Heat-Dependent Hairpin Melting Drives TRPV1 Opening

Running title: Digital codes in heat-sensing TRPV1

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Abstract

The capsaicin receptor TRPV1 is a bio-thermometer in a primary afferent sensory neuron for noxious heat detection. However, the underlying molecular mechanism is unclear. Here, a hairpin thermodynamic model, together with graph theory, was used to examine a role of temperature-dependent little hairpin sizes and strengths in controlling the temperature threshold and sensitivity and gating states of TRPV1. The results showed that heat-evoked melting of the biggest little hairpin with a minimal loop size and weak non-covalent interactions initiates a temperature threshold for channel opening while the smaller little hairpins with strong H-bonds may stabilize the open state and increase heat efficacy. The temperature sensitivity may be determined by a change in the total minimal sizes of little hairpins upon a change in the total little hairpin-dependent non-covalent interactions along the gating pathway from a closed state to an open one. Therefore, this model may provide a broad structural basis for the thermo-gated TRP channels. (155 words)

Keywords: desensitization; hairpin thermodynamics; non-covalent interaction; temperature sensitivity; threshold; TRP channel

1. INTRODUCTION

Temperature is important in everyday life. Transient receptor potential (TRP) vanilloid-1 (TRPV1) is well-known as a noxious heat sensor in a primary afferent neuron of the dorsal root and the trigeminal ganglia. Its activation allows sodium or calcium to be permeable to a channel pore. With a temperature activation threshold (~42°C) above a normal human body temperature 37°C and a temperature coefficient Q_{10} (defined as the ratio of equilibrium constants measured 10°C apart) higher than 20, this channel can work as a bio-thermometer to monitor a noxious heat signal above 42°C and to regulate the human body temperature. [1-4] In addition to the physical stimuli, capsaicin can also activate TRPV1, producing hot sensation. As cold can decrease the capsaicin-evoked activity of TRPV1, [5] the same gating pathway seems to be shared by both stimuli. On the other hand, residues sensitive to both physical and chemical stimuli are different in TRPV1, suggesting their different gating pathways. [6-9]

TRPV1 is a homotetramer with each monomer consisting of six transmembrane helices. The first four segments (S1–S4) form a voltage-sensor-like domain (VSLD) while the last two (S5 and S6), together with the intervening loop and the pore helix and the extracellular cap-like pore turret between S5 and S6, are folded as a pore domain. The pore domain is surrounded by the VSLD in a domain-swapped arrangement. The cation conductance pathway centers in the pore domain. A hydrophobic constriction around I697 forms a lower S6 gate while the segment from G643 to D646 (^{643}GMGD^{646}) between S5 and S6 (S5-P-S6) in rat TRPV1 (rTRPV1) acts as a short selectivity filter. The S4–S5 linker, which is a short amphipathic helix and almost parallel to the cytoplasmic membrane, couples the VSLD with the pore domain for channel gating. Thus, the overall structure of the TRPV1 channel resembles that of the voltage-gated ion channel. [10-11] However, TRPV1 has a conserved TRP domain, which runs parallel to the inner leaflet of the membrane by virtue of a sharp bend after S6, not only linking the S6 gate crossing bundle but also interacting with the S4–S5 linker and the pre-S1 domain in favor of allosteric coupling between different gating domains. In addition, the characteristic ankyrin repeats within the cytoplasmic N terminus can tether cytoplasmic N- and C-terminal domains for channel assembly. [11-14] Although the structural basis for the chemical stimuli such as capsaicin has been clear, [15-16] the heat-evoked gating pathway is still unknown for TRPV1 regarding the temperature threshold and sensitivity and desensitization.

Since 2013 a DNA hairpin thermal sensor with a limited poly-A loop size has been created to monitor a change in a biological microenvironment. [17] Its initial hairpin melting curve is similar to the temperature-dependent activation curve of the thermosensitive TRPV channels. Its hairpin melting temperature is also in a threshold range of thermosensitive TRPV channels despite an increase with the smaller or less-flexible loop, the shorter duplexed stem, the more H-bonding pairs at the ends, or the higher ionic strength in an aqueous solution. [1, 6, 18-23] In this regard, it is fitting to ask if the thermosensitive TRPV channels exploit a series of little hairpins with minimal loop sizes and weak noncovalent interactions in the loops to control the temperature thresholds and sensitivity or Q_{10} and gating states. In this computational study, graph theory was used as a novel tool to examine this hypothesis by analyzing the cryo-electron microscopy (cryo-EM) structural data of rTRPV1 with or without phosphatidylinositol (PI)-bound at the capsaicin site around the temperature threshold. This hairpin thermodynamic model generates several melting temperatures (T_m) and structural thermo-sensitivity (Ω_{10}) values which are comparable to the experimental thresholds (Tth) and functional thermo-sensitivity Q_{10} values, respectively. Therefore, these results are consistent with the proposals that the melting temperature of the biggest little hairpin with a minimal loop size and weak noncovalent interactions in the loops may be responsible for the temperature threshold for rTRPV1 activation, and that Q_{10} may be controlled by a change of the total minimal loop sizes of the little hairpins in response to a change of the total non-covalent interactions along the gating pathway from a closed state to an open one within 10°C apart.

2. RESULTS

2.1. The PI-free rTRPV1 Had the First Biggest Little Hairpin in the VSLD at 4°C

When capsain competes off the PI lipid at low and high temperatures, this vanilloid compound did not not bridge any two amino acid chains in rTRPV1 to cause any little hairpin in the presence of MSP2N2 under the given nanodisc condition. [14] Therefore, the state-dependent cryo-EM structures of PI-free rTRPV1 at different temperatures could be used to examine the role of temperature-dependent little hairpin melting in the gating pathway. At 4°C, several kinds of non-covalent interactions between amino acid side chains along the gating pathway from D388 in the pre-S1 domain to K710 in the TRP domain formed a little hairpin topological loop network in the closed state.

First, H-bonds between different hydrophilic amino acid side chains contributed to the formation of little hairpins. In the pre-S1/TRP interface Y401-D707 and D411-N695 and K425-E709 were paired for H-bonding. In the VSLD and its interfaces with the TRP domain and pre-S1 domain and the S4-S5 linker, these H-bonding pairs were Q423-D427/R701, D424-R428, D427-K431, Y441/N551-Q519, T449-W549, Y463/Y537-K535, D471-R474, Y487-R491, Q494-Q498, R534-E536, R557-Q700, and E570-K571. In the pore domain and its interface with the TRP domain, the H-bonding pairs included D576-R579/T685, Y584-T641/Y666-T641, and Q691-N695 (**Fig. 1A**).

Second, π interactions involving aromatic side rings also brought about little hairpins. In the VSLD and its interfaces with the pre-S1 domain and TRP domain and the S4-S5 linker, these π-interacting pairs covered Y401-E397/R499, H410-I696, W426/W697-R701, F434-W426/F438/Y555, F438-Y555, Y441-F488/F516/Y555, Y444-F448/F488, M445-W549, Y453-Y454, V486-F490, Y487-F488, F488-F516, F489-I493/L524, Y495-R499/E513, F507-I514, F516-N551/Y554, F517-L521, F522-F543, Y530-F531/Y537, Y554-Y555/R557 and Q560/K698-W697. In the pore domain, the π-interacting pairs had F580-F584/L678, F589-T593, F591-Y666, Y627/E636-F649, F640-Y666, F659-Y653/F649/L663, and Y666-T670 (**Fig. 1A**).

Third, a salt bridge between E636 and K639 in the outer pore also shaped a little hairpin. In addition, the charged H-bonding pairs were also involved in the salt bridges. For example, K425-E709, D424-R428, D427-K431, D471-R474, R534-E536, E570-K571, and D576-R579 (**Fig. 1A**).

Taken together, along the gating pathway from D388 to K710, seventy non-covalent interactions produced the total number of minimal little hairpin sizes as 97 (**Fig. 1A**). Among the multiple little hairpins, although several smaller ones had 0 to 8 minimal residues in the loops, the biggest one was outstanding in the VSLD. It had a minimal 24-residue loop from T449 to Y463, K535, Y537, W549 and back to T449 to control the Y463-K535 H-bond (**Figs. 1B-C**). Because one and half equivalent H-bonds formed this little hairpin loop, the predicted melting temperature was 21°C (**Table 1**).

2.2. The Second Biggest Little Hairpin Appeared in the VSLD/pre-S1/TRP/VSLD interfaces of PI-free rTRPV1 at 25°C

If the first biggest little hairpin with a minimal 24-residue loop in rTRPV1 carries out the single-step melting reaction like DNA hairpins, the calculated melting temperature 21°C should be enough to open it or to change its size above 21°C (**Table 1**). In agreement with this assumption,

when the temperature raised from 4°C to 25°C, a new R455-Y537 H-bond was born so that the Y463-K535 H-bond could be controlled by a smaller little hairpin with a minimal 7-residue loop (**Figs. 2A-B, 2D**). This change rearranged some non-covalent interactions along the gating pathway (**Fig. 2A**).

Table 1 New parameters of the rTRPV1 bio-thermometer based on the hairpin thermodynamic model

Construct	**Control**	**rTRPV1**				
Lipid environment	MSP2N2	MSP2N2				native
PDB ID	7LPE	7LPA	7LPB	7LPD	7LPC	7LPC
Lipid PI	free	free	free	free	bound	bound
Sampling temperature	**48°C**	**4°C**	**25°C**	**48°C**	**48°C**	
Gating state	open	closed	closed	inactivated	closed	closed
# of the biggest little hairpin	3	1	2	4	6	5
Biggest little loop size (L_{max}), a.a	9	24	10	9	12	16
Equivalent H-bonds in L_{max}	1.0	1.5	1.0	2.0	2.5	2
Total non-covalent interactions	65	70	76	71	78	75
Total little hairpin sizes, a.a.	73	97	96	61	92	108
Calculated melting temperature	46°C	**21°C**	**44°C**	56°C	**55°C**	**42°C**
Measured threshold T_{th}		**<25°C**	**<48°C**		**>48°C**	**42°C**
Calculated $\Omega_{10,\,min}$ at E=0.5 kJ/mol			7.74		6.49	12.1
Calculated $\Omega_{10,\,mean}$ at E=1.0 kJ/mol			**17.4**		**14.9**	**26.9**
Calculated $\Omega_{10,\,max}$ at E=3.0 kJ/mol			63.0		55.7	95.1
Measured Q_{10}			**16.4**			**22-28.2**
Ref. for T_{th} and Q_{10}			[14]			[1, 14]

In the VSLD, the Q494-Q498 H-bond and the F507-E513 π interaction were broken while the K466-D471 salt bridge, the R491-E513 and S505-D509 H-bonds, and the Y495-F496 and Y511-L515 π interactions were added. In the meanwhile, the H-bond and the π interaction exchanged in the Y487-R491 and Y495-R499 pairs. In the VSLD/pre-S1 interface, when the D427-K431 H-bond and the E397-Y401 π interaction were disconnected, the critical R409-D509

salt bridge emerged. In the VSLD/TRP interface, R491 formed an H-bond with D707. As a result, in the pre-S1/TRP interface, the H410-I696 and W697-K698 π interactions and the D411-N695 H-bond were disrupted. In the S4-S5 linker/TRP interface, the R557-T704 and Q560-K694 H-bonds were present. In the pore domain and its interfaces with the S4-S5 linker and the TRP domain, the new non-covalent interactions included the E648-K656 salt bridge and the T650-N652 and K688-E692 H-bonds. This global conformational change demonstrated that the pore domain was coupled to the VSLD via the interactions between the S4-S5 linker and the TRP domain.

Along the gating pathway from D388 to K710, although the total number of all minimal little hairpin loop sizes slightly decreased from 97 to 96, the total number of all non-covalent interactions significantly raised from 70 to 76 (**Fig.2A**). When the R409-D509 salt bridge and the D509-S505 and the Y401-R499 π interaction in the pre-S1/VSLD/TRP interfaces generated the second biggest little hairpin with a minimal 10-residue loop from Y401 to R409 and D509 and S505 and R499 and back to Y401 (**Figs. 2C-D**), one equivalent H-bond produced a melting temperature 44°C based on the single-step melting reaction like DNA hairpins (**Table 1**).

2.3. PI-free rTRPV1 Opened at 48°C in the Absence of the Second Biggest Little Hairpin in the TRP/VSLD/pre-S1 Interfaces but Presence of the Third One in the VSLD/S4-S5 Linker/TRP Interfaces

When PI-free rTRPV1 opened at 48°C upon the melting of the second biggest little hairpin with a minimal 10-residue loop in the TRP/VSLD/pre-S1 interfaces, several changes occurred in non-covalent interactions (**Fig. 3A**).

The R409-D509 salt bridge and the R491-E513/D707 and K425-E709 and the Q423-D427/R701 and D424-R428 H-bonds were broken. In the meantime, in the presence of the Y401-R499 π interaction, Y401 formed a new H-bond with Q498 so that the smallest little hairpin with no residue in the loop bridged the VSLD with the pre-S1 domain. When the Y554-Y555 π interaction was disrupted, Y554 also H-bonded with T704 to stabilize the S4-S5 linker/TRP interface together with the R557-Q700 H-bond and the Q560-W697 π interaction. As a result, in the VSLD/S4-S5 linker interface, the π interaction moved from Y555-F438 to F559-F438 with the formation of the R557-E570 H-bond.

When this conformational change extended to the VSLD, the D471-K466 salt bridge, the D471-R474 and S505-D509 and R534-E536 and K535-Y463/Y537 and Q519-Y441/N551 H-bonds, and the Y441-F488 and Y530-F531 π interactions were disconnected. In the meanwhile,

the new Y495-S510 H-bond and the new R499-D509 salt bridge and the new F448-S481 and Y454-R474 π interactions emerged.

When this conformational change extended to the pore domain and its interfaces with the S4-S5 linker and the TRP domain, the Q560-K694 and E570-K571 and D576-R579 and K688-E692 and Q691-N695 H-bonds, and the E648-K656 salt bridge were disrupted. In the meanwhile, the new D601-N628/N652 and E651/Y653-K656 H-bonds were born.

By all accounts, the total numbers of all non-covalent interactions and minimal little hairpin sizes along the gating pathway from D388 to K710 dramatically decreased from 76 and 96 to 65 and 73, respectively (**Fig. 3A**). As a result, the structural thermo-sensitivity Ω_{10} was about 17.4, which was similar to the experimental Q_{10} value (~16.4). [14] On the other hand, even if one equivalent H-bond sealed the third biggest little hairpin with a minimal 9-residue loop in the VSLD/S4-S5 linker/TRP interfaces to control the R557-E570 H-bond, the melting temperature was about 46°C (**Fig. 3C, Table 1**).

2.4. PI-free rTRPV1 Inactivated at 48°C with the Fourth Biggest Little Hairpin in the Pore Domain

When E570 in the S4-S5 linker further formed an H-bond with Q700 in the TRP domain at 48°C, the smallest little hairpin with no residue loop replaced the third biggest little hairpin to stabilize the R557-E570-Q700 H-bonding network up to the melting temperature 64°C. However, the formation of the H410-E692 π interaction and the N695-H410/Q691 H-bonds in the pre-S1/TRP/S6 interfaces locked the channel in a new state with other changes (**Figs. 4A-C**).

In the S4-S5 linker/TRP interface, the E570-K571 salt bridge appeared with the W697-K698 π interaction. When the F559-F438 π interaction was broken in the S4-S5 linker/VSLD interface, the Y401-Q498 H-bond was also disrupted in the pre-S1/VSLD interface. In the meanwhile, the E397-Y401 π interaction was restored with the new R420-Q423 H-bond in the pre-S1 domain. In the VSLD, the F448-S481 and Y454-R474 and Y495-F496 and F517-F496/L521 π interactions were broken with the R499-D509 salt bridge. In the meanwhile, the F438-Y555 and Y444-F488 and F489-L524 and Y530-F531 and Y554-Y555 π interactions and the Y464-K535 and Q494-Q498 and R534-E536 H-bonds were restored with the new Y463-Y537 and R491-Q494 H-bonds and the new Y463-P456 π interaction. Finally, in the outer pore, the D601-N628/N652 and T650-N652 and Y653-K656 H-bonds were disconnected. In the meanwhile, the Y627-F649/E636 π interactions were present.

Taken together, although the total number of all non-covalent interactions along the gating pathway from D388 to K710 slightly increased from 65 to 71, the total number of the minimal little hairpin loop sizes significantly decreased from 73 to 61 residues (**Fig. 4A**). In other words, this new state was much more like a closed state but more stable than the open state and thus served as an inactivated state to desensitize the channel activity (**Figs. 1A, 2A**). In this case, a minimal 9-residue loop from D576 to F580, L678, T685 and back to D576 in the S5-S6 interface became the fourth biggest little hairpin (**Figs. 4C, 4E**). When two equivalent H-bonds sealed it, the resultant melting temperature was about 56°C (**Table 1**).

2.5. The Replacement of the Fifth Biggest little Hairpin in the VSLD/pre-S1 Interface with the Sixth One in the VSLD Prevented the PI-bound rTRPV1 in MSP2N2 from Opening at 48°C

When the resident PI occupies the well-known vanilloid site, rTRPV1 in MSP2N2 cannot open even at 48°C. This closed state of PI-bound rTRPV1 is almost identical between 4°C and 48°C. [14] However, compared to PI-free rTRPV1 at 25°C, there were several differences once the PI lipid linked R409, D509, R557, E570 and Q700 together via H-bonds (**Fig. 5A-B**).

In the pore domain and its interfaces with the S4-S5 linker and the TRP domain, the E648-K656 salt bridge and the T650-N652 and K688-E692 H-bonds were disrupted with the formation of the Y627-E636 and E692-K571/R575 hydrogen or salt bridges and the F587-F591 and D654-F655 π interactions. In the VSLD, the K466-D471 salt bridge, the R455-Y537 and R491-E513 and Y495-R499 and S505-D509 and K535-Y537 H-bonds, and the Y495-F496 and Y511-L515 π interactions were disrupted. In the meanwhile, the P456-Y463 and F489-F490 π interactions and the Y463-Y537 H-bond were formed with the shift of the π interaction from F496-F517 to Y495-E513. In addition, the Y487-R491 π interaction became an H-bond.

When the H-bond shifted from R557-T704 to Y554-T704 pairs in the VSLD/TRP interface, the R491-D707 H-bond was replaced with the R499-D707 salt bridge in the VSLD/TRP interface. In this case, although the smallest little hairpin with no residue in the loop could control the Y401-R499 π interaction and the R499-D707 salt bridge and the D707-Y401 H-bond, the fifth biggest little hairpin with a minimal 16-residue loop from Y401 to R409 and D509 and R499 and back to Y401 should provide a melting temperature 42°C as a threshold to release the nearby PI lipid from the vanilloid site for rTRPV1 opening (**Table 1, Figs. 5A, 5C, 5E**). Even if the D424-R428 H-bond was disconnected in the VSLD/pre-S1 interface, the total number of the non-covalent

interactions and minimal little hairpin loop sizes along the gating pathway would be 75 and 108. If the same open state was employed, the structural thermo-sensitivity Ω_{10} would be about 26.9, which was comparable to the Q_{10} value (21.9-26) (**Table 1**). [1, 14]

However, the tight interaction of rTRPV1 with MSP2N2 allowed H410 in the pre-S1 domain to form an H-bond and a π interaction with N695 and I696 in the TRP domain, respectively. In that case, the sixth biggest little hairpin in the VSLD replaced the fifth one in the pre-S1/VSLD interface. It had a minimal 12-residue loop from D509 to F516, Y554, R557 and back to D509 via the PI lipid (**Figs. 5A, 5D, 5E**). If that sixth biggest little hairpin was also subjective to the single-step melting reaction like the DNA hairpin, the melting temperature was as high as 55°C. That may be why the PI-bound rTRPV1 in MSP2N2 cannot open at 48°C. [14] Of special note, although the total number of the non-covalent interactions along the gating pathway slightly increased from 75 to 78, the decrease of the total minimal little hairpin loop sizes from 108 to 92 still lowered down the structural thermo-sensitivity Ω_{10} from 26.9 to 14.9 for rTRPV1 in such a tight lipid environment (**Table 1**).

3. DISCUSSION

TRPV1 is designed and created as a bio-thermometer in the thermostable system to detect noxious heat and to protect a human or animal body from heat damage. [1-4] To this end, it should meet several protein engineering requirements. First, it should be an integral membrane protein with a relative high physicochemical stability or heat capacity to avoid thermal denaturation. Second, it should be an ion channel that can directly couple a temperature signal to an electronical signal reporter for a readout by a primary afferent sensory neuron. Third, its opening from the closed state within 10°C apart should have a high Q_{10} with a steep response to a small change in a surround predetermined temperature. Fourth, it should have a heat switch with two temperature threshold limits for two gating states, one for activation to sense noxious heat and the other for inactivation to alleviate pain by exploiting use-dependent desensitization upon constant noxious heat stimuli. In the biological and chemical world, a hairpin topological loop structure may be an ideal tool for thermosensitive TRP channels to achieve them.

The DNA hairpin with the limited loop sizes has been designed as a temperature switch, a thermometer and an ionic detector with Q_{10} in the range from 1.8 to 2.7. Its oligonucleotides with complementary nucleotide sequences at the ends form a duplex region as the hairpin stem upon intra-strand hybridization, and its nucleotides in between as the hairpin loop. When a temperature

increases above a threshold, the DNA hairpin melts and thus becomes single stranded. Its melting temperature threshold sharply depends on the H-bonded base pairs in the stem, the size and flexibility of the loop and the ionic strength in a solution. Generally, a stable stem or a small and rigid loop or a high salt concentration in the solution allows the DNA hairpin to have a high threshold to sense and to respond to highly specific and small changes in an environmental temperature. When the loop length is decreased from 20 to 10 A's or the H-bonded G-C base-pairs raise from 2 to 4, the melting temperature threshold can be increased more than 20 degrees. Clearly, a short stem favors sharp hairpin melting rather than gradual melting. Since a change in the loop sequence has no significant effect on the melting temperature threshold of the DNA biosensor, [17] once noncovalent interactions create a hairpin topological loop, the hairpin thermodynamics should be comparable between DNA and protein.

Like the DNA hairpin thermal biosensor, at a given physiological salt concentration (150 mM NaCl), when non-covalent interactions made the biggest little hairpin with a minimal loop size along the gating pathway from D388 in the pre-S1 domain to K710 in the TRP domain of rTRPV1, the melting temperature increased with a decreased loop size and an increased number of equivalent H-bonds to seal the loop. In the absence of the PI lipid at the vanilloid site, rTRPV1 had the first biggest little hairpin with a minimal 24-residue loop from T449 to Y537 and K535 and Y463 and back to T449 in the VSLD to control the Y463-K535 H-bond at 4°C (**Fig. 1**). One and half equivalent H-bonds to seal the loop allowed the melting temperature to be about 21°C (**Table 1**). However, the Y463-K535 H-bond was not broken at 25°C. Instead, Y537 formed another H-bond with R455, decreasing the minimal loop size from 24 to 7 residues to hold the Y463-K535 H-bond. As a result, the second biggest little hairpin was born with a minimal 10-residue loop from Y401 to R409, D509, S505, R499 and back to Y401 to control the R409-D509 salt bridge (**Fig. 2**). As one equivalent H-bond kept this loop, the melting temperature was about 44°C (**Table 1**). Therefore, its melting at 48°C further disrupted the R409-D509 salt bridge in the loop and resulted in the formation of the third biggest little hairpin with a minimal 9-residue loop from R557 to E570, Q560, W697, R701, Q700 and back to R557 (**Fig. 3**). However, the presence of one equivalent H-bond to control the R557-E570 bridge set a melting temperature threshold at about 46°C (**Table 1**). Therefore, at 48°C the formation of the E570-Q700 H-bond generated the smallest little hairpin with no residue in the loop to increase the melting temperature up to about 64°C via one equivalent H-bond to seal the loop. In this case, the fourth biggest little hairpin

appeared in the S5-S6 interface with a minimal 9-residue loop from D576 to F580 and L678 and T685 and back to D576 (**Fig.4**). This loop had two equivalent H-bonds so that the melting temperature was about 56°C (**Table 1**). On the other hand, in the absence of the H410-I696/N695 bridges in a native lipid environment, the fifth biggest little hairpin would have a normal melting temperature 42°C in the presence of two equivalent H-bonds to seal a minimal 16-residue loop from Y401 to R409 and D509 and R499 and back to Y401 (**Table 1, Figs. 5A, 5B, 5C, 5E**). However, once PI-bound rTRPV1 was reconstituted in MSP2N2, the presence of the H410-I696/N695 bridge allowed the sixth biggest little hairpin in the VSLD to use 2.5 equivalent H-bonds to maintain a minimal 12-residue loop from D509 to F516 and Y554 and R557 and back to D509 via the PI lipid bridge. The resultant melting temperature 55°C could prevent the D509-PI-R557 bridge from melting at 48°C for channel opening (**Table 1, Figs. 5A, 5B, 5D, 5E**). Therefore, like a DNA hairpin, a single-step melting reaction of the biggest little hairpin topological loop may be enough to initiate the temperature-dependent threshold in rTRPV1. In accordance with this proposal, in the putative presence of the seventh biggest little hairpin with a minimal 15-residue loop from Y408 to R415 and D517 and R507 and back to Y408 (**Fig. 5E**), platypus (*Ornithorhynchus anatinus*) TRPV1 (pTRPV1) decreases an activation threshold by about 3°C. [24]

Despite six biggest little hairpins in rTRPV1, their roles in gating states may be different. The additional R455-Y537 H-bond decreased the loop size of the first biggest little hairpin from 24 to 7 residues to hold the Y463-K535 H-bond above 21°C. All these happened in the VSLD so that the channel was still closed (**Table 1, Figs. 1-2**). In stark contrast, the second one was closely coupled to the lower S6 gate via the R409-D509 salt bridge in the VSLD/pre-S1 interface and the R491-D707 H-bond in the VSLD/TRP interface. Therefore, its melting disrupted these two bridges, allowing PI-free rTRPV1 to open above 44°C (**Table 1, Figs. 2-3**). Although the third biggest little hairpin may favor the open state by uplifting the S4-S5 linker against the S6 gate via the stimulatory R557-E570 H-bond in the VSLD/S4-S5 linker interface, it had an upper melting temperature limit 46°C. Accordingly, when another E570-Q700 H-bond decreased the minimal loop size from 9 residues to zero for a higher melting temperature 64°C to protect the R557-E570 from the disruption above 46°C, the fourth biggest little hairpin appeared in the S5-S6 interface increasing the upper melting temperature limit from 46°C to 56°C. In the meanwhile, the presence of the H410-E692 π interaction and the N695-H410/Q691 H-bonds in the pre-S1/TRP/S6

interfaces may inactivate the channel, alleviating pain upon constant noxious heat stimuli-induced desensitization (**Table 1, Figs. 3-4**). [1, 8, 14] In agreement with this proposal, the absence of a serine residue between S407 and E408 may not allow pTRPV1 to have inactivating interactions between H416 in the pre-S1 domain and E700 or N703 in the TRP domain so that pTRPV1 has no inactivation (**Figs. 4E, 5E**). [24] In this regard, the deletion of S404 or the H410A mutation may stop inactivation of rTRPV1. On the other hand, the cryo-EM structure of pTRPV1 is expected to have closed and open states but not an inactivated state around 40°C in the presence of 150 mM NaCl.

Since the fourth biggest little hairpin factually had the melting temperature 56°C (**Table 1**), the upper threshold limit for the rTRPV1 bio-thermometer may be 56°C. In the native lipid system, the fifth putative biggest little hairpin with a minimal 16-residue loop in the VSLD/pre-S1 interface may have a normal melting temperature 42°C (**Table 1, Figs. 5C, 5E**). Like the second one, its melting above 42°C may disrupt the R409-D509 salt bridge in favor of channel opening (**Fig. 6A**). When the salt concentration was lowered down from 150 mM to 130 mM, the threshold may also decrease from 42°C to 37.7°C. [8, 24] On the other hand, when rTRPV1 was reconstituted in MSP2N2, the sixth biggest little hairpin had a minimal 12-residue loop in the VSLD to control the D509-PI-R557 bridge. The very high melting temperature 55°C in such a lipid system may be beyond the physiological range for the detection of noxious heat unless a vanilloid compound like capsaicin could bind near D509 and R557 to remove the PI lipid (**Table 1, Figs. 5D, 5E**). [14] Taking all these into account, rTRPV1 may have a bio-thermometer range from 42°C to 56°C to monitor the noxious heat (**Fig.6A**). Further experiments are necessary to examine the role of the Y401-R499 π interaction and the R409-D509 salt bridge in securing the normal temperature threshold 42°C for rTRPV1 opening.

When PI-free rTRPV1 opens at 48°C from a closed state at 25°C, the opening of multiple little hairpins may increase entropy but decrease enthalpy as a result of the decrease in the total non-covalent interactions from 76 to 65 along the gating pathway from D388 to K710 (**Figs 2-3**). [25] Thus, a small decrease in the free energy may be enough for intra-molecular gating rearrangements to activate TRPV1. However, even if several short little hairpin stems were included, such a small decrease may be insufficient to produce a high Q_{10} for noxious heat detection. Therefore, a decrease in the total minimal loop sizes of little hairpins to control the decrease in all the non-covalent interactions and the resultant increase in heat capacity along the

gating pathway should be considered. In direct line with this proposal, when the total number of all minimal hairpin sizes dramatically decreased upon the decrease of the total non-covalent interactions, the resultant structural thermo-sensitivity (Ω_{10}) was comparable to the functional thermo-sensitivity (Q_{10}). For PI-free rTRPV1, the calculated Ω_{10} was about 17.4 while the experimental Q_{10} is about 16.4. For PI-bound rTRPV1, the predicted Ω_{10} was about 26.9 while the experimental Q_{10} is in a range from 21.9 to 26. [1, 14] The presence of the PI lipid at the vanilloid site apparently increased both Ω_{10} and Q_{10} values by about 1/2. [14] When the intensity of a non-covalent interaction ranged from the minimal 0.5 kJ/mol to the maximal 3 kJ/mol, the normal Ω_{10} could be in a range from 12.1-95.1 (**Table 1**). Therefore, any measured Q_{10} values may be reasonable in this range.

Since a decrease in the total minimal loop sizes of little hairpins upon a decrease in the total non-covalent interactions along the gating pathway may be responsible for heat-evoked channel opening from a closed state and the related high Q_{10}, in addition to the biggest little hairpins to start the temperature threshold of TRPV1, the third biggest little hairpin and other smaller little hairpins may play other roles.

The third biggest little hairpin had one equivalent H-bond to seal a minimal 9-residue loop for the open state of rTRPV1 (**Figs. 3, 6**). As the mutation R557E/A/L or E570L/A can disrupt the stimulatory R557-E570 H-bond, it is reasonable that the R557E or E570L mutant leads to a complete loss of function, or mutants R557A/L and E570A have low heat sensitivity, or E570A increases the threshold from 41.5°C to 44.3°C. [26, 27]

The first smaller little hairpin had no residue in the loop from Y441 and though F516 and Y554 and Y555 and back to Y441. It was shared by both closed and open states (**Figs 2-3, 6**), and the related aromatic residues were highly conserved in thermosensitive TRPV1-4 but not in non-thermosensitive TRPV5-6. [28] The tight π interactions may allow it to serve as a relatively stationary anchor against which the S4–S5 linker can uplift in favor of TRPV1 opening. In support of this notion, the Y441S, Y444S, Y554A or Y555S mutation leaves the channel not functional. [26, 29]

The second smaller little hairpin had a minimal 6-residue loop from D576 to K579 and F580 and L678 and T685 via the D576-T685 and D576-R579 H-bonds and the F580-L678 CH-π interaction (**Figs. 1, 2, 4, 5**). The D576-R579 H-bond was disrupted in the open state (**Fig. 3**). It has been reported that the mutant R579E is not functional and the D576N/R or R579A mutant is

less sensitive to heat than wild type rTRPV1. [26] Since the residues for this smaller little hairpin are conserved in TRPV1-6, it may serve as a key common anchor in the pore domain. [28]

The third smaller little hairpin had no residue in the loop from Y584 to T641 and F640 and Y666 and back to Y584 (**Fig. 6B**). The residues for this smallest hairpin were also highly conserved in TRPV1-4 but not in TRPV5-6. [28] In agreement with its importance, the Y666A mutation is not functional while the F640L or T641S mutation decreases the temperature threshold to allow them to be constitutively active at room temperature. [30-31]

The fourth smaller little hairpin had no residue in the loop from Y627 to N628, D601, N652, T650, F649 and back to Y627 to stabilize the open state (**Figs. 3A, 6B**). It has been reported that the N628K or N652T or Y653T or combined mutation increases the temperature threshold, and the triple mutant N628K/N652T/Y653T has the longer opening durations [7] Furthermore, the N628L mutation greatly decreases the heat sensitivity (Q_{10}~2.6) but the N628D mutation has the opposite effect (Q_{10}~86), [14] possibly by mediating the heat stablity of this smaller little hairpin. On the other hand, the turret deletion or replacement with glycine between G603 and N626 in mouse TRPV1 (mTRPV1) decreases the temperature threshold to a different extent, [8] possibly by weakening the corresponding R410-D510 salt bridge in the VSLD/pre S1 interface or generating another new biggest little hairpin with a minimal size larger than 16 residues in the loop along the gating pathway from D389 to K711. Notably, although the deletion of the turret (Δ604-626) still allows the TRPV1 mutant construct to be activated by heat, the binding of external double-knot toxin or vanilla-toxin (DkTX) to the outer pore prevents heat activation, [9, 15] possibly by affecting the smallest little hairpins in the outer pore (**Fig.6**).

The fifth smaller little hairpin had no residue in the loop linking W426 on pre-S1, F434 on S1, Y554 and Y555 and R557 on S4, and Q700 and R701 in the TRP domain together. This little hairpin topological structure was highly conserved in different gating states and thus may also act as a solid anchor upon which the S4–S5 linker can uplift to facilitate TRPV1 opening (**Figs. 1-6**). The sixth smaller little hairpin had no residue in the loop from Y401 to Q498 and R499 and back to Y401 to hold the high Q_{10} value (**Figs. 3, 6**). The seventh smaller hairpin had no residue in the loop from Y444 to M445, W549, T449, F448 and back to Y544. It was highly conserved in all the gating states and thus may serve as a static anchor (**Figs. 1-6**). The eighth smaller hairpin had no residue in the loop from W426 to F434, F438, F559, Q560 W697, R701 and back to W426,

possibly in favor of the open state (**Figs. 3A, 6**). Further experiments are necessary to test these possibilities.

4. CLOSING REMARK

The little hairpin topological loop structures can be used to design a thermosensitive bio-thermometer with a high Q_{10} value. In addition to the ionic strength in the physiological range, the little hairpin size and the number and the intensity of the hairpin-forming non-covalent interactions between amino acid side chains can be used to tune the little hairpin strength for different melting temperature thresholds to detect distinct small environmental temperature changes. These non-covalent interactions include salt bridges, aromatic side chain π interactions and H-bonds. Both the biggest and smaller little hairpins are necessary to control the temperature threshold and the thermal efficacy. Their opening and closure should be reversible and fast enough to have a very large effect on channel gating, which can be easily coupled to an electrical readout in the primary afferent sensor neuron. In this regard, this study may provide a broad structural basis for thermosensitive TRP channels to work as a bio-thermometer.

5. MATERIALS AND METHODS

In this *in silico* study, the little hairpin topological structural aspects of rTRPV1 channels below and above the temperature threshold were investigated and analyzed with graph theory as the bio-thermometer basis for noxious heat detection. The *in silico* protomer models were based on the cryo-EM structural data of closed rTRPV1 in MSP2N2 with PI-bound at 4°C (PDB ID, 7LP9, model resolution= 2.63 Å) and 48°C (PDB ID, 7LPC, model resolution= 3.07 Å), capsaicin-bound at 4°C (PDB ID, 7LPA, model resolution= 3.37 Å) and 25°C (PDB ID, 7LPB, model resolution= 3.54 Å) and 48°C (PDB ID, 7LPD, model resolution= 3.55 Å), open rTRPV1 with capsaicin-bound at 48°C (PDB ID, 7LPE, model resolution= 3.72 Å), [14] UCSF Chimera was used to assess non-covalent interactions in TRPV1. All the stereo- or regioselective diagonal and lateral non-covalent interactions such as salt-bridges and lone pair/CH/cation-π interactions and H-bonds between amino acid side chains along the gating pathway from D388 in the pre-S1 domain to K710 in the TRP domain of rTRPV1 were included to test their potential roles in forming the little hairpins with minimal loop sizes, controlling the temperature threshold and stablizing the heat afficacy for the higher temperature coefficient Q_{10}. The standard definition of non-covalent interactions was employed.

The minimal loop sizes in the little hairpin to control the non-covalent interactrions were calculated using graph theory. The primary amino acid sequence line from D388 to K710 was marked in black. An amino acid side chain involving a non-covalent interaction along the gating pathway of rTRPV1 was defined as a vertex and marked with an arrow in different colors. The same kind of non-covalent interactions between two amino acid side chains was marked in the same color for an edge. A hairpin topologicval loop was considered significant when an edge forms a sealed topological ring with the amino acid sequence between two ends of the edge or other edges. The minimal loop size L was definded as the number of residues that do not participate in any non-covalent interctions in the little hairpin loop. Only the minimal loop sizes of the little hairpins were marked in black. The biggest and smaller little hairpin sizes for channel gating were shown in red circles. The total number of all noncovalent interactions and minimal hairpin loop sizes along the gating pathway were shown in black and blue circles, respectively.

The temperature threshold was calculated from the melting temperature T_m of the biggest little hairpin along the gating pathway using the following equation: [17]

$$T_m\ (°C) = 34 + (n-2)\times 10 + (20-L_{max})\times 2$$

where, n is the total number of the non-covalent interactions equivalent to H-bonds in the biggest little hairpin, and L_{max} is the loop length of the biggest little hairpin.

If a thermosensitive ion channel changes from a closed state to an open state within a temperature range ΔT, $\Omega_{\Delta T}$ was used to evaluate the structural thermo-sensitivity of a single ion channel as calculated using the following equation:

$$\Omega_{\Delta T} = [(L_o-L_c)E/2]^{(Nc/No)}$$

where, L_o and L_c are the total numbers of all the minimal lengths of little hairpins along the gating pathway in the open and closed states, respectively; N_c and N_o are the total numbers of all the non-covalent interactions along the gating pathway in the closed and open states, respectively; E is the energy intensity of a non-covalent interaction in a range of 0.5-3 kJ/mol. Usually, E=1 kJ/mol.

When ΔT=10°C, Ω_{10} could be comparable to the functional thermo-sensitivity (Q_{10}) of a single ion channel. Q_{10} was calculated using the following equation:

$$Q_{10} = (R2/R1)^{10/(T2-T1)}$$

where, R1 and R2 are rates of a reaction at two different temperatures T1 and T2 in Celsius degrees or kelvin, respectively.

Conventions and Abbreviations: cryo-EM, cryo-electron microscopy; DkTx, double-knot toxin or vanillotoxin; PI, phosphatidylinositol; TRP, transient receptor potential; TRPV1, TRP vanilloid-1; TRPVi (i=1; 2, 3, 4, 5, 6); hTRPV1, human TRPV1; mTRPV1, mouse TRPV1; pTRPV1, platypus TRPV1; rTRPV1, rat TRPV1; VSLD, voltage-sensor-like domain

Acknowledgements

The author's own studies cited in this article were supported by NIDDK Grant (DK45880 to D.C.D.) and Cystic Fibrosis Foundation grant (DAWSON0210) and NIDDK grant (2R56DK056796-10) and American Heart Association (AHA) Grant (10SDG4120011 to GW).

Conflict of Interest: The author declares no conflict of interest.

Data Availability Section: This study includes no data deposited in external repositories

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Figure legends

Figure 1. **The little hairpin topological structural network along the gating pathway of PI-free rTRPV1 in the closed state at 4°C. A,** The little hairpin topological loop map. The cryo-EM structure of closed rTRPV1 with capsaicin bound at 4°C (PDB ID, 7LPA) was used for the model.[14] The pore domain, the S4-S5 linker, the TRP domain, the VSLD and the pre-S1 domain are indicated in black. Salt bridges, π interactions, and H-bonds between pairing amino acid side chains along the gating pathway from D388 to K710 are marked in purple, green, and orange, respectively. The minimal loop sizes in the little hairpins required to control the relevant non-covalent interactions are labeled in black. The total numbers of all minimal hairpin loop sizes and non-covalent interactions along the gating pathway are shown in the blue and black circles, respectively. **B,** The structure of the 1st biggest little hairpin with a minimal 24-residue loop in the VSLD to control the Y463-K535 H-bond. **C,** The sequence of the 1st biggest little hairpin with a minimal 24-residue loop. The Y463-K535 H-bond is marked in a blue box.

Figure 2. **The little hairpin topological structural network along the gating pathway of PI-free rTRPV1 in the closed state at 25°C. A,** The little hairpin topological loop map. The cryo-EM structure of closed rTRPV1 with capsaicin bound at 25°C (PDB ID, 7LPB) was used for the model.[14] The pore domain, the S4-S5 linker, the TRP domain, the VSLD and the pre-S1 domain are indicated in black. Salt bridges, π interactions, and H-bonds between pairing amino acid side chains along the gating pathway from D388 to K710 are marked in purple, green, and orange, respectively. The minimal loop sizes in the little hairpins required to control the relevant non-covalent interactions are labeled in black. The total numbers of all minimal hairpin loop sizes and non-covalent interactions along the gating pathway are shown in the blue and black circles, respectively. **B,** The structure of the little hairpin with a minimal 7-residue loop in the VSLD to control the Y463-K535 H-bond. **C,** The structure of the 2nd biggest little hairpin with a minimal 10-residue loop in the VSLD/pre-S1 interface to control the R409-D509 salt bridge. **D,** The sequences of two little hairpins with minimal 7- and 10-residue loops to control the Y463-K535 H-bond and the R409-D509 salt bridge in the blue boxes, respectively.

Figure 3. **The little hairpin topological structural network along the gating pathway of PI-free rTRPV1 in the open state at 48°C. A,** The little hairpin topological loop map. The cryo-EM structure of open rTRPV1 with capsaicin bound at 48°C (PDB ID, 7LPE) was used for the model.[14] The pore domain, the S4-S5 linker, the TRP domain, the VSLD and the pre-S1 domain are indicated in black. Salt bridges, π interactions, and H-bonds between pairing amino acid side chains along the gating pathway from D388 to K710 are marked in purple, green, and orange, respectively. The minimal loop sizes in the little hairpins required to control the relevant non-covalent interactions are labeled in black. The total numbers of all minimal hairpin loop sizes and non-covalent interactions along the gating pathway are shown in the blue and black circles, respectively. **B,** The structure of the little hairpin with no residue in the loop in the VSLD/pre-S1 interface to disrupt the R409-D509 salt bridge. **C,** The structure of the 3rd biggest little hairpin with a minimal 9-residue loop in the VSLD/S4-S5 linker interface to control the stimulatory R557-E570 H-bonding bridge. **D,** The sequences of two little hairpins with minimal 0- and 9-residue loops to control the Y401-Q498/R499 bridges and the R557-E570 bridge in the blue boxes, respectively.

Figure 4. **The little hairpin topological structural network along the gating pathway of PI-free rTRPV1 in the desensitized state at 48°C. A,** The little hairpin topological loop map. The cryo-EM structure of inactivated rTRPV1 with capsaicin bound at 48°C (PDB ID, 7LPD) was used for the model.[14] The pore domain, the S4-S5 linker, the TRP domain, the VSLD and the pre-S1 domain are indicated in black. Salt bridges, π interactions, and H-bonds between pairing amino acid side chains along the gating pathway from D388 to K710 are marked in purple, green, and orange, respectively. The minimal loop sizes in the little hairpins required to control the relevant non-covalent interactions are labeled in black. The total numbers of all minimal hairpin loop sizes and non-covalent interactions along the gating pathway are shown in the blue and black circles, respectively. **B,** The structure of the little hairpin with no residue in the loop in the VSLD/S4-S5 linker/TRP/VSLD interfaces to govern the R557-E570-Q700 H-bond network. **C,** The structure of the smallest little hairpin in the pre-S1/TRP interface responsioble for the inactivation and desensitization. **D,** The structure of the 4th biggest little hairpin with a minimal 9-residue loop in the S5-S6 interface to control the D576-T685 H-bond. **E,** The sequences of three little hairpins

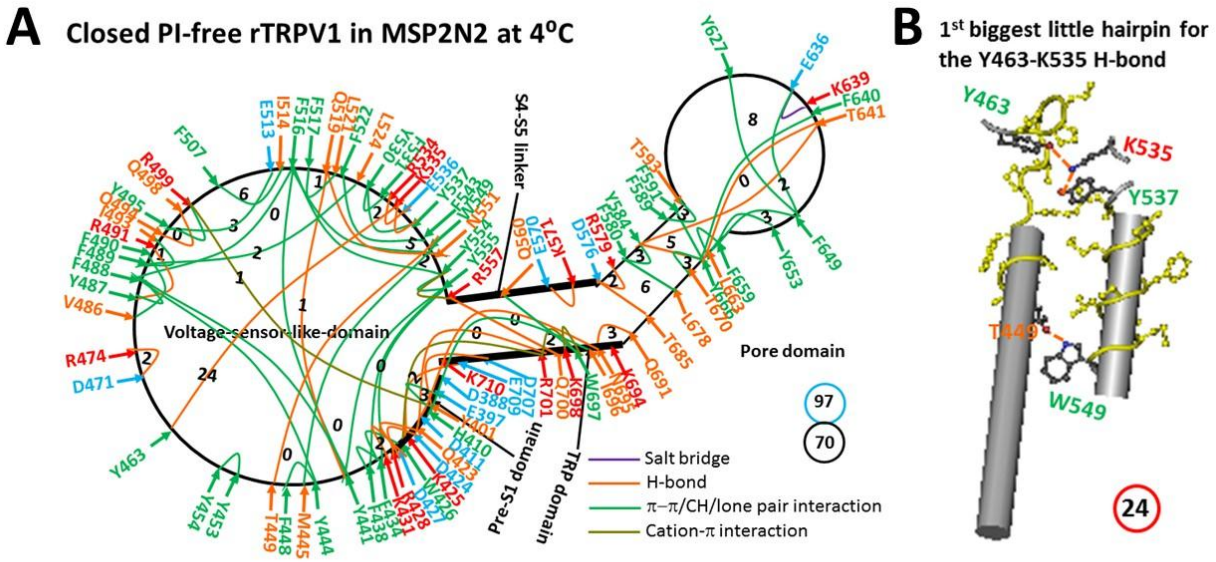
with minimal 0-, 7-, and 0-residue loops to control the R557-E570-Q700 H-bond network, the D576-T685 H-bond, and the H410-E692/N695-Q691 bridges in the blue rectangles, respectively.

Figure 5. **The little hairpin topological structural network along the gating pathway of PI-bound rTRPV1 in the closed state at 48°C. A,** The little hairpin topological loop map. The cryo-EM structure of closed rTRPV1 with PI bound at 48°C (PDB ID, 7LPC) was used for the model.[14] The pore domain, the S4-S5 linker, the TRP domain, the VSLD and the pre-S1 domain are indicated in black. Salt bridges, π interactions, and H-bonds between pairing amino acid side chains along the gating pathway from D388 to K710 are marked in purple, green, and orange, respectively. The minimal loop sizes in the little hairpins required to control the relevant non-covalent interactions are labeled in black. The total numbers of all minimal hairpin loop sizes and non-covalent interactions along the gating pathway are shown in the blue and black circles, respectively. The dashed lines are absent in the native lipid system. The putative biggest little hairpin loop size in the native lipid system is labled in grey. **B,** The structure of the PI pocket. **C,** The structure of the 5th biggest little hairpin with a minimal 16-residue loop in the VSLD/pre-S1 interface to control the D509-R409 salt bridge near PI. **D,** The structure of the 6th biggest little hairpin with a minimal 12-residue loop in the VSLD to control the D509-PI-R557 bridge. **E,** The sequences of the 5th, 6th, and 7th biggest little hairpins with minimal 16-, 12-, and 15-residue loops to control the D509-R409 salt bridge in rTRPV1, the R557-PI-D509 bridge in rTRPV1, and the R415-D517 salt bridge in pTRPV1, respectively. The controlled bridges are marked in the blue rectangles.

Figure 6. The tentative model for TRPV1 opening and desensitization upon the heat-evoked melting of the biggest little hairpin. A. The cryo-EM structures of rTRPV1 in the closed and open and desensitized states (PDB ID: 7LPC, 7LPE, 7LPD, respectively) were used for the model.[14] In the presence of PI at the capsaicin site, rTRPV1 is closed below the threshold 42**°**C and has the biggest little hairpin with two equivalent H-bonds sealing a minimal 16-residue loop (red) to control the VSLD/pre-S1 interface. When the temperature increases above the threshold 42^0C, rTRPV1 is open upon the decrease of the minimal hairpin size from 16 residues to 0 in the loops in the VSLD/pre-S1 interface. In the meantime, the biggest little hairpin with 1.5 equivalent H-bonds sealing a minimal 9-residue loop (red) is formed in the VSLD/S4-S5 linker/TRP interfaces

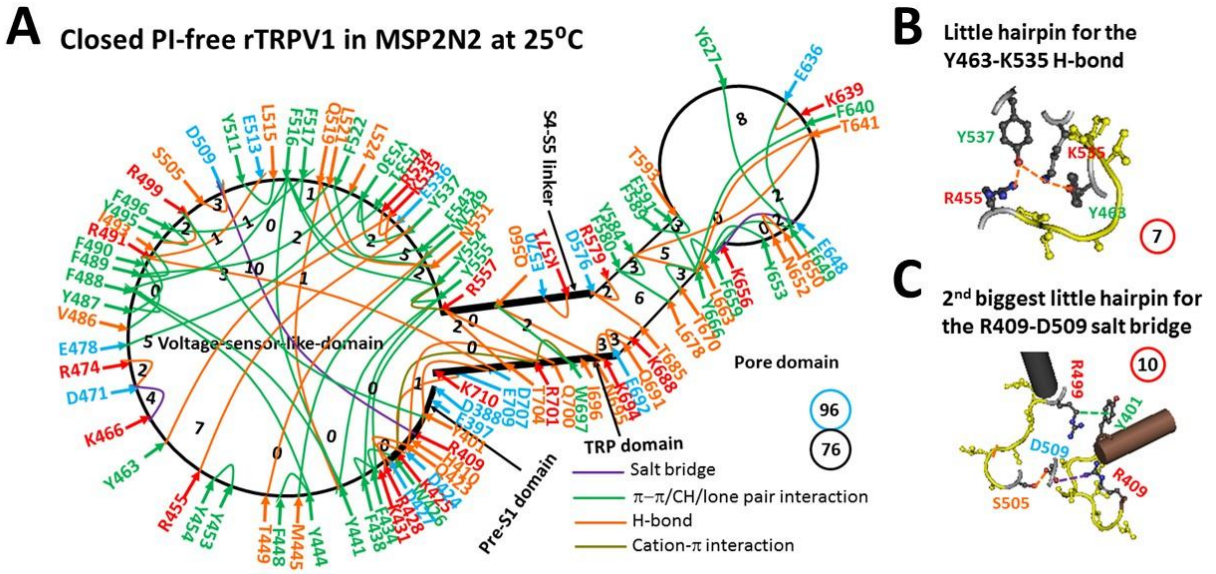
for the open state. However, in the desensitized state, rTRPV1 further inactivates with the presence of the fourth biggest little hairpin with 2.0 equivalent H-bonds sealing a minimal 9-residue loop in the S5-S6 interface in the temperature range from 42°C to 56°C. **B,** The proposed smaller little hairpins for TRPV1 gating. The minimal loop sizes in little hairpins are shown in the red circles.

Figures



C 1st biggest little hairpin sequence : ^{449}TAAAYYRPVEGLPPY^{463}–^{535}K–^{537}YVASMVFSLAMGW^{549}–^{449}T

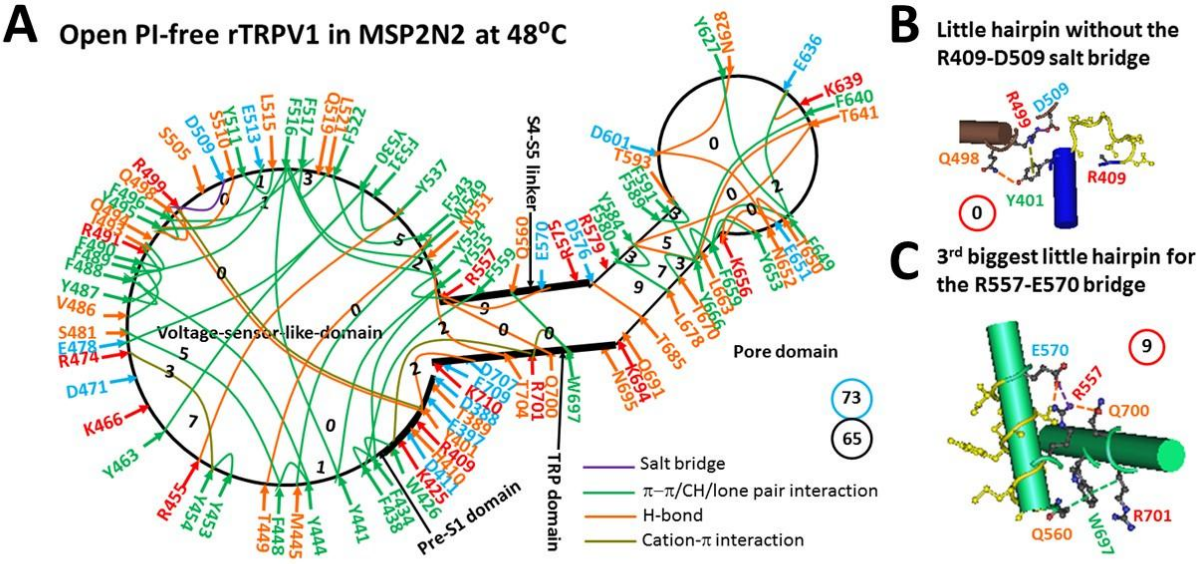
Figure 1



D Little hairpin sequence : ^{455}RPVEGLPPY^{463}–^{535}K–^{537}Y–^{455}R

2nd biggest little hairpin sequence : ^{401}YSSSETPNR^{409}–^{509}D–^{505}SKLSPRR^{499}–^{401}Y

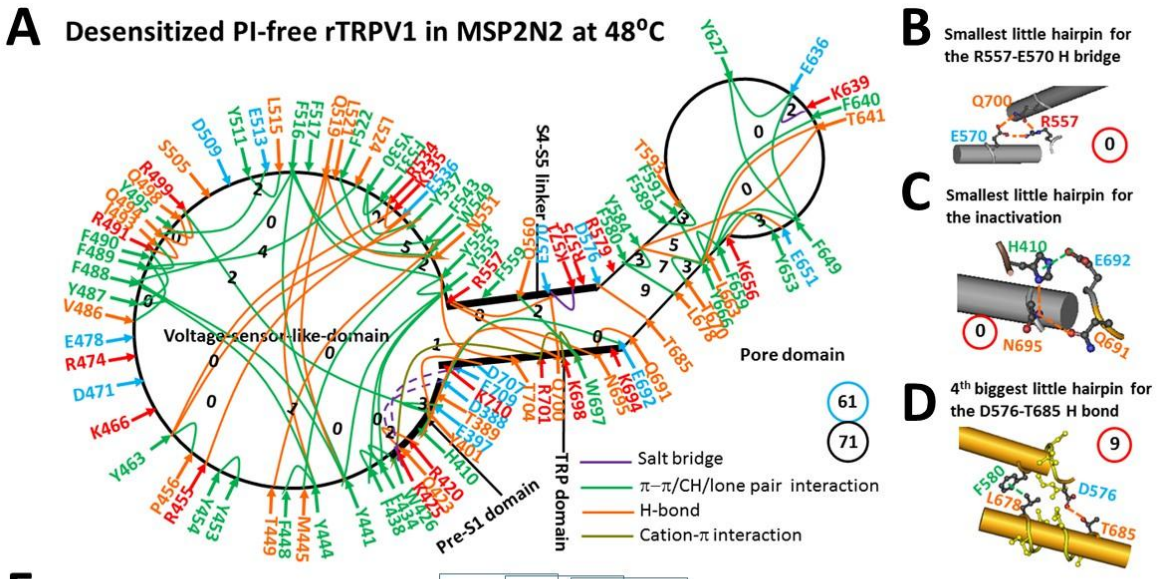
Figure 2



D Little hairpin sequence : ^{401}Y—^{498}QR^{499}—Y^{401}

3rd biggest little hairpin sequence : ^{557}R—^{570}EIMVAYIGMQQ^{560}—W^{697}—^{701}RQ^{700}—^{557}R

Figure 3



E Smallest little hairpin sequence : ^{557}R—^{570}E—Q^{700}—^{557}R

Inactivating little hairpin sequence : ^{410}H—^{692}EQ^{691}—^{695}N—^{410}H

4th biggest little hairpin sequence : ^{576}DLCRF^{580}—^{678}LIALMGET^{685}—D^{576}

Figure 4

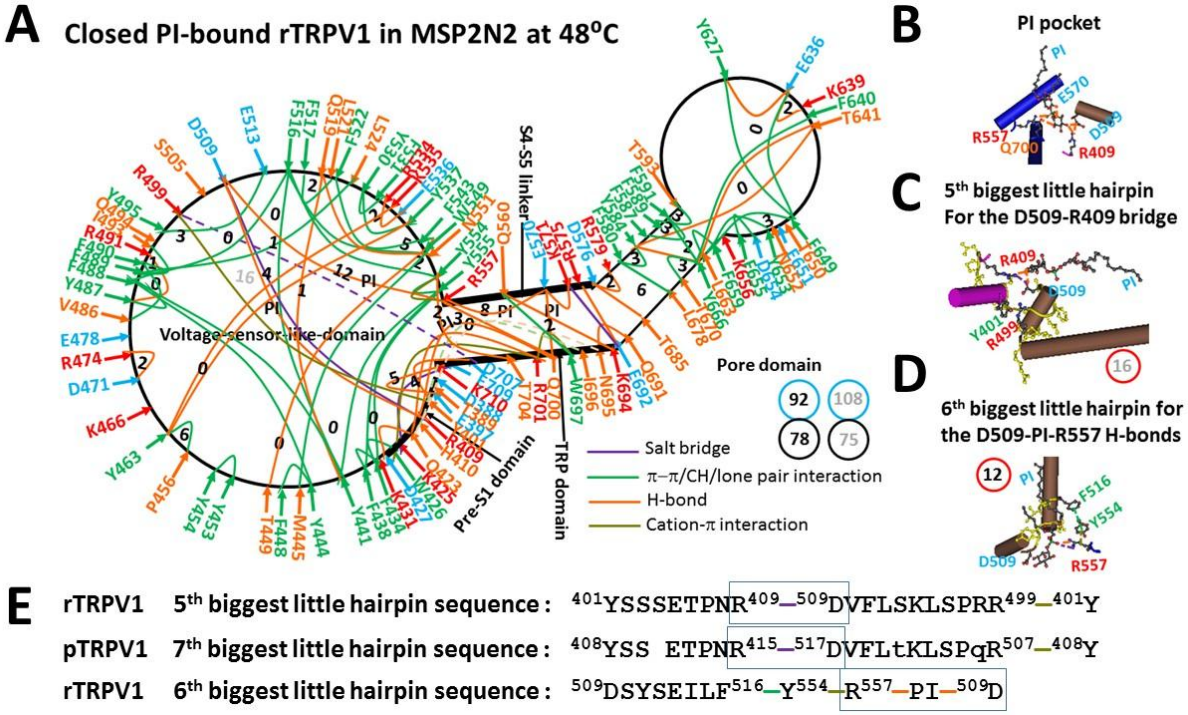


Figure 5

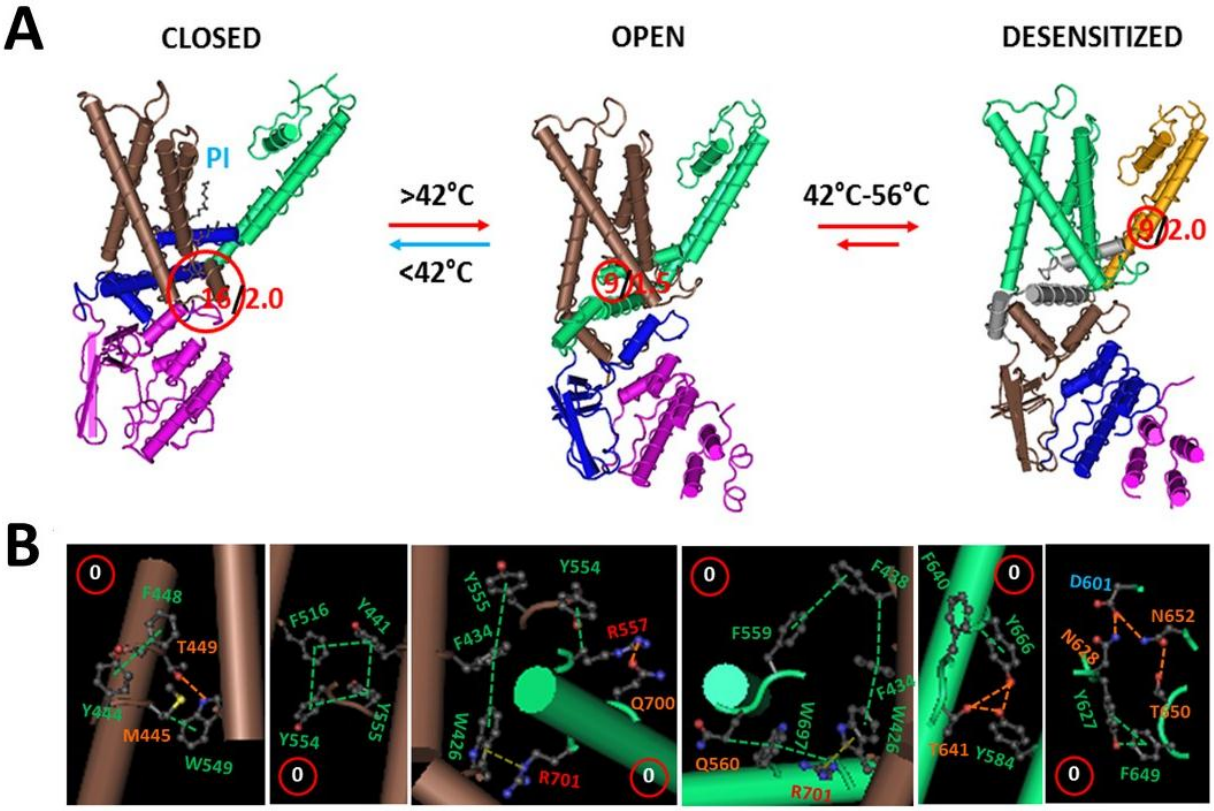


Figure 6