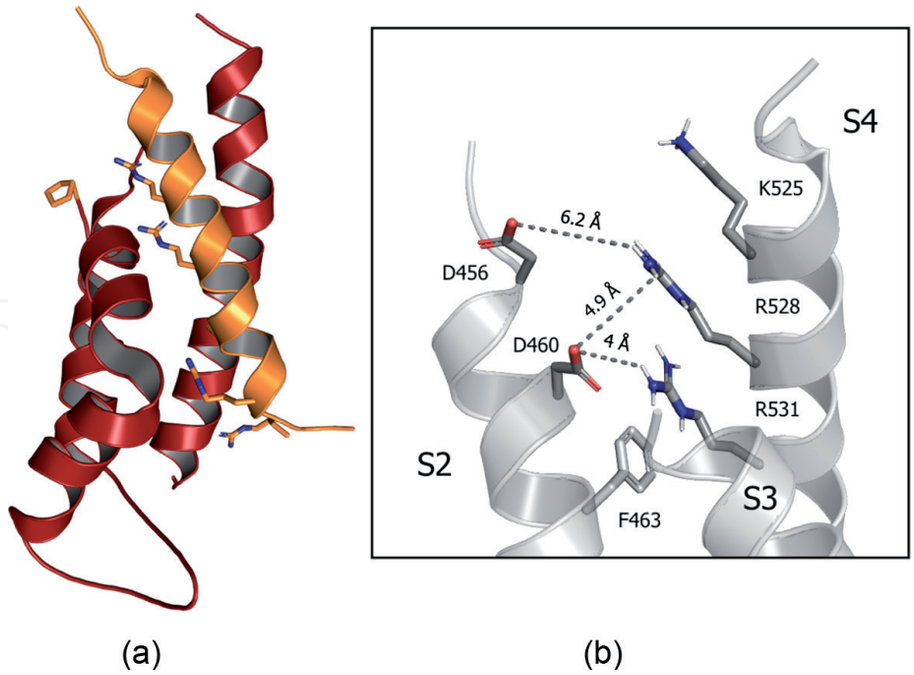

Figure 2.
The gating charge transfer center (CTC) in the voltage sensors of Kv channels. a. Ribbon representation of the four segments (S1–S4) making one VSD of the human ether-a-go-go-related gene (hERG) channel (PDB code: 7cn0). b. Close-up view of the CTC highlighting the gating charges on the S4 segment. Side-chains of the positively charged residues on S4 (labeled as R and K) and negative residues D456 and D460 interact forming salt bridges. The hydrophobic residue F463 controls the energy barrier of the final gating transition. Figures containing structures were prepared with Pymol (http://www.pymol.org/).

flexibility of the S3a region facilitates movement of the segment S3b-S4, which in turn exhibits an even higher flexibility profile due to its composition rich in residues with small side-chain (Gly, Ser, Thr) and basic residues (**Figure 1**). Notably, these predictions have been experimentally confirmed elsewhere [9, 10] and some reports also indicate that abnormal S4 movements cause pathological effects related for example to the development of epilepsy [11]. Therefore, it is becoming increasingly clear that the VSD is a flexible dynamic structure with evident relevance in physiological disorders.

The mechanisms of gating in ion channels have been intensively studied. On activation, outward S4 motion is associated with specific interactions with conserved negative countercharges (Asp and Glu) in transmembrane segments S1, S2, and S3 by forming sequential salt bridges with the positively charged residues in S4 inside an aqueous pore (**Figure 2**). Such interactions facilitate the translocation of the S4 segment in an energetically unfavorable membrane environment promoting the sequential salt-bridge formation and the electromechanical activation of the S4-S5 linker, which directly couples voltage sensor movement to the activation gate [12]. These negative countercharges are well-conserved in S1, S2, and S3 transmembrane segments in Kv channels (**Figure 3**). Besides, several VSD countercharge mutations associated with disease phenotypes including neural, cardiac, or skeletal muscle disorders have also been identified [14]. Despite channelopathies often affect ion channel gating, many of these pathologies have yet to be functionally or biophysically characterized. In this regard and given the diverse physiological and pathophysiological functions played by members of the VGIC superfamily, the VSD becomes a promising target for rational drug design.

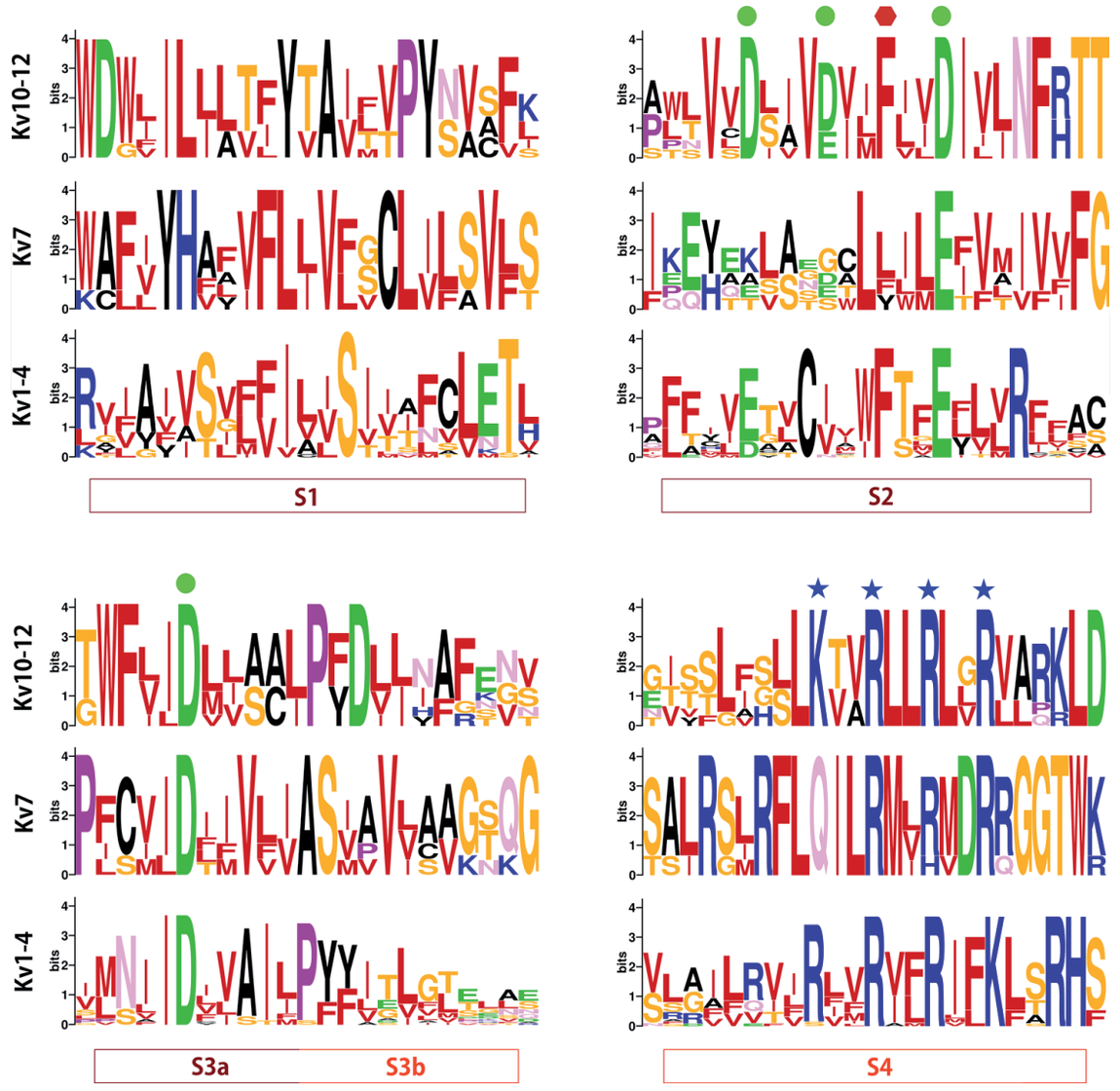

Figure 3.
Sequence logos from the three main subfamilies of voltage-gated potassium channels using the webserver WebLogo (http://weblogo.threeplusone.com/). The bars below the sequence logos represent the extent of transmembrane segments S1–S4. The consensus sequences generated are represented statistically, showing the relative conservation of each residue at that position. The height of each letter in every position indicates the maximum theoretical entropy for protein sequences (measured in bits) [13] which is determined by the number of aligned sequences and the degree of ambiguity in the alignments for each residue. Blue stars indicate basic residues involved in voltage sensing on S4. Green circles indicate conserved negative countercharges in S2 and S3. Red hexagon depicts a well-conserved aromatic residue that controls the transfer of the more inner gating charge, shaping the electric field inside the voltage sensor. Conserved residues addressed in this study are color-coded as: Red (hydrophobic); green (acidic); blue (basic); orange (small side-chain); pink (flexible side-chain); and black (rigid side-chain).

2. The voltage sensor domain as a pharmacological target

There are many reports on the interactions of different intracellular ligands with ion channels and particularly important is the well-understood interaction of ligands with the cytosolic tail domain (CTD) in large-conductance calcium-activated potassium channels (BK_{Ca}), which contain several binding sites. Also relevant are the studies of the interaction of cGMP or cAMP with the cyclic nucleotide-binding domain (CNBD) in cyclic nucleotide-gated (CNG) and hyperpolarization-activated (HCN) channels. Structural and functional information has shown that frequently the ligand-binding sites in those channels are clustered located at the interface

between the cytosolic domain and the VSD, acting synergistically to activate the gate at the PD [15, 16]. In other studies, ligands have been found directly coupled to the VSD influencing the channel activation, opening, closing, or inactivating the pore, such as some protein toxins from tarantula do [17, 18] or the binding of vanilloids, monoterpenoids, and related compounds to the S1–S4 domain in the transient receptor potential (TRP) channels [19].

From a structural perspective, the study of interactions between specific chemical ligands and the VSD in ion channels opens the possibility to rationally design both agonist and antagonist drugs. Let us have a look at three specific cases—(1) the voltage- and lipid-gated potassium channel KCNQ2 (**Figure 4**), (2) the cold/

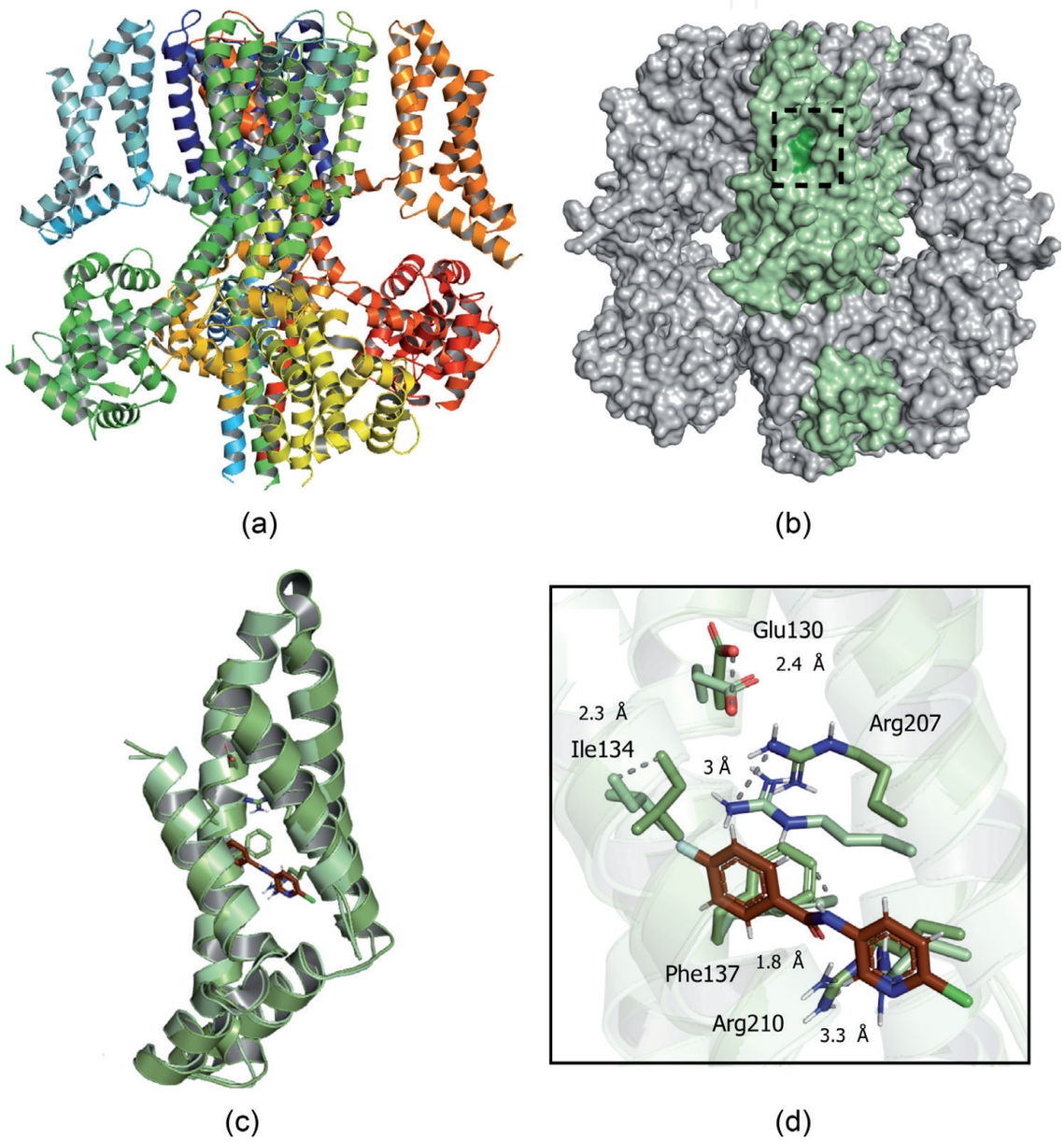

Figure 4.
Structure of the human Kv7.2 (KCNQ2) channel (PDB accessions codes 7cr0 (apostate) and 7cr1 (in complex with ztz240). a. Ribbon representation of side view showing the VSDs exposed to the lipid bilayer (not shown here). b. Surface representation showing the ligand-binding pocket as a box. c. the unliganded structure of the VSD (highlighted in pale green) is superimposed with the one in complex with ligand (highlighted in smudge green). d. Three-dimensional stick representation of the Kv7.2 pocket in complex with ligand ztz240 (dark brown). The distances traveled by the side-chain conformational rotamers are shown as dashes. The overlapping residues correspond to the side-chains involved in ligand binding.

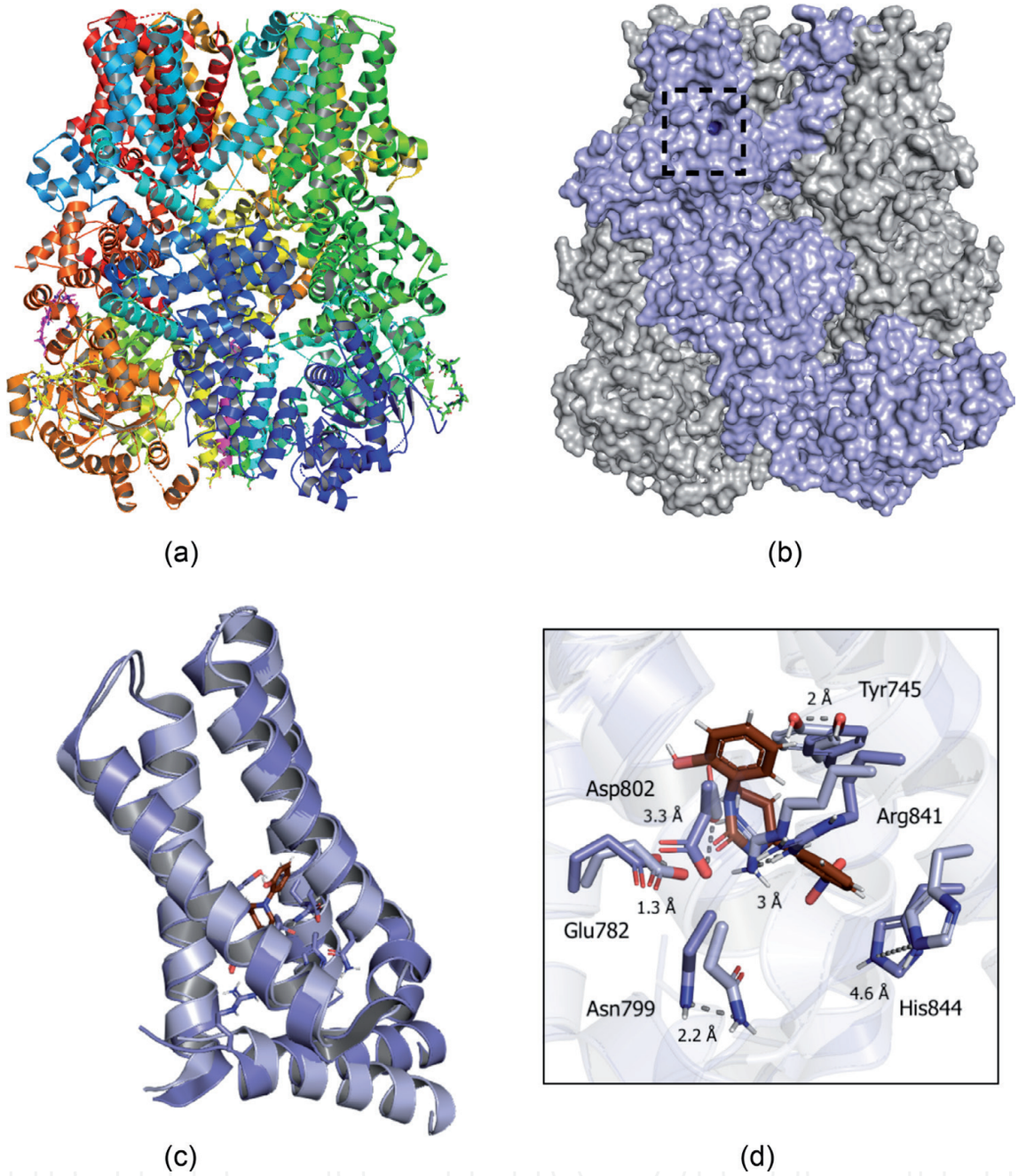

Figure 5.
Structure of the TRPM8 ion channel from the collared flycatcher (Ficedula albicollis) (PDB codes 6bpq (apostate) and 6nr3 (in complex with icilin, PI(4,5)P2, and Ca^{2+}). a. Ribbon representation of side view. b. Surface representation showing the ligand-binding pocket as a box. c. The unliganded structure of the VSLD is highlighted in light blue and superimposed with the one in complex with icilin (semi-dark blue). d. Overlay of both structures to visualize the side-chain conformation changes (see **Figure 3** *for details).*

menthol activated TRPM8 channel (**Figure 5**), and (3) the capsaicin receptor TRPV1 (**Figure 6**). Studies on the KCNQ2 (Kv7.2) potassium channel show that the aromatic amide ztz240, a derivative of niclosamide, binds to the open configuration of the VSD. This interaction directly couples such a chemical ligand with a binding pocket of 170 $Å^3$ located between some specific residues at segments S2 (Glu130, Ile134, Phe137) and S4 (Arg207 and Arg210), i.e. precisely in the gating charge pathway of this ion channel [20]. This raises the possibility to design drugs using the channel gating pore in voltage-dependent channels as a therapeutic target [21]. In this case, ztz240 and some derived chemotypes have demonstrated important

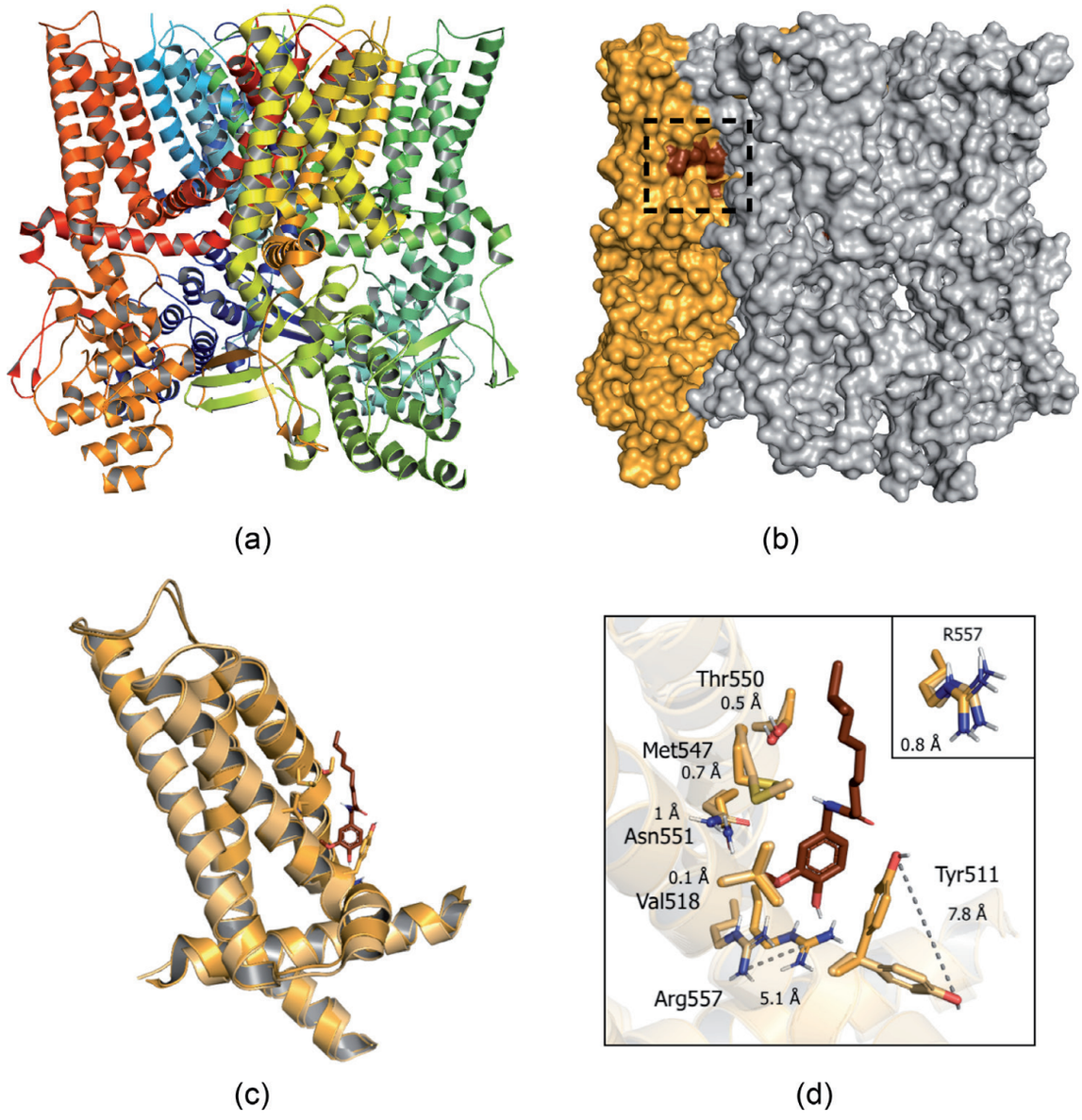

Figure 6.
Structure of the TRPV1 channel from rat (PDB codes 7lp9 (apostate, 4°C), 7lpe (in complex with capsaicin, 48°C), and 7lpa (in complex with capsaicin, 4°C). a. Side view of the tetramer in ribbon representation. b. Surface representation showing the ligand-binding pocket as a box. c. The unliganded structure of the VSLD is highlighted in light orange and superimposed with the one in complex with capsaicin (bright orange). d. Overlay of structures at 4°C (apostate) and 48°C (in presence of capsaicin) to visualize two different rotamers by residue (see **Figure 3** *for details). Inset: Superposition of Arg557 side-chain obtained at 4°C for apo- and holo-structures.*

anti-epileptic activity, which might be valuable for the treatment of epilepsy (see below). Remarkably, this interaction in the gating charge pathway of KCNQ2, considering the induced-fit model, demonstrates that this pocket may accommodate different activators [20].

In the case of TRPM8, an analog binding pocket has been described as highly adaptable to accommodate diverse chemical structures in distinct orientations. Both agonists and antagonists are dynamically recognized in this promiscuous pocket making the whole S1–S4 domain conformationally dynamic and transmitting these rearrangements to the TRP helix, but without inducing important changes in its overall structure [22]. Menthol, the main compound of mint, is the clue activator to understand how TRPM8 channels are ligand-activated. It binds to the cavity formed between S1 and S4 by a so-called "grab and stand" mechanism. The hydroxyl group

of menthol works as a hand to specifically grab with Arg842 (segment S4) through a hydrogen bond, while its isopropyl "legs" stand on residues on S4 through electrostatic interactions. Thus, menthol binding induced widespread conformational rearrangements in the S1–S4 domain which open the S6 bundle gate to allow ion permeation [23].

On the other hand, since TRPV1 channels participate in several pathways of neuronal inflammatory signaling, it also represents an attractive therapeutic target for the treatment of neuroinflammation, neurodegenerative diseases, and chronic pain. Feng et al. [24] have studied diverse diarylurea compounds by molecular docking and dynamics, finding that specific residues located in the interface between the VSD and the PD are implicated in several van der Waals interactions. Particularly important are residues Tyr511, Leu518, Leu547, Thr550, Asn551, Arg557, and Leu670. Besides these observations, residues at the base of the interface between the VSD and the PD (segments S3, S4, S5, and the S4-S5 linker) are important binding sites for N-(3-fluoro-4-methylsulfonamidomethylphenyl)urea. Docking analysis of this compound with human TRPV1 has revealed that hydrogen bonding and π–π interactions with Tyr511 (segment S3) and hydrophobic interactions with two pockets in the S3 and S4 segments (residues Met514, Leu515 and Leu547, Thr550, respectively) are critical for its activity. Flexible docking studies have also revealed that N-benzyl phenylsulfonamide derivatives of 2-(3-fluoro-4- methylsulfonamidophenyl)propanamide specifically bind to the same pockets, being again critical for the potent activity of these antagonists [25, 26]. Notably, conformational analyses have revealed that the S1–S4 domain in TRPV channels remains relatively static during opening [27–29].

These three examples are mechanistically different, but they all share something in common, and this is the specific association of their respective ligands at the VSD, as well as their direct association with the potential difference across the membrane. However, even though TRP channels have frequently been treated as strictly ligand-dependent, it is increasingly clear that their voltage sensitivity could be underestimated [30]. These reports support the idea that the increasingly available detailed structural information, as well as detailed functional studies, greatly simplifies the search for chemical modulators with agonistic or antagonistic action. In consequence, the identification of potential ligand-binding sites in the VSD makes the rational design of new drugs, the goal of several research efforts.

2.1 Side-chain flexibility and ligand accommodation

Proteins are intrinsically flexible. This property derives from the two bonds associated with the carbon α (Cα), which can freely rotate and contribute to the flexibility of the main backbone. The torsion angles Phi (Φ) and Psi (ψ) represent the rotation around the Cα-N bond and the one around the Cα-carbonyl bond, respectively. However, this rotational capacity also depends on the steric and conformational effects of the associated side-chains. In these terms, one classical structural parameter to estimate the mobility of each atom into a protein structure is the so-called B-factor, which reflects the degree of thermal motion and static disorder. This parameter, also called the Debye–Waller factor, represents the atomic displacement of the macromolecule and is used in protein crystallography to describe the attenuation of X-ray or neutron scattering caused by thermal motion, which reflects the uncertainty in atom positions [31, 32]. Therefore, proteins are intrinsically rigid or flexible ultimately based on their primary sequence.

From this perspective, in addition to their known physical and chemical properties, if their relative location parameters are considered, amino acids can also be classified in terms of their contribution to the flexibility of a given segment within a protein. The amino acids that are considered rigid generally consist of those that exhibit bulky side-chains, generally cyclic or aromatic, those that have heavy heteroatoms, and are generally hydrophobic. On the other hand, amino acids considered flexible are those having polar side-chains, have charges, or whose side group is only a proton, i.e. glycine [33]. Proline deserves a separate discussion, as this amino acid has been considered both rigid and flexible in terms of its kinking effect on alpha helices [34].

Typically, flexibility in proteins has been visualized in terms of local and global motions, which include—(i) the multiple conformations that a certain residue can acquire in the polypeptide chain, (ii) local-scale fluctuations in the conformation of the side chains with respect to the backbone, and (iii) massive movements of subdomains with respect to another part of the protein [35]. In this last regard, a pioneer study of protein crystal structures shows that intrinsic flexibility can be distinguished in terms of hinge motions and shear displacements between close-packed segments of the protein [36]. It is becoming clearer that studying protein flexibility and the multiple side-chain conformations during molecular docking is very relevant since it may contribute to a favorable change in the Gibbs binding free energy by optimizing the van der Waals interactions between the protein and the ligand. This favors a change in enthalpy and minimizes the decrease in entropy [37, 38], albeit protein flexibility also depends on several other factors, including heat capacity, conformational entropy, salt bridge networks, electrostatic interactions, and the hydrophobic effect [39]. In any case, the study of side-chain flexibility in ion channels and how it contributes to ligand accommodation could be critical to understand molecular recognition events and predict ligand binding. This could open novel therapeutic strategies for the treatment of diverse neuropathic disorders.

2.2 Three cases of study: Kv7.2, TRPM8, TRPV1

Since flexible regions in proteins can be predicted from the primary sequence through the evaluation of the normalized B-factor for a determined structure [40], we have implemented an easy algorithm in Excel based on the procedure carried out by Smith and cols. [32, 33]. In general terms, B-factor normalization, Bn, depends on (1) the atomic thermal factor, B, reported on the PDB, (2) the sample mean value of B-factors, Bm, for a dataset of protein structures, and (3) the standard deviation of the sample distribution of such factors, $B\sigma$, for a determined structure [41]. In sum, normalized B-factors are indicative of each local residue flexibility that can be calculated as:

$$Bn = \frac{B - Bm}{B\sigma} \tag{1}$$

Based on these theoretical principles, we implemented an algorithm to predict local side-chain flexibility, which correlates the composition of amino acids in a protein sequence in the context of its N- and C-terminal neighbors. The program assigns a weighted normalized B-factor value based on a stiffness classification for each amino acid, in accordance with previously published results [33]. This software, so-called FlexiProt, includes Trp, Tyr, Phe, Cys, Ile, Val, His, Leu, and Met as rigid amino acids

and the rest of them, *i.e.* Gly, Thr, Arg, Ser, Asn, Gln, Asp, Pro, Glu, and Lys as flexible. The program incorporates a graphical generator of local flexibility profiles, based on primary sequence, to help the user better visualize this disorder parameter for its subsequent structural evaluation. Flexiprot is friendly and interactive since although it runs automatically, it allows at all times the possibility of evaluating local subsequences for a better prediction of the internal flexibility parameter, based on structural aspects associated with the theoretical degrees of freedom of the side-chains.

Through this type of sequence analysis, we show in **Figure 7** the local flexibility profiles of three distinct channels for the segments S3 to S4—(1) human KCNQ2 (hKv7.2), (2) mouse TRPM8 (mTRPM8), and (3) rat TRPV1 (rTRPV1). As in the Kv1.2-2.1 *paddle* chimera (**Figure 1**), in these three cases, local flexibility for segment S4 is always higher than the one for segment S3. However, the profile of the S3 segment in the TRPV1 channel is significantly stiffer than the other two, which have similar flexibility profiles for the same segment, although the S4 segment of the voltage-dependent channel Kv7.2 is considerably more flexible compared to their thermosensitive counterparts. This notorious flexibility is mainly determined by the highly conserved Arg residues, responsible for transporting the gating charges in the voltage sensor during the activation mechanism of these proteins [5]. Considering the new structures available for this channel in the presence of the ztz240 modulator, whose binding site is precisely in the intimacy of the voltage sensor, it becomes interesting to analyze the role of side-chain flexibility for each of the interacting

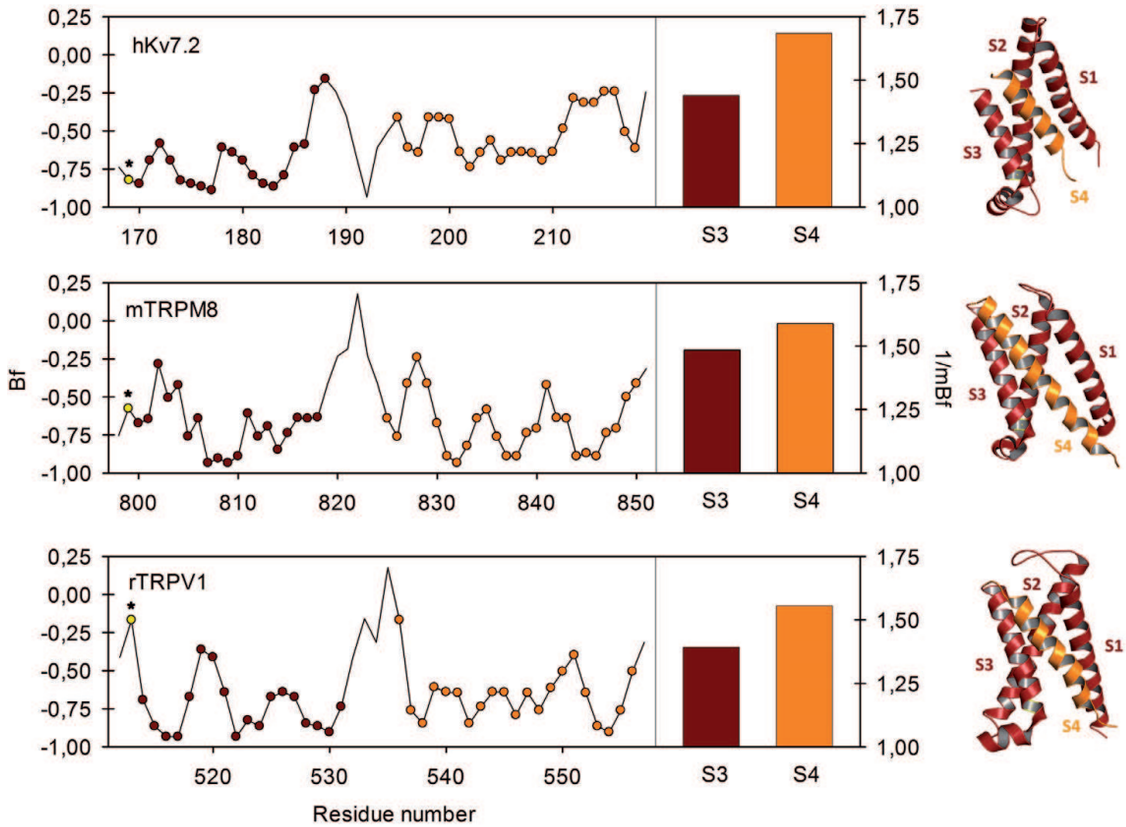

Figure 7.
Predicted flexibility for segments S3 and S4 in human Kv7.2, TRPM8 (mouse), and TRPV1 (rat). Flexibility (defined by B-factor values) using the FlexiProt algorithm according to Ref. [5] shows a higher flexibility profile for segment S4 compared to S3. Predictions also indicate that the three channels follow the flexibility ranking TRPV1 < TRPM8 < Kv7.2. Asterisk indicates the start of the first turn of the α-helix at the N-terminal part of segment S3a. mBf: mean B-factor.

residues and that has been mentioned elsewhere [20, 42]. **Figure 4** show this interaction in two conformational states for the VSD of the Kv7.2 channel, in the absence and presence of the state-dependent modulator ztz240. For a segment of 145 residues that encompass the integrity of the VSD, a root-mean-square deviation (RMSD) of 1.159 Å is indicative of a fine accommodation for this drug without representing a significant conformational change of this protein domain. It is clearly noted that the intrinsically flexible residues Arg207 and Arg 210 at the S4 segment undergo an important conformational rearrangement that allows the ligand to be adequately accommodated through van der Waals interactions. The analysis of the structures also indicates that these residues are displaced 3.0 and 3.3 Å respectively. Another important residue, implicated in the potentiation of the activity of ztz240 is Glu130, an amino acid considered even more flexible than Arg [33], which is found as a countercharge in the S2 segment and whose side group moves 2.4 Å during the interaction. In contrast to these data, two other amino acids also important for the interaction, Ile134 and Phe137—amino acids of a rigid nature—show a rearrangement of 2.3 Å and 1.8 Å, respectively. These data suggest that the significant local flexibility of the S4 segment in this channel strongly contributes to ligand accommodation with minor effects on the large movements that the VSD experience during activation and that some of the main residues interact with this ligand move in a range of 1.8–3.3 Å.

In TRPM8, a channel described also as sensitive to voltage [43, 44], a similar effect to Kv7.2/ztz240 is observed. According to recent structural studies of this channel, icilin, a compound derived from tetrahydropyrimidine-2-one and more potent than menthol as the agonist, binds to the voltage-sensor-like domain (VSLD) mainly through van der Waals interactions to residues Tyr745 (S1), Glu782 (S2) Asn799 (S3), Asp802 (S3), Arg841 (S4), and His844 (S4) [28, 45, 46]. Analogously to that seen in the Kv7.2 channel, the significant flexibility exhibited by the S3 and S4 segments in the TRPM8 channel contributes to a fine accommodation of icilin through conformational rearrangements of these amino acids in a range from 2 to 4.6 Å (**Figure 5D**). These displacements occur in the context of an RMSD of 0.89 Å over 123 Cα atoms which integrate the VSLD of this cold-sensitive channel. Similarly, the interaction of the antagonist TC-I 2014 in the same cavity of the VSLD [28] induces small rearrangements of the corresponding side-chains implicated in ligand sensitivity, with displacements of around 1–3 Å and an average RMSD of 0.427 Å (data not shown). Taken together, these observations suggest that the high flexibility profile in the S3 and primarily the S4 segments of these transmembrane domains facilitates a fine repositioning of the participating side-chains, which are implicated in ligand accommodation.

The case of the transient receptor potential vanilloid subtype 1 channel is slightly different. **Figure 6** shows the interaction of capsaicin with the VSLD of TRPV1. Thanks to the recently released structural data of TRPV1 channels in presence of this ligand, a more detailed exploration of such interactions as a function of the associated local flexibility contribution of the VSLD contributes to a better understanding of this process. For a segment of 166 residues encompassing the whole VSLD and part of the TRPbox, an RMSD of 0.63 Å suggest an almost null conformational change for this part of the protein in the course of the closed-to-open transition during the ligand interaction, as it has been previously reported [25]. In this case, residues Val518, Met547, Thr550, and Asn551 move their side-chains less than 1 Å when they interact with capsaicin, whereas Tyr511 and Arg557 experience a significant displacement of 7.8 Å and 5.1 Å, respectively. These observations are consistent with the low predicted local flexibility for segment S3 in this channel (**Figure 7**, **Table 1**) [5]. Furthermore, they are also in good agreement with the vision that the VSLD acts as a rigid body during TRPV1 activation [25].

Channel	S1–S4 length (Cα)	S3 (mBf)	S4 (mBf)	RMSD (Å)	Side-chain displacement (Å)
Kv7.2	145	1.44	1.68	1.16	2.4 (E130) 2.3 (I134) 1.8 (F137) 3.0 (R207) 3.3 (R210)
TRPM8	123	1.48	1.59	0.89	2.0 (Y745) 1.3 (E782) 2.2 (N799) 3.3 (D802) 4.6 (H844)
TRPV1	166	1.39	1.55	0.63	7.8 (Y511) 0.1 (V518) 0.7 (M547) 0.5 (T550) 5.1 (R557)

mBf, mean B-factor (1/mBf).

Table 1.
Flexibility parameters for the studied channels upon ligand interaction.

Our analysis shows that in this case, the low flexibility profile of the S3 segment contributes to creating a rigid crevice. This structure accommodates the catechol/vanilloid ring of capsaicin at the base of the VSLD where the bulky side-chain of Tyr511 residue rearranges with a displacement of almost 8 Å during a transition from 4 to 48°C. It is very significant that this tyrosine, which frequently is classified as a rigid side-chain with low conformational entropy, in the context of the structure of this channel, carries out a significant rearrangement even greater than Arg557, which has been frequently quantified as much more flexible [33, 47]. Besides, the side-chain of Arg557 at S4 undergoes a conformational rearrangement of 5.1 Å during the interaction but an almost null displacement (0.8 Å) if this is carried out at 4°C (**Figure 6D**, *inset*). Indeed, despite its low flexibility, tyrosine has been considered very effective for mediating molecular recognition maybe because changing the orientation of its side-chain from *gauche* negative (g–) to *trans* (t) conformation is equivalent to moving the hydroxyl group around 9 Å, which is the length of an average drug molecule [48, 49]. On the other hand, the motion of Arg557 is associated with the formation of a hydrogen bond with the Glu570 residue on the S4-S5 linker, leading to its swivel [50]. This also confirms that arginine often participates in molecular recognition events. The terminal positively charged guanidinium group of this residue affords multiple geometries due to its long side-chain can retain substantial residual conformational entropy occupying several rotameric states, while maintaining specific interactions through its charged functional group [51]. In sum, we hypothesize that, in contrast with the mechanism dependent on the large local (S3–S4) flexibility of the voltage-dependent Kv7.2 channel or the cold/menthol-activated TRPM8 channel, these large conformational changes in specific residues compensate for the low mobility that the whole transmembrane domain (i.e. the VSLD), as a rigid body, undergoes during TRPV1 activation.

3. Side-chain flexibility and the dynamic nature of protein-ligand interactions

Despite the large increase in deposition of crystallographic, NMR, and cryo-electron microscopy structures in recent years, little dynamic information regarding the conformational degrees of freedom of protein structures is currently available. *In silico* local flexibility theoretical prediction together with molecular dynamics algorithms are likely to be useful in helping to solve this limitation. Diverse computational strategies have been developed to explore the side-chain rotameric states as a function of the primary sequence, backbone structure, and ligand interaction by molecular docking in specific protein motifs [52–54]. Besides, the prediction of protein flexibility [32, 33, 40, 41], as well as its identification and visualization [55], have been a constant goal in protein research. Side-chain flexibility represents an intrinsic property of amino acids, as it correlates with configurational entropy differences and indeed is related to the generation of dynamic rotamers, which are defined as a particular combination of side-chain dihedral angles [38].

Given the dynamic and multifactorial nature of flexibility in proteins, trying to predict the binding mode of any ligand is an inspiring challenge. However, the use of predictive tools, dynamic simulations, and specific experimental tests will facilitate a better understanding of the molecular mechanisms underlying ligand-dependent modulation of ion channels. This could be of great impact on the rational design and discovery of novel drugs. Therefore, in the case of the study of the VSD as a ligand-binding motif, side-chain flexibility is especially relevant and must be always considered in light of the induced-fit model and conformational selection mechanisms [56]. In these terms, since side-chain and also frequently the backbone are subject to rearrangements upon ligand interaction (*see* our previous analysis above), we suggest that any experimental approach to develop novel drugs should be designed from the perspective of a dynamic target. In this sense, the study by Li and cols. is very relevant since they identify a hydrophobic pocket inside the charge transfer center (CTC) of the Kv7.2 channel which can accommodate different chemical ligands [20]. This enables such openers to regulate ion channel activation and offers new therapeutic strategies for the treatment of several hyperexcitability disorders, such as epilepsy and neuropathic pain. Since the VSD exhibits important conformational freedom during the gating process, it is important to note that this class of ligands preferentially binds to specific conformational states. The compound ztz240, for example, is accommodated to a hydrophobic pocket only when the VSD is in its activated conformation. This interaction stabilizes the activated state of the channel, thus contributing to its antiepileptic activity [57]. Therefore, it is conceivable to consider the so-called gating pore as an important target for ion channel research given its potential adaptability to new openers or inhibitors.

In the **Figure 8** included at the end of this study, the workflow for the development of drugs with therapeutic potential is shown sequentially. *In silico* and structural studies, from the perspective of the "induced fit" model and the "conformational selection" hypothesis [58, 59], both contribute to a better understanding of the dynamic aspects of the protein/ligand interactions. In these terms, the role of side-chain flexibility becomes pivotal, and methods to predict it, such as the one performed herein, as well as methods to visualize it, such as those reported elsewhere [55] are

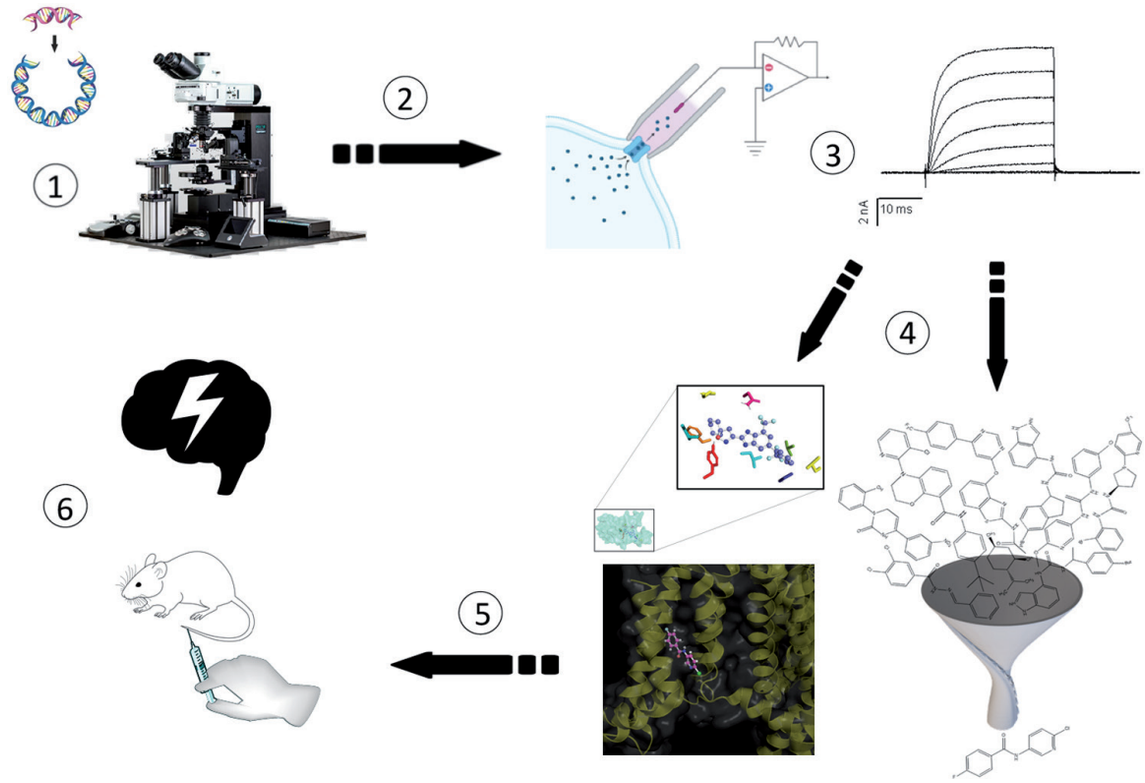

Figure 8.
Compounds that act on the voltage sensor could be a good alternative for the development of new analgesic drugs and provide a complement to pain therapy. After synthesis of a compound with pharmacological potential, ion channel mutagenesis (1) and associated electrophysiological tests (2) are performed. The study of the activation/inactivation properties of ionic channels with therapeutic interest (3) is decisive for establishing correlations between the adaptability of the molecular target and the candidate drug (4). Considering side-chain intrinsic flexibility and the degree of pocket disorder during these molecular recognition events allows the identification of residues crucial for drug activity. Finally, the new active compounds are tested for their in vivo validation using animal models (5). This strategy could be applied to the discovery of several modulators capable of dealing with diverse neurological disorders (6).

indispensable analysis tools. By the appropriate selection of chemical candidates with pharmacological potential, ion channels with defined mutations can be experimentally evaluated, shedding some light on the involvement of specific residues in ligand accommodation and their effects on voltage sensor regulation. Furthermore, thanks to the correct study of the flexibility profiles for a given segment, it is also possible to evaluate the nature of these interactions, providing additional information about the degrees of freedom necessary for an adequate ligand accommodation. With this background, experimental testing in animal models and eventual clinical studies becomes an achievable goal for the treatment of neurological disorders and problems of acute pain.

From this perspective, the predictive analysis of local flexibility that we have performed here on three different ion channels clearly shows how the characteristic ligands of each protein are accommodated in the binding sites generating important conformational changes in the side-chains of specific residues. In a very revealing way, we found that the Kv7.2 channel and the TRPM8 have a VSD and a VSLD with high S3-S4 flexibility profiles (mBf of 1.44/1.48 for S3 and 1.68/1.59 for S4, respectively) (**Table 1**). These domains show small local conformational changes according to the corresponding RMSD values calculated (1.16 Å and 0.89 Å respectively), while on the other hand, a channel such as TRPV1, whose flexibility profile is rather rigid (mBf = 1.39 for S3 and 1.55 for S4) exhibits a still minor conformational change

(RMSD = 0.63 Å for the equivalent segment) during the interaction with its specific ligand. Likewise, the conformational changes that we observe in the participating side-chains are also revealing, since the rotamers generated during the ligand interaction in flexible VSDs (Kv7.2 and TRPM8) are the result of rotations less than 3 Å on average, while in the case of the rigid S1-S4 segment of the TRPV1 channel, the conformational changes of the side-chains are less than 1 Å but two residues, in particular, Tyr511 (S3) and Arg557 (S4), undergo rotations of 7.8 Å and 5.1 Å, respectively, which suggest a different mechanism for ligand accommodation. This is even more revealing when it is considered that the conformational changes observed in the side-chain of Arg557 were obtained at 48°C [48] while the conformational rotamer for that residue at 4°C is less than 1 Å and that corresponding to Tyr 511 is even higher (8.2 Å) with a side-chain angle rotation of ~101°, which also suggest that motion of this residue is critical for ligand binding (**Figure 6D**).

After this analysis, we speculate that two different mechanisms for ligand accommodation in ion channels exist, which seem to be dependent on the conformational freedom of the VSD. These mechanisms could be interpreted as VSDs that have higher degrees of freedom, i.e. flexible and more prone to fine induced-fit mechanisms, which adapt better to the ligand through small displacements of multiple participating side-chains. On the other hand, more rigid VSDs follow the classical lock and key model for enzyme-substrate interactions, in which the ligand is accommodated directly but with important conformational changes in certain very specific residues. The conformational freedom of these specific residues would compensate for the low mobility observed in the rest of the structure.

4. Conclusions

Flexible regions in proteins are critical elements for the recognition of macromolecular interactions and induced molecular flexibility is essential to understand the principles of molecular recognition between ligand and receptor. However, the nature of side-chain flexibility is elusive and dynamic processes involving this flexible component are among the most difficult to characterize. Given its direct participation in the activation of voltage-dependent channels, the voltage-sensing domain is a very attractive target from the therapeutic point of view. As side-chain flexibility represents an intrinsic property of amino acids which is correlated with configurational entropy differences, it is now known that rotamer changes in specific residues during ligand interaction are finely synchronized [38]. Our analysis has confirmed this claim. According to our predictive algorithm, the local flexibility in S3–S4 segments which are implied to ligand binding in three different channels, correlated well with the adaptability of specific residues through the generation of side-chain rotamers during each interaction. However, what is intriguing is the fact that segments exhibiting high flexibility profiles (i.e. Kv7.2 and TRPM8) are correlated with small-scale changes and the generation of side-chain rotamers that are more homogeneously and subtly accommodated among the participating residues during ligand binding, while those which are slightly more rigid (i.e. TRPV1) remain practically immobile during the interaction, except for one or two residues that undergo a very pronounced conformational rearrangement to accommodate the drug. Since it is assumed that these new conformations are energetically favorable states [60], in terms of drug design these observations might not be trivial when considering the induced-fit model [58, 59] in combination with the conformational selection hypothesis [61]. In

agreement with them, the dynamic binding of drugs to a specific protein target may lead to chemoselectivity, high ligand affinity as well as the favoring of long residence times in the binding site. There are many potential interactions if those factors are considered and reasonably well understood. In these terms, the identification of side-chain rotamer rearrangements upon ligand binding in combination with the use of the global RMSD comparison between two protein conformers is more informative. Thus, the incorporation of predictive tools of side-chain flexibility in protein/ligand interactions is key to infer dynamic aspects in molecular docking. Besides, the experimental evaluation of new drugs from this perspective becomes pivotal in the rational design of therapeutic strategies to control several physiological disorders and face emerging channelopathies.

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Notes

FlexiProt 2.0 is a software designed for the prediction of flexibility profiles in primary sequences. Today, our group is working to share it in the public domain. For more information or in case of interest, contact the corresponding author at: daniel.bm@veracruz.tecnm.mx.

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