1 **The Role of Ion Transition from the Pore Cavity to the Selectivity Filter in the**
2 **Mechanism of Selectivity and Rectification in the K_v1.2 Potassium Channel:**
3 **Transfer of Ion Solvation from Cavity Water to the Protein and Water of the**
4 **Selectivity Filter**

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12 **Abstract:**

13 Potassium channels generally have a selectivity filter that includes the sequence
14 threonine-threonine-valine-glycine-tyrosine-glycine (TTVGYG). The last five amino
15 acids are conserved over practically the entire range of evolution, so the sequence
16 obviously is necessary to the function of the channel. Here we show by quantum
17 calculations on the upper part of the channel "cavity" (aqueous compartment
18 between the gate and selectivity filter) and lower part of the selectivity filter (SF)
19 how the channel with two sets of four threonines (the channel is fourfold
20 symmetric) effects rectification and selectivity. The threonines are at the location
21 in which the ion transfers from the cavity into the SF; in this calculation they play a
22 key role in selectivity. The channel is also a rectifier. The wild type channel with
23 K^+ and three other cases are considered: 1) the upper set of four threonines is
24 replaced by serines. 2) and 3) Related computations with the Na^+ and NH_4^+ ions help
25 to clarify the important factors in moving the ion from the cavity to the SF. In
26 particular, one set of angles (not bond angles, **O**(T373–C=O) − **O**(T374–OH) −
27 **H**(T374–OH)) flips a hydrogen into and out of the ion path, allowing the K^+ to go
28 forward but not back. This is essentially a ratchet and pawl mechanism, with the
29 ratchet driven by electrostatics. This also allows a clear path forward for K^+ but not
30 for Na^+ or NH_4^+, nor for K^+ in a T$\rightarrow$S mutant. Atomic charges in the lowest positions
31 in the SF are the driving force moving the ion forward, but the O - O - H angle just
32 specified is key to making the "knock-on" mechanism move the ions forward only,
33 using the ratchet with the pawl formed by the hydrogen in the bonds that flip. A
34 water interacts with threonine hydroxyls to allow ion passage, and another water
35 moves together with the K^+.

INTRODUCTION: Potassium channels are found in all cells, and in particular are required as part of excitable tissue, including nerve and heart. They have been extensively studied both for their intrinsic importance and because of the diseases that result when the channels do not function normally. There are several properties that practically all channels have, and are certainly important in the voltage gated potassium channels we consider here: gating, selectivity, conductivity, and inactivation. Selectivity is a function of a selectivity filter in the channel pore. The well-known sequence threonine-threonine-valine-glycine-tyrosine-glycine (TTVGYG) in potassium selectivity filters (SF) is found in essentially all potassium channels, certainly voltage gated channels, plus virus (1) and bacterial channels(2, 3) that are not voltage gated. Save for occasional minor mutations of the valine, and sometimes the first threonine, this has been conserved through half a billion years, and probably more, of evolution. Nature having solved the problem of K^+ selectivity, it has saved the solution.

The first step controlled by the SF is transfer of the ion from the channel cavity which is in the center of the channel pore through which the ion must travel, where it is generally hydrated, to the SF where it must be cosolvated by the amino acids, starting with threonine (we follow customary usage, and call the intracellular end of the SF the lower end). The function of both threonines is generally assumed to have to do with the co-solvation of the ion by some combination of the hydroxyl group and backbone carbonyl groups, but the atomic level details are of critical importance. The problem has been considered by a number of groups, but still is not solved. The reason for the conservation of the threonine, which is rarely substituted by serine, for example, suggests the importance of the geometry and location of the hydroxyl, and requires determination of the exact structure. The wild type (WT) X-ray structure not only of KcsA, a bacterial channel with a small cavity in its pore, but of the eukaryotic, and larger, voltage gated channel $K_v1.2$, is known from the work of MacKinnon and coworkers (4, 5). A number of cases have been calculated for the KcsA bacterial channel. We have done such a study for that channel some time ago (6, 7). Many studies of this channel have also been carried out by other workers. Ban and coworkers(8) have presented the most relevant example, in which they attempted to solve two problems: transfer from the hydrated cavity to the lowest section of the SF, and the exit of the ion at the other end, using quantum mechanical (QM) calculations similar to those we use). Their

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model of the filter consisted of a section at the cavity to filter boundary, but without any of the cavity or its waters, and a section at the extracellular end, with a cylinder without atoms between. They assumed that serine and threonine should be essentially equivalent, and did not compute the actual cavity+lower section structure, but calculated energy assuming full hydration of the ion in the cavity. They did calculate free energy at room temperature for both Na^+ and K^+, but based on their hydration assumptions. We obtained somewhat different results in our earlier work (6, 7) as well as in this calculation. Varma et al (9), find the basis for selectivity still not solved a decade ago. Since then, a number of papers that present a variety of calculations and experiments (10-16) have made attempts to consider the problem; this selection is far from exhaustive, as the literature is voluminous, and the approaches varied. Several authors approach the problem from a thermodynamic point of view, some from a structural point of view. The principal experimental techniques include X-ray diffraction and fluorescence resonance energy transfer (FRET). However, a recent solid state NMR study used $^{15}NH_4^+$ as a proxy for K^+ to determine ion binding density (17) in the SF, to obtain ion binding dissociation constants, with pH dependent occupancy. However, results presented here suggest that NH_4^+ is not a legitimate proxy for K^+; it behaves more like a charged water molecule. A single molecule FRET study finds the SF switching from constrained to dilated conformation, with the constrained conformation, present with K^+, essential for K^+ selectivity. Langan and coworkers (18) used anomalous X-ray diffraction to find an average occupancy of 0.25 per site, which they interpreted as meaning that all four sites are fully occupied; as they noted themselves, this contradicts data showing cotransport of water (19, 20). Their claim was that this supports the direct "knock-on" model in which an ion pushes the ion ahead of it forward. More recently, Langan et al used neutron diffraction to clarify the ion to water ratio in the SF (21). Tilegenova et al (22) used two mutants of the KcsA filter to support the ion-water-ion-water sequence in the filter. Kratochvil and coworkers used 2D IR, with MD simulations, in a semisynthetic KcsA channel, to conclude that water separated the ions in the SF(23). However, Strong et al(24)(24) now show that the Kratochvil et al result fails to settle the matter. Weingarth et al combined solid state NMR with molecular dynamics (MD) simulations on the KcsA channel, and found buried ordered water molecules, primarily in the upper part of the selectivity filter, and outside the pore (25). This is a section of the channel we do not consider in our calculations. However, they also found some ordered water, or

105   possibly weakly ordered water, that they attributed to connections of the SF to the
106   remainder of the pore, and these findings are consistent with our calculations,
107   although they cannot be taken as direct confirmation.

108   There are a large number of simulations and other calculations as well; we consider
109   quantum calculations (QM) first. Cifuentes and Semiao (26) proposed the existence
110   of two paths through the filter, using a time-dependent external field, producing
111   resonances and interference. Summhammer and coworkers (27) carry this one step
112   further in considering the delocalization of the ions. They state that the deBroglie
113   wavelength is not much smaller than the Coulomb potential periodicity. For K^+ we
114   find this unlikely, although for H^+ with a deBroglie wavelength $\lambda_D \approx 0.3$ Å, assuming
115   momentum at room temperature equal to $h/\sqrt{(2mk_BT)}$ (h= Planck's constant,
116   m=ionic mass, k_B=Boltzmann constant, T=temperature). However, for the much
117   heavier, and more relevant, K^+ ion, λ_D is about 6 times smaller, and thus should be
118   out of range of the periodicity of the potential. Summhammer et al argue that
119   transient coherence among ions is required to explain the rate of transport of
120   K^+ ions. Vaziri and Plenio (28) also argue that quantum coherence is important for
121   the SF, and suggest experiments to test this hypothesis. De, March et al (29) extend
122   this argument by including Coulomb repulsion among ions. De et al(30) carried out
123   a QM/MM (MM = molecular mechanics) calculation on the KcsA SF, starting with
124   the lowest threonine, T75 in the 1k4c numbering. They do not include the cavity at
125   all, and the discussion of the role of water is limited. They conclude that each of
126   the amino acids in the TVGYG sequence (the preceding T is not as conserved) has a
127   role to play, with Y78 having key importance in selectivity. Pichierri (31) has
128   calculated the dipole of the KcsA channel and found that the full length KcsA dipole
129   is 403 D (D=Debye), from which he concludes that the channel dipole has a role to
130   play in transport, something no other author has emphasized. Illingworth and
131   Domene, and Illingworth et al (32, 33) have reviewed results on the effects of
132   polarizability. Pless and coworkers (34) emphasized hydrogen bonds, with
133   connection to inactivation. Miloshevsky and Jordan (35) carried out an energy
134   minimization study, also with relevance to inactivation. Bucher and coworkers (36)
135   studied the effects of coordination number, using a QM/MM method. One paper
136   (Cifuentes and Semiao (37)) actually looked at quantum interference of the ions in
137   the SF, a very different matter than a standard QM calculation, which optimizes
138   structure by finding an energy minimum, although it seems unlikely that such large

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139   ions as K^+ would show interference effects if separated by as much as 5 Å (see the
140   comment on de Broglie wavelength above).

141   Several authors have considered the role of entropy in selectivity (14), (38-42).
142   Generally, this is attributed to the conformational shrinking or expanding of the
143   filter, as the amino acids make more or less room for the ion. When the ion is
144   constrained the entropy is lower. However, not all studies have found this type of
145   constriction. While the experimental evidence does not entirely rule it out, there
146   does not seem to be very strong experimental support for this type of constriction
147   either (see also Weingarth et al (25) for example for a possible role for ordered
148   water in producing these effects; this said, they allow for alternating conformations
149   of the SF). These authors find several ways in which the constriction of the SF
150   presents entropic barriers to the ion in the SF. They do not agree in detail, but the
151   general conclusion is that constriction lowers entropy, and this can lead to a barrier
152   to the advancement of the ion. Dixit and coworkers (43) considered coordination
153   of the ion, also with consequences for the entropy. QM approaches give accurate
154   optimizations of a section of the protein, but this may not be a section sufficient to
155   provide a good representation of the actual performance of the channel. It also
156   ignores entropy.

157   The most appropriate form of computation, QM as against MD, is still controversial.
158   Questions have been raised about QM, for example by Roux and others, concerning
159   local minima, the contribution of the surroundings being small but not negligible
160   (44, 45); thermal averaging gives more accurate results, and the local contribution
161   to the energy may, in principle, be important for the free energy(43). In this work,
162   the energy results suggest that local minima are not a problem (further discussion
163   on this point below), surroundings are subtracted in taking differences, but we still
164   do not have results at a finite temperature. The most obvious disadvantage of QM
165   is that the calculation gives the structure at 0 K. It therefore omits thermal
166   averaging and entropic effects. This means that the QM approach, when used for
167   comparison between different ions or mutations, implicitly assumes that the
168   contribution of vibrations of the protein is either neglected, or assumed to be very
169   similar in the cases compared so that it subtracts out in the differences used for
170   data here. It is small compared to the main energy terms, but large enough that it
171   can make a difference in some cases, as has been pointed out, for example, by Yu
172   and Roux (46). Here, the number of degrees of freedom affected by the differences

173 in structure is much smaller than the total, so the differences should be small.
174 However, differences of a few kJ cannot be ruled out, and this may be greater than
175 $k_B T$ (≈2.5 kJ at 300 K). Most of our conclusions are based on structural calculations;
176 energy calculations are consistent with these, but the main conclusions do not hang
177 on them.

178 The most common form of calculation on the filter, as on the rest of the channel, is
179 MD (44, 46-48). Various results, and models, have resulted from these calculations,
180 depending on the starting conformation, on the choice of force fields, and other
181 parameters. Several of the experimental papers have been supplemented by MD
182 calculations. Bucher and Rothlisberger commented on MD from a quantum point
183 of view. Because the results are so variable, interpretation is not obvious. Bucher
184 et al(36) showed, using a QM/MM calculation, that the correct coordination
185 numbers of ions in the channel are not the same as those derived from MD
186 calculations. We will not consider MD calculations further, as the results do not
187 seem consistent, the force fields may not be accurate, and polarization along with
188 electron shifts that alter local charges as the ions move is usually neglected.

189 We may briefly compare the K^+ selective channels to the non-selective NaK
190 channel, which allows all monovalent, and, apparently, some divalent, ions to pass.
191 There is a large literature on this channel as well, much of it devoted to trying to
192 determine the reason for its non-selectivity. The TVGYG sequence has one
193 mutation, to TVGDG, with the consequence that the upper two sites are replaced
194 by a second aqueous vestibule. The open and closed structures have been
195 determined at high resolution (36, 49, 50). In this channel, both Na^+ and K^+ maintain
196 a symmetric configuration. The reason for the loss of selectivity has been variously
197 interpreted in terms of small structural adjustments by a number of authors,
198 without a general agreement (51-53). These citations are not comprehensive, but
199 are sufficient to see that there is still not a general agreement as to how the NaK
200 channel helps elucidate selectivity in selective channels.

201 A controversy that remains concerns the order of ions and water in SF. There are
202 proponents of what Strong et al (24) refer to as "hard" and "soft" knock on
203 mechanisms, where "hard" means that ions can be adjacent to each other in the SF
204 (earlier referred to as "direct"), while "soft" would have a water molecule between
205 ions.

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206   Despite the extensive effort by multiple groups to understand ion selectivity, and
207   the passage of ions through the SF, the subject still requires further work. Our
208   calculation shows how an ion moves from the cavity in the $K_v1.2$ channel into the
209   lowest position in the SF, and compares, at an atomic level, the charges on the K^+
210   ion and the neighboring solvating oxygens, to those when Na^+ approaches the filter,
211   and to those in a mutant in which threonine is replaced by serine. $K_v1.2$ being
212   larger, hence more difficult to understand, it has been less studied than KcsA. The
213   calculations in the literature for the most part do not examine the transition from
214   the cavity to the filter in atomic detail, or, as with MD simulations, there are serious
215   problems that remain. They do note that the filter may be flexible, and some show
216   a relation to inactivation, to ordered water, and sometimes to entropy. These
217   calculations do not consider the reason serine is so rare, with threonine almost
218   always the lowest residue in the SF. Some models are severely truncated, but done
219   at QM level, and others are complete but done by MD with classical potentials. The
220   KcsA channel pore differs from the voltage gated K_v channels, such as Shaker or
221   $K_V1.2$, not only in lacking a voltage sensor, but also in that the KcsA cavity contains
222   less water, with the volume of the cavity being appreciably smaller. However, the
223   SF is the same, and there is sufficient water even in KcsA to participate in solvating
224   the ion at the entrance to the SF. De and coworkers (30) have used ONIOM 2-layer
225   calculations, with the center of the filter in the QM section, to sort out the
226   contributions of the individual amino acids in the filter sequence. They do consider
227   hydration at the filter entrance, finding it endothermic, hence the entrance to be
228   kinetics dependent. They do not consider the serine mutation. Channels with T→S
229   mutations show somewhat reduced polarizability, and possibly reduced
230   conductivity (52); Rossi et al attributed stronger binding of the K^+ ion to the
231   polarizability of the four extra methyl groups in the WT compared with the serine
232   pore (54).

233   *Reasons for using quantum calculations:*   This said, there are a number of
234   advantages to a QM calculation. The charges on the atoms, and the bond orders,
235   can be determined. The energies, even though at 0 K, are more accurate, as
236   exchange and correlation energy terms that have no classical analogue are
237   included. These are larger than the room temperature value of k_BT, and probably
238   larger than the entropy terms that are lost. Also, the channel must go through tens
239   of thousands of cycles in its lifetime. Most calculations do not test whether this is

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240   possible under the assumptions of that calculation. MD calculations, to the best of
241   our knowledge, have not been shown to return to the starting conformation (we
242   omit steered MD calculations here, which do not bear upon this point). We have
243   been able to test one case for the stability of the calculation, using our QM
244   calculations on a section of the voltage sensing domain (53), and found that it is
245   possible to get back to the same minimum from different starting positions. Of
246   course, one instance is not conclusive, but it does suggest that the QM results are
247   not necessarily caught in local minima. The calculations presented here also show
248   that the differences in energy that are found are at least reasonable; if there were
249   serious problems with traps in local minima, we would expect large differences in
250   the energy between different conformations, and the results should be
251   inconsistent with the overall expectations. This is not found, although each
252   minimization (optimization) is an entirely independent calculation. We believe this
253   is significant evidence that the calculations avoid falling into inconsistent local
254   minima. In our calculation, we use a large enough section of the protein that the
255   interaction of the ion with the protein includes at least a second ring of atoms
256   around the ion. Thus most of the surroundings contribution to the energy is
257   included; Dixit and Asthagiri (43) have shown that the surroundings contribution is
258   not large in general, and the fact that we have included at least all of the first shell
259   of protein atoms and much of the second means that this calculation should be
260   accurate, at least from this point of view. The more distant protein would be
261   essentially the same for all ions, and all cases, removing one source of error when
262   taking differences; errors of this type would cancel to a rather high degree of
263   accuracy. All energy values used here are differences.

264   It is clear that there has been a huge amount of interest in the question of how ions
265   traverse the pore of potassium channels, (as well as other channels, e.g., Na^+, Ca^{2+},
266   not discussed here). Each possible effect on the ion's path has been considered,
267   not always together. Most of the approaches either use classical calculations or
268   simplified models. Taken together past work has helped in understanding aspects
269   of the conductivity of the pore of potassium channels, but still leaves much more
270   to be done. We present QM calculations here that show in detail how an ion enters
271   the SF at the lower end of the SF. This too is a subset of the entire problem, but by
272   including all relevant atoms in the region, examining a K_v channel which can be
273   compared to the smaller KcsA channel which we had studied earlier, and paying full

274 attention to the transfer of the ion from hydration in the cavity to cosolvation by
275 the threonine at the beginning of the TVGYG sequence in the SF, all using QM
276 calculations to insure the inclusion of the electrostatic, polarizability, and van der
277 Waals effects, that have previously often been considered separately (55) as well
278 as the exchange and correlation energy totally omitted in classical calculations. In
279 some cases, we will see that electrostatics dominates, but it is worth making sure
280 that all are included, as a difference of even 5 kJ ($\approx$2 k_BT, where T is assumed to be
281 about room temperature) can be significant, and this much or more can result from
282 non-electrostatic interactions. The water takes an interesting configuration with
283 the ion in the cavity, which appears to be that which is seen in the X-ray structure.
284 Oakes et al (56) in a new MD simulation of KcsA, including two mutations (T75A,
285 T75C, where T75 corresponds to T374 in the K_v1.2 channel) consider variations on
286 a knock-on mechanism, with some conformations KWK (ion-water-ion) and some
287 in which the potassium ions are directly apposed (KK). They do not, however,
288 consider specific bonding or bond angles for hydrogen bonds. Their conclusions are
289 similar to those of previous work, in which the KWK conformation is more common
290 than KK; they have cavity water spacing in general agreement with X-ray electron
291 density. They do not observe the "basket" of water first reported by Kariev et al for
292 the KcsA pore (57) , and found again in this work with the K_v1.2 channel.

293 There has been considerable electrophysiological data in the older literature that
294 is also very informative, especially now that calculations give atomic level results
295 that should match the data, at least qualitatively, and actually almost
296 quantitatively. Nimigean and Miller (58) studied the transmission of K^+ through the
297 channel, and concluded that there were barriers both in the channel cavity and in
298 the selectivity filter. Our calculations show how this must be, in atomic detail. They
299 also studied the transmission of other ions. Much earlier Hille (59) found that the
300 ratio of permeability for K^+ to NH_4^+ was 1.00:0.13; quantitative differences among
301 Na^+, K^+ and NH_4^+ in the channel pore that appear in our calculations provide
302 mechanistic insight. Our calculations on the T$\rightarrow$S mutation (T374S in the 3Lut
303 numbering) concern particularly the bottom of the SF; differences include
304 hydrogen bonding and ion coordination, both in KcsA and K_v1.2, with its additional
305 water. It is known that this mutation remains selective for K^+ (16), so that the most
306 serious question is how the transfer from the cavity to the SF is arranged. The actual
307 current for the mutation has not been reported, and we expect the current in the

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308 mutant to be reduced. Our calculated energy suggests that the transfer to the filter
309 becomes more difficult by perhaps an order of magnitude in the mutant. We will
310 show how the K$^+$ ion is symmetric in the WT channel, while the channel with serines
311 substituted for the four tyrosines becomes slightly asymmetric, with the ion moving
312 towards a crevice that forms when shorter side chains of the serines leave four
313 potential wells, instead of the single well for the WT. Our results agree, for the WT,
314 with the structure, including in particular the water "basket" mentioned earlier; a
315 very similar structure has been reported (as an antiprism) by Zhou and Mackinnon
316 (3,4). We have also calculated the atomic charges which help in understanding the
317 behavior of the ion, as well as the reason for the loss of symmetry. Our results in
318 this sense include results beyond those of Rossi and coworkers (54), although we
319 do not obtain thermodynamic quantities, as theirs do.

320 Many papers cite a "knock-on" mechanism, in which ions push the ion ahead in the
321 transmission sequence forward. However, it is rarely clear why there should not be
322 a "knock-back" mechanism, in which the ion ahead, which exerts the same force
323 on the ion following as that ion exerts on the forward ion, pushes the following ion
324 back. The channel is known to be a rectifier; why does it rectify? We show how the
325 solvating oxygen groups have charges that pull the ions from the cavity into the
326 filter. The ion is then held by the charges on the solvating oxygens, which may
327 include the water ahead of the ion in the pore, so that the ion is not pushed back,
328 and any repulsion with the forward ion serves to push that ion forward. Without
329 determining the charges, the initial step into the SF is hard to understand. This still
330 does not completely explain rectification. Most important, we find that a set of four
331 waters behaves as the pawl in a ratchet and pawl mechanism that does not allow
332 the ion to come back, accounting for the rectification implied by the knock-on
333 mechanism. The results on Na$^+$ ion show that there is no reason, in principle, for an
334 ion to always move forward; Na$^+$ actually takes a backward step.

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A   **B**
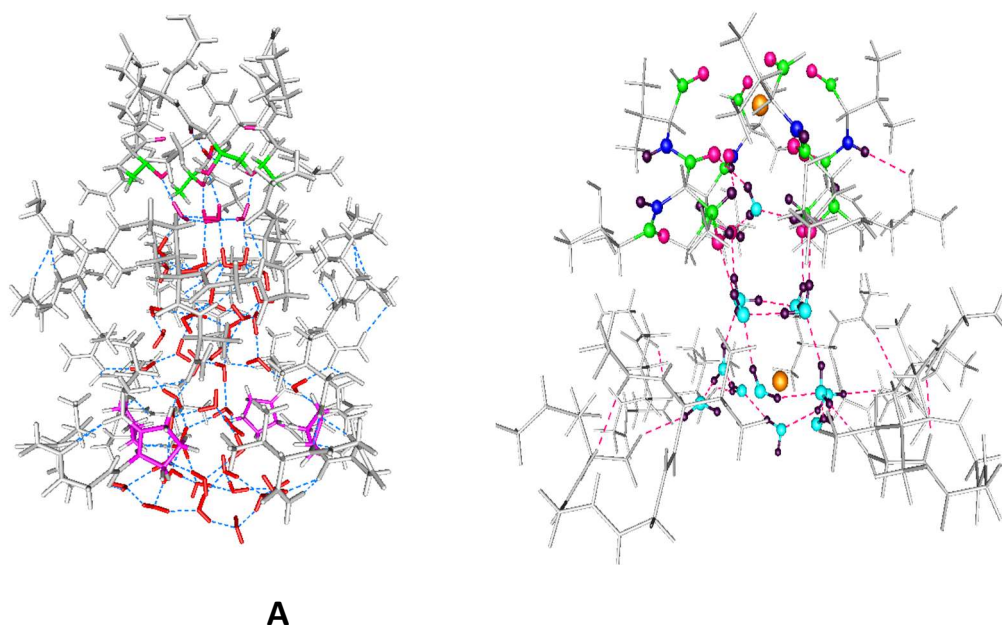
Fig. 1: Fig. 1A shows the system with 870 atoms, but no K$^+$, from which the starting conformation 1 (Figure 1B, for the K$^+$ ion case) was derived.

Methods: The system is truncated from a calculation with 870 atoms and no ions, as shown in Fig. 1A. Fig. 1B is an example of a starting configuration derived from that in Fig 1A, with 457 atoms, and shows the arrangement of the oxygens and two ions at the top of the cavity and the bottom of the selectivity filter, together with the surrounding protein atoms. In figure 1A five levels of oxygens are shown as large spheres. There are two K$^+$ ions (gold) in Fig. 1B. The lowest level of oxygens (light blue) are in the cavity. In the case shown here the lowest K$^+$ is in the cavity, just above this level of water molecules. Four water molecules (dark purple) above this level are hydrogen bonded to a level above in which the oxygens (small light purple spheres) are from the lowest threonines in the TVGYG sequence of the selectivity filter. These are the waters mentioned above, with their hydrogen bonds to the threonine oxygens, which form a "basket" similar to the structure found by Zhou and Mackinnon (4), which they described as an antiprism; the symmetry is less exact here, so we do not label it as an antiprism. There is a water molecule above the threonine oxygens (light blue sphere with two dark hydrogens). The second K$^+$ above this is solvated by carbonyl protein backbone oxygens (light purple). This constitutes the S4 location in the selectivity filter The system in each case was optimized using HF/6-31G*, using Gaussian09 (60). The energy reported is calculated by single point calculations using B3LYP/6-31G**, which was also used

359   for the NBO calculations (61, 62) that give the atomic charges. The calculation for
360   Fig. 1A began from the X-ray structure (3Lut (63)), including 720 protein atoms from
361   the pore, starting with the gate and extending to the second SF position (S3). 50
362   water molecules were added to this, for a total of 870 atoms, and the result was
363   optimized. From this structure, 38 water molecules were deleted, leaving 12. 419
364   of the protein atoms were kept, the rest deleted; two ions were added, for a total
365   of 457 atoms. This was the fundamental starting structure for the wild type (WT)
366   cases (Fig 1B). With the T374S mutation, there were 12 fewer atoms, and with
367   NH_4^+ as the ion, 8 more atoms. The calculations consisted of optimization of the
368   upper section of the cavity, with the first two positions of the SF, generally
369   designated S4 and S3, the optimization starting from the 3Lut structure. In each
370   case, 52 atoms were frozen to prevent overall motion of the system; all the frozen
371   atoms were protein backbone atoms that did not border the pore. The locations of
372   the end of the serine chain in the initial structure were adjusted by hand.

373

374   *Definitions: We use "conformation" to include the complete system, with both ions;*
375   *"position" refers to the location of individual ions. We use "location" (or*
376   *occasionally "position", where the latter is unambiguous), to refer to parts of the*
377   *protein, such as the threonine –OH, or the S3 or S4 locations in the SF. There are 4*
378   *"cases": K^+(WT); K^+ (T374S); Na^+(WT); NH_4^+ (WT). Each case was calculated in three*
379   *conformations.*

380   The three conformations which were optimized were defined as follows:

381   *Conformation 1*, all cases: Initial configuration: one ion placed close to the S3
382   position from the X-ray structure, a water molecule in S4, and the other ion placed
383   below the basket, in the cavity. This was then optimized. Figure 2 shows the
384   position of the most relevant atoms for K^+, Figure 3 for Na^+, making it clear how
385   different the K^+ and Na^{-+} structures are after optimization. In each case, in
386   conformation 1, the ion was started from a position on the pore axis symmetric
387   with respect to the solvating oxygens. The lower ion was initially solvated by the
388   basket water molecules for all cases. S3 is near the top of system as calculated, the
389   pore being truncated one layer of atoms above this.

390 *Conformation 2*, all cases: The calculation began from the optimized conformation
391 found from conformation 1, except that the lower ion was moved up approximately
392 3 Å to a location between the basket and S4; it was optimized from this start.

393 *Conformation 3* started from the optimized conformation 2, with the ions moved
394 such that one ion was below the basket, which is in all cases partially broken (see
395 Figures) and the position of the other ion depended on the case, as the positions
396 of the ions were not always the same after conformation 2 optimization. The point
397 is to start with what would be the consequence of the upper ion moving up and out
398 of the calculated range, the pore water below moving into this position, and a new
399 ion coming up from below in the cavity. It would be a third step after the ions had
400 gone through the first two conformations. The essential characteristic of this
401 conformation for K^+(WT) has one ion fairly near the S4 position, with water in the
402 upper (S3) position. The NH_4^+ and mutant cases had also diverged enough after
403 conformation 2 optimization to require specific description, which follows in the
404 results section, and is illustrated in the figures. Except for K^+ (WT) some asymmetry
405 developed during optimization, which appears to have been especially important
406 for Na^+. NH_4^+ was asymmetric, but moved by a rather different mechanism, more
407 like a water molecule in that it formed hydrogen bonds.

408 **RESULTS**:

409 *Conformation 1:* With the K^+ ion, there was hardly any change in WT from the
410 essentially X-ray position start. With the Na^+ ion, however, the ion in S3 (upper ion)
411 actually moved **down**, back towards the cavity, to a position in which it was held by
412 four solvating groups, including the one pore water. Because the S2 location is not
413 present, the interpretation is limited. The Na^+ result is clearly different than the
414 results with K^+. For the K^+ ion in the T374S mutant, the result in conformation 1 is
415 the same as WT, as neither ion is near the serines. The NH_4^+ behaves somewhat like
416 a charged water molecule; it can form hydrogen bonds, and seems to take the place
417 of water in conformations 2 and 3. The charge drives it forward, however. We have
418 not tabulated the results from conformation 1, in which the lower ion is below the
419 basket in all cases, and the lower ion does not move.

420 The results from the optimizations are expressed quantitatively in Tables.
421 Tables 1 and 2 correspond to conformations 2 and 3. Conformation 1 is not
422 included, in which one ion is placed below the basket, as the K^+ ion moved very

423 little. There are two important results that begin to be understood from this
424 conformation:

425 Selectivity: The *upper* Na^+ ion, starting in S3, moves **down**, as noted above. The K^+
426 ion starting in this position holds its place; it cannot move up, as the section we
427 calculate does not have S2, but it does not move down, as Na^+ does; the Na^+ is held
428 by three waters in the SF, between the ions, at a distance of 2.36 Å. A fourth oxygen
429 from threonine completes the solvation of this ion. This suggests at least part of
430 the reason for the difficulty Na^+ has in making progress toward the extracellular
431 surface in going from conformation 1 to conformation 2 (but see also large positive
432 ΔE, Table 3). Specifically, the energy that is required to break the solvation
433 occasioned by the short 2.36 Å oxygen-ion distance is great enough for the ion to
434 be effectively trapped. The short distance is possible because the ion moves off
435 axis; a symmetrically placed ion, like K^+, is sufficiently distant from four oxygens
436 that it is not held as tightly. This appears to have a major role in selectivity, because
437 the short ion – oxygen distances hold the ion tightly.

438 Rectification: There is one other important difference in the arrangement of both
439 the T373 backbone carbonyl oxygen and T374 –OH groups. These oxygens can
440 either point into the pore or flip back away from the pore. With K^+ the –OH forms
441 a hydrogen bond with the water in the S4 position for three out of four –OHs. One
442 flips to form an H-bond with a T373 carbonyl. With Na^+ three OH flip, while one
443 forms an H-bond with the S4 water. However, as the lower ion moves up, the K^+ WT
444 case shows these –OHs all flipping out of the way, and this is the only case in which
445 this happens. See the discussion of the O – O – H angle below as a ratchet and pawl
446 (not a bond angle: the first O is from a carbonyl). Rectification must also be
447 explained, because the force exerted on an ion rising into the selectivity filter is the
448 same as the force exerted by the ion to push the ion in the selectivity filter forward.
449 Hence, an ion could be pushed back, reversing the current. This mechanism
450 appears to account for the fact that ions must move in one direction.

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455   TABLE 1

456   Distances (Å)$^+$ of *lower* ion to nearest 8 oxygen atoms in **conformation 2**;

++ →	OH 1	OH 2	OH 3	OH 4	W 1	W 2	W 3	W 4
K$^+$ WT	**2.68**	**2.79**	**2.82**	**2.87**	**2.88**	**2.93**	4.03	4.07
Na$^+$ WT	**2.40**	**2.65**	3.93	4.02	**2.30**	**2.40**	**2.44**	3.75
NH$_4$$^+$ WT*	**2.96**	3.80	4.64	5.15	**2.85**	**2.86**	**2.93**	**3.07**

457

458    $^+$ The distances apply to the final position of the lower ion, starting (pre-
459   optimization) between the basket and S4; the upper ion starts in S3
460   ++ T374 OH first four columns, basket water oxygens, last four columns
461   **NH$_4$$^+$ the distance is given to the N atom. Boldface distances show solvating*
462   *oxygens*

463

464   The lower ion described in Table 1 (conformation 2) moves up, and becomes the
465   upper ion in conformation 3; the lower ion in conformation 3 is below the basket.
466   In effect, this assumes the former lower ion in going from conformation 2 to
467   conformation 3 has been "knocked-on", becoming the upper ion, while a new ion
468   enters the system as the new lower ion. The upper ion in conformation 2 has moved
469   into the upper part of the SF, therefore no longer within the computed section. (as
470   quantum particles, the ions are not actually distinguishable, so describing the ions
471   as though they could be labeled assumes a somewhat classical viewpoint, but it
472   should be clear that the overall conformations and transitions between them are
473   being described.) Table 1 shows that only K$^+$ in the WT has symmetric distances to
474   the next level up, defined by the four threonine –OH distances. The total number
475   of solvating oxygens (boldface) for all cases is 5 or 6, except in the mutant in which
476   only 4 oxygens are within the appropriate distance to solvate the ion. The K$^+$ in the
477   WT has its solvation symmetric in this plane. In the other three cases, only one or
478   two of the –OH oxygens are within solvating distance, making the ion take an
479   asymmetric position in this plane, or just below it. The K$^+$(WT) case has the ion
480   breaking the basket, so that the distances are unequal in that plane, but the ion
481   remains essentially on the pore axis. This is not true of any of the other cases. In
482   the mutant, the ion makes a little progress, while the Na$^+$ case has the ion
483   asymmetric, and close to its initial position vertically, unlike the K$^+$ ion. In other
484   words, only the K$^+$ ion makes forward progress into the SF. Alternatively, one can

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485 think of a competition between the basket water and the threonine –OH, which is
486 won by the threonine –OH only for the K^+(WT) case. The NH_4^+ ion hardly moves
487 from its initial position, remaining largely solvated by the basket waters. Its
488 tetrahedron is oriented with three H atoms down, one up.

489 TABLE 2

490 Distances (Å) of the *upper* ion, now in position S3, to the nearest 8 oxygen atoms,
491 in **conformation 3**.

	+NCO 1	NCO 2	NCO 3	NCO 4	OH 1	OH 2	OH 3	OH 4
K^+ WT	**2.89**	**3.08**	**2.86**	**2.85**	3.95	**3.29**	**3.22**	4.70
Na^+ WT	4.81	4.53	4.49	3.73	**2.88***	**2.29***	**2.90***	**2.47***
NH_4^+ WT**	5.27	4.09	5.28	5.28	**2.81**	**3.08**	**2.88**	**2.79**
K^+ T374S	5.48	5.29	4.63	5.12	**2.78**	**2.79**	**2.85**	**2.75**

492 *+ Distances to T374 carbonyl oxygens, except for NH_4^+*
493 ** Distances to basket waters below the SF; Na^+ has failed to advance*
494 ***NH_4^+: the distance is given to the N atom. One basket water remains in its*
495 *solvation shell*
496 *For the mutant with K^+ two basket waters remain in its solvation shell, in addition*
497 *to the T374 OHs*
498 **Boldface distances show solvating oxygens**
499 Table 2 gives distances for the same ion for which the distances were given in Table
500 1, but with the ion in a different position. It gives the corresponding distances for
501 the upper ion in the transition from the threonine oxygen plane to what is
502 essentially the S3 position. The distances in the first four columns are to the
503 oxygens of the backbone carbonyl groups.

504 The lower ion in this conformation starts in the cavity below the basket.
505 Examination of the solvation shells shows Na^+ stuck with strong solvation by basket
506 water from the cavity, and failure to advance to the SF. The NH_4^+ and K^+ mutant
507 advance, but not completely. This set of distances shows a clear difference
508 between Na^+ and K^+ in the pore. Only K^+ (WT) has strong solvation from the
509 carbonyl oxygens, and no solvation from basket water. None of the other cases has
510 the ions optimizing close to the carbonyls.

511

512 Table 3 shows the transition energies between the various structures, as the
513 differences in total energy of the system with the different ions, with the mutation,
514 in kJ mol^{-1}. For comparison, k_BT is approximately 2.5 kJ mol^{-1}, so 20 kJ mol^{-1} in the
515 Table corresponds to a factor of exp(8) ≈ 3000; that is, a barrier of 20 kJ mol^{-1} would
516 slow the ion by a factor of roughly 3000.

517   TABLE 3

518   Total system energy change (kJ mol^{-1}) for the transfer of the ion from
519   conformation 1 to 2, and from 2 to 3

	K^+ (WT)	Na^+ (WT)	NH_4^+ (WT)	K^+ (T374S)
ΔE 1→2	-32	+35	+3	-21
ΔE 2→3	-11	-32	+22	+50

520

521 Table 3 shows the change in energy is negative both for a K^+ ion to go from the
522 cavity to the plane at the bottom of the lowest position in the SF (S4), and then on
523 to the top of S4. All other cases show at least one large energy increase, which
524 would suggest that there is a substantial barrier to the progress of the ion, but this
525 must be understood with caution. Lack of an energy barrier is not sufficient to allow
526 the ion to progress; for one thing, for the mutant, and especially for Na^+, the ion
527 goes backward instead of to the top of S4. The energy goes down, but this does not
528 necessarily mean the ion goes forward. Energy can go down if the next
529 conformation is of lower energy, with the ion going backward as with Na^+ going
530 from conformation 2 to 3. Also, there is a ratchet and pawl mechanism that can
531 block progress, and appears to do so in every case except K^+ (WT). We will discuss
532 the ratchet and pawl mechanism for insuring K^+ goes forward below. Unlike Na^+,
533 the K^+ at the S3 position moves forward out of the computed region; it is replaced
534 by a water, which moves up from below. This is the only case in which there is no
535 barrier to either step, either energetically or through the ratchet and pawl
536 mechanism (Table 5). Na^+ and NH_4^+ behave quite differently from K^+ and each
537 other. NH_4^+ has a substantial energy barrier in the last step, but, as we see below,
538 behaves more like a charged water molecule than like metal ions, and it can move
539 up, albeit not as well as K^+; its conductivity is about 0.13 that of K^+ (59). The NH_4^+
540 conductivity mechanism is different from that of the metal ions, and it is possible
541 to understand how this ion moves forward in spite of asymmetry, or the O – O - H

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542 angles, by examining the selective hydrogen bonding. Finally, the T374S mutant
543 shows the ion essentially unable to move past the first step. In this case, the result
544 is consistent with the one large energy barrier. The ion in this case breaks the water
545 basket below the SF, and retreats, somewhat as Na^+ does in the conformation 2 to
546 conformation 3 transition. The hydration of the ion in the first step switches from
547 the basket to the threonine hydroxyls (except that in the T374S case, it is serine
548 hydroxyls). We know from experimental results that the selectivity for K^+ over Na^+ is
549 large, that it is less for NH_4^+, and that the serine mutant allows K^+ to pass, but not
550 as well as the WT.

551 *Error limits:* These energy results are qualitatively consistent with the observed
552 behavior, even if the energy differences, especially for the mutant second step,
553 seem somewhat too large. Because the absolute values of all calculations are those
554 corresponding to the same outer section of protein atoms in the calculation, with
555 presumably the same errors due to truncation of the system, the differences are
556 likely to be more accurate than the absolute values, due to cancellation of errors in
557 nearly identical systems. Interaction of the center of the system with the boundary
558 may differ in the several cases by enough to account for only a small error. An error
559 of >20 kJ mol^{-1} would be required to invalidate any conclusion stated here (the
560 mutant conformation 2→3 transition may be so large as to be an exception). The
561 most important difference is that between K^+ and Na^+. From conformation 1 to
562 conformation 2, it is 67 kJ mol^{-1}, which seems reasonable. While the errors are
563 almost certainly not large enough to affect conclusions, they might be on the order
564 of 10 kJ, and qualitatively the same results would be obtained for errors up to 20
565 kJ. This said, selectivity can be better understood by examining structure than from
566 these energy values; they are, however, qualitatively consistent

567 There is also the fact that these are 0 K energies, and they may not all have the
568 same heat capacities, hence the ΔG values at room temperature may differ
569 somewhat from the 0 K ΔE. However, the differences should be small, as the
570 structures are similar except for the ion's positions and immediate solvation shells.
571 Unfortunately, it is not possible to use our present computer resources to obtain
572 these values for two reasons: thermodynamic quantities are computed assuming a
573 normal mode analysis, which, with a reasonable basis set requires too much
574 memory, and more fundamentally, a normal mode analysis is likely to be in error.
575 The non-linearities occasioned by the coupling of modes is already enough to

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576   produce errors larger than the differences between the different cases. That the
577   order of magnitude is reasonable is important to assure us that the calculations are
578   not badly in error; the differences are between completely independent
579   calculations. If the calculations were inconsistent, they would show this in huge
580   values of energy differences, but that does not happen (Table 3); if inappropriate
581   energy minima were reached by different calculations, this kind of error would be
582   expected; each calculation requires a separate minimization, so if significantly
583   different minima were found by the optimization, that would be apparent. We are
584   therefore justified in taking structural results as basically reliable.

585   TABLE 4

586   Ion charge for the relevant cases*

	K$^+$ (WT)	Na$^+$ (WT)	NH$_4^+$ (WT)	K$^+$ (T374S)
Conformation 2	**0.80 / 0.76	0.79 / 0.75	0.92 / 0.84	0.78 / 0.81
Conformation 3	0.75 / 0.82	0.74 / 0.73	0.84 / 0.83	0.77 / 0.78

587   *Recall that conformation 3 has the ion which was originally the lower ion rising to
588   the S4 position of the SF; conformation 2 has the lower ion in the position in which
589   it optimizes in the first transition from below the basket, as shown in the Figures.
590   However, for Na$^+$ the ion moves backward, so the ion position in conformation 3
591   differs from that of K$^+$; (see Figures). However, this leads to only limited difference
592   in charge transfer
593   ** The left hand number in each cell is the charge on the ion in the upper position
594   (e.g., for K$^+$(WT), in S3 for conformation 2); the right hand number is the charge on
595   the ion in the lower position, in the conformations shown. The NH$_4^+$ charge is the
596   sum of charges on all five atoms.

597

598   It is difficult to separate the effects of electrostatics, van der Waals terms, and of
599   charge transfer back to the ions; this may not even be a meaningful statement, as
600   the charge transfer leads to electrostatic consequences. The strictly quantum terms
601   (exchange and correlation energy) can be determined separately, but not easily
602   interpreted; they are smaller than the electrostatic terms, but not negligible
603   compared to k$_B$T, which is relevant. Almost certainly, the van der Waals terms are
604   smaller than the electrostatic terms, but they may not be negligible, as the
605   differences may be of the order of a few kJ mol^{-1}; again comparable or slightly larger

606 than $k_B T$. We observe (Table 4) that the K^+ and Na^+ ions both have charge of about
607 $0.78 \pm 0.04\ q$ (q is the electronic charge) in most conformations and positions. This
608 seems to remain roughly constant in the conformations that we calculate here. The
609 total charge on the NH_4^+ ion (lower position) is somewhat larger. The charge for the
610 K^+ ion (lower position) in the mutant is still approximately 0.81 in conformation 2.
611 We can see that there is some charge transfer, with a fraction of an electron moving
612 to the ion, but that there is not a great deal of difference as the ion moves from
613 one position to another. Electrostatics remains the largest term in driving ions
614 forward, however, especially for K^+ in the WT channel because of the charges on
615 the protein groups. At the top of the S4 SF position, the four atoms at the corners
616 of the positions (H-N-C=O of T374) have charges of -0.161 ±0.05; the bottom
617 corners of the S4 position (H-C-O-H, also of T374), +0.058 ±0.08. Summing the four
618 corners, there is then a net charge difference of 0.876 (average) driving the ion
619 upward. The thickness of this site is approximately 3 Å, the diagonal 6 Å(top), 5.3 Å
620 bottom). The consequent field is sufficient to drive the ion from the bottom to the
621 top of this section, helping in the rectification. However, there is another
622 component in the rectification, the ratchet and pawl mechanism that we have
623 referred to earlier.

624 *Ratchet and Pawl: Hydroxyl Rotation Is a Key To Rectification:* There is one
625 arrangement of three atoms that is critical for the progress of the ion through the
626 selectivity filter: O(T373 –C=O) – O(T374 –OH) – H(T374 –OH). Using the T373
627 carbonyl oxygen as reference, the T374 side chain OH can flip its hydrogen into
628 the ion path, or back away from it.

629 TABLE 5

630 Number of angles (O(T373 –C=O) – O(T374 –OH) – H(T374 –OH) in 3 ranges

631

	K^+ (WT)	Na^+	NH_4^+	K^+ (T374S)
Angle*	<25°/>90°/M+	<25°/>90°/M	<25°/>90°/M	<25°/>90°/M
Conformation 1	1/3/0	2/2/0	1/3/0	2/2/0
Conformation 2	4/0/0	2/1/1(35°)	1/3/0	1/2/1(53°)
Conformation 3	3/0/1(35°)	3/1/0	3/1/0	2/2/0

632 * O(T373 –C=O) – O(T374 –OH) – H(T374 –OH)

633 + angle: 25°< M <90°

20

634   Table 5 shows that the T374 hydroxyl proton either bends back out of the pore
635   pathway (the O(T373 –C=O) – O(T374 –OH) – H(T374 –OH) angle is usually ≈18°,
636   always < 25°), or rotates into the path (angle >90°); of the 48 angles in the Table,
637   only 3 take intermediate values, two of 35°, one of 53°, as shown. While the great
638   majority of angles shown as <25° are actually ≈18°, there are two in conformation
639   3, K$^+$ (WT), that are larger, ≈ 24°. The essential point is that angles <25° do not
640   obstruct the path of the ion, while angles >90° do. Only K$^+$(WT) has no obstruction
641   going from conformation 1 to conformations 2 and 3. On the other hand, the
642   conformation 1 angles show that the ion cannot move backward. This set of angles
643   in the three conformations makes this section of the channel a rectifier. As the ion
644   moves to conformation 2, the hydroxyls flip back out of the way, allowing the next
645   ion to move forward. In effect, the hydroxyls act as a pawl in a linear ratchet and
646   pawl mechanism. Combining Table 5 and the electrostatic charges of the S4 cube
647   allows us to see how an ion moves forward. There is a charge difference going from
648   the bottom plane of the S4 position to the top plane, in total, as noted above,
649   averaging 0.876 q, spread out over approximately 60 Å^2 and separated by roughly
650   4 Å. This constitutes the force driving the ion forward, analogous to the crank in a
651   mechanical ratchet and pawl. Here, the molecular equivalent reorients with each
652   passing K$^+$, allowing it to pass. The knock-on mechanism relies on electrostatic
653   interaction between ions, but the force pushing back the rising ion equals the force
654   pushing the upper ion forward, so that we must account for the reason that the
655   pair of ions always moves forward through the selectivity filter. Our calculations
656   are not sufficient to give the details of the return to the initial configuration from
657   the final step in which the upper ion moves ahead, but it is not hard to see
658   qualitatively how the next ion, moving in below the basket, could restore the initial
659   configuration.

660   For all the cases other than K$^+$ (WT), the hydroxyls are not entirely cleared for the
661   transition from conformation 1 to 2. The obstructions are not absolute, and do not
662   alone account for selectivity, but do suggest that the channel is most easily
663   traversed by K$^+$ in the WT channel. The ratchet and pawl mechanism in the other
664   cases interferes with the progress of the ion by having the pawl partially intrude
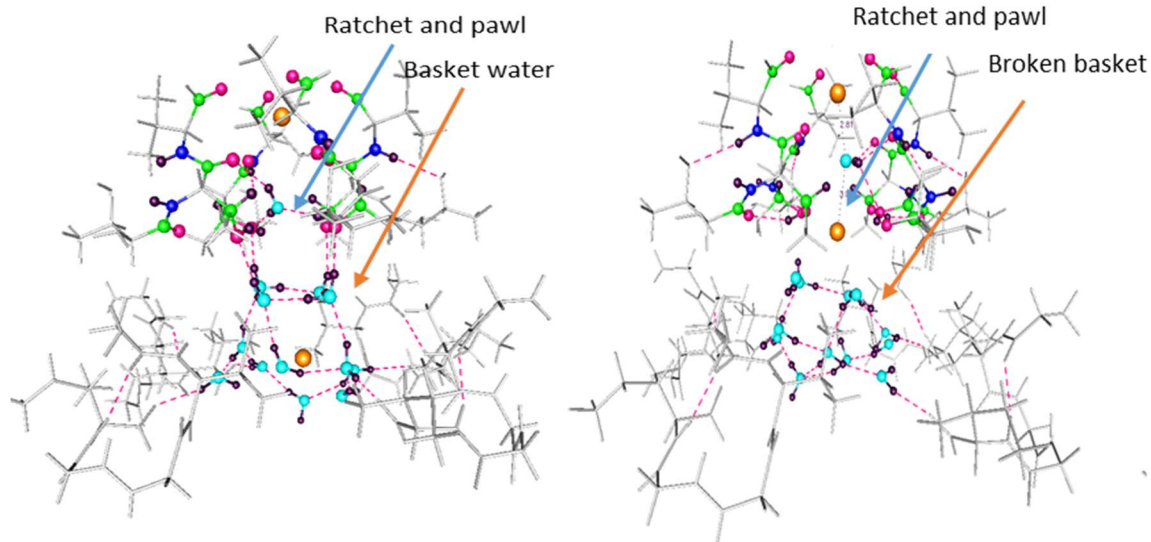665   into the ion path ahead of the ion, rather than behind.

666   We next need to compare the ion-pore interactions with specific detail, including
667   the protein.

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668 ### 1) K^+ and Na^+:

669 We have seen in Table 3 that the K^+ ion goes downhill in energy in both transferring
670 from conformation 1 to 2 and from 2 to 3. The angle formed by the upper ion/water
671 oxygen/lower ion is almost the same for both ions ($167°$ for Na^+, $162°$ for K^+), so in
672 both cases the pore is slightly bent. However, there is a substantial barrier for Na^+
673 in going from conformation 1 to 2, and as we noted it retreats instead of moving
674 forward. The water molecule in S4 rotates as the ion passes.

675 With K^+ in conformation 2, Table 5 shows when the hydrogen of the T374 hydroxyl
676 is bent back out of the way of the ion, or remains in the ion path.

677 A) K^+ (WT): *Conformation 1.* The hydrogen forming the hydrogen bond (T373
678   carbonyl—T374 –OH) forms three such bonds in conformation 1 in the WT,
679   with K^+. In conformation 1 the ion is out of the way of the hydroxyl, so this is
680   not a problem for conduction. Only one out of four hydrogens is bent back.
681   *Conformation 2*: All four H from the T374 –OH are bent back (average $O - O$ -
682   H angle $\approx 18°$). Now there are no hydrogen bonds between the threonine and
683   the pore SF water, leaving a clear path for K^+. The plane defined by the central
684   water molecule is almost orthogonal to the pore axis (water plane angle to
685   pore axis = $98°$), (*cf.* Na^+ for which the corresponding angle is $116°$). With the
686   water tipped down in this manner, the hydrogens are positioned to make
687   hydrogen bonds with the T374 carbonyl oxygens, while with the K^+ the
688   hydrogens are not able to make such hydrogen bonds. *Conformation 3*:
689   Essentially as in conformation 2, no interfering hydrogen bonds; the $O - O$ - H
690   angles are a little larger, about $24°$. The distance from the upper ion to the
691   water oxygen is 2.81 Å, meaning that they are closely associated; the distance
692   to the lower ion, 3.08 Å, is enough larger that the association is appreciably
693   weaker. In conformation 3, the water-ion distance is down to 2.75 Å, but this
694   is water to the next ion, which has jumped up through the entire S4 position,
695   with the four $O - O - H$ angles we have been discussing all bent back out of
696   the way. The ion that was above the water in conformation 2 has moved to
697   the upper part of the SF, out of the calculated section.

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CONFORMATION 1	CONFORMATION 2

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CONFORMATION 3

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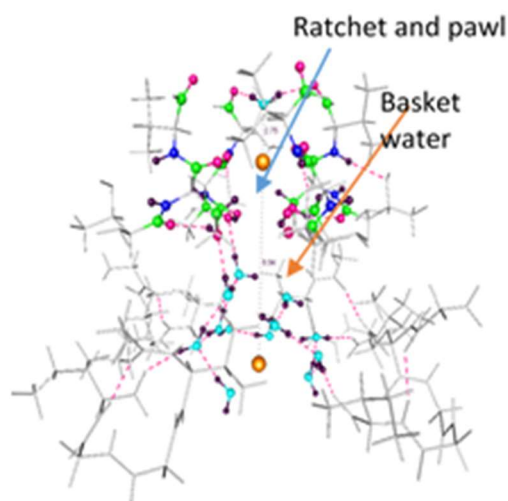
Fig. 2: The three conformations with K$^+$ in WT pore. Ions are large gold spheres. The basket of water is indicated for conformation 1 and for the partial recovery in conformation 3, while the broken basket that exists as the ion passes through is shown in conformation 2 (indicated by orange arrows). The level of the ratchet and pawl mechanism is indicated by the blue arrows; the relevant hydrogen atoms from the hydroxyls are shown in black (small spheres). C atoms, green; N, dark blue; solvating water O, light blue; carbonyl O atoms solvating the ion at bottom and top of S3, magenta; all other atoms, gray, including the 52 frozen atoms. The surrounding atoms of those relevant to the ion progress (gray) remain nearly constant throughout the calculation.
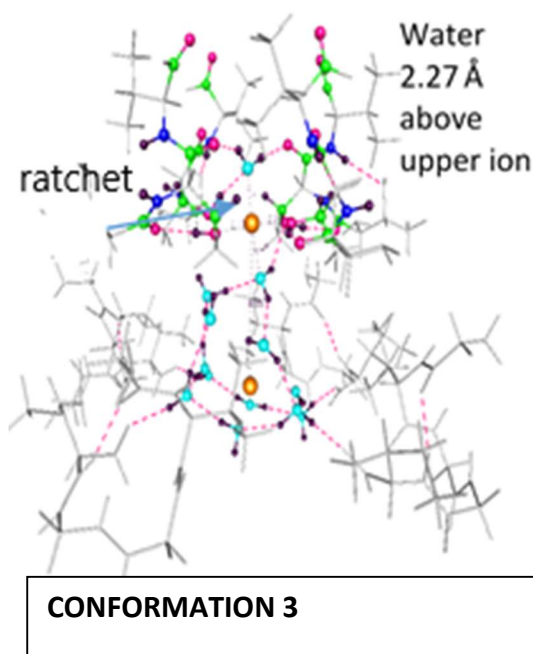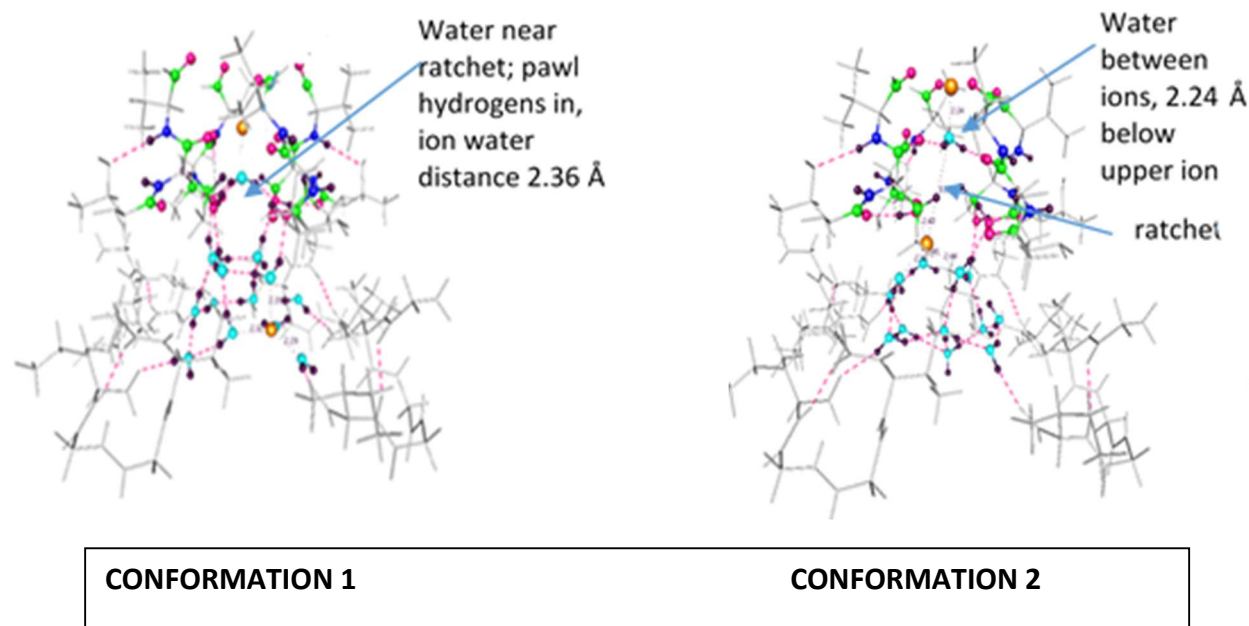
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| CONFORMATION 1 | CONFORMATION 2 |

| CONFORMATION 3 |

Fig. 3: Na$^+$: The corresponding Na$^+$ conformations: Same colors and conditions as Fig. 2. Note the off axis position of the lower Na$^+$ in conformations 1 and 2. In conformation 3, the Na$^+$ has retreated below the S3 position, has a water above it, and waters below at hydrating distances, so that it cannot move. In conformation 1, the water designated as "near ratchet" is oriented with protons down, in contrast to all other cases, and the upper ion-water distance is 2.36 Å, much less than in the K$^+$ case (3.97 Å), so it solvates Na$^+$ strongly. The basket is intact. In conformation 2, with the lower ion still off-axis, and tied via electrostatic interactions to the pore wall, the upper ion is strongly solvated, and a water is only 2.24 Å below, thus tightly hydrating it; in going to conformation 3, the upper ion

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738   actually retreats, and a water *above* is 2.27 Å away, and tightly hydrogen bonded
739   to the protein as well.

740   **B)** Na$^+$ (Fig. 3): *Conformation 1*: There are two obvious differences with K$^+$ in
741   conformation 1. First the lower Na$^+$ ion is off axis. Second, there is a water molecule
742   in the pore between the ions. In the Na$^+$ case, it hydrates the upper ion rather
743   strongly (distance to oxygen, 2.36 Å), and the hydrogens are below the oxygen. This
744   is the only case in which the water takes this orientation, blocking the pore and
745   interacting with the ratchet hydroxyls (three of the T374 residues). With K$^+$ the
746   distance to the upper ion is 3.97 Å, too great to contribute to the hydration of the
747   ion. The lower Na$^+$ ion interacts primarily with cavity water, below the basket.
748   *Conformation 2*: Asymmetry, and one interfering H bond, remain and make it
749   difficult to advance, as the ion's positive charge is close to the negatively charged
750   oxygen on one side of the pore (Table 1). The combination of the asymmetry in the
751   Na$^+$ - O distances, with the O – O – H angles shown in Table 5 adding somewhat to
752   the effect, helps explain the barrier as shown in the energy increase in going from
753   conformation 1 to conformation 2 (Table 3). Thompson et al found an X-ray
754   conformation for KcsA with Na$^+$ very much like what we calculated, save that they
755   did not find the asymmetry. It is not clear that they allowed for the possibility of
756   asymmetry; the placement of the ions and water is otherwise very similar to our
757   calculation for conformation 2; they also found a large barrier for the ion to enter
758   the SF, in agreement with our result for going from conformation 1 to conformation
759   2 (Table 3) (64). *Conformation 3*: This is the only case in which the ion went
760   *backward* after conformation 2, which accounts for the negative energy for the
761   transition (Table 3); it is not the case that the negative energy indicates lack of a
762   barrier to forward motion, but lack of a barrier to backward motion. The ion is
763   blocked in going from 1 to 2, so overall there is no progress for the ion in the SF.
764   *Summary:* The location of the hydrogen bonds partially determines whether the
765   ion can progress through the SF but it is mainly the asymmetry and, secondarily,
766   the O – O – H bonds that constitute the pawl for the ratchet that impede forward
767   motion (Table 5), so the ion is blocked by the hydrogens that remain in the path.
768   For K$^+$, but not for Na$^+$, the pawl - O – O – H bonds bend all the hydrogens out of
769   the path of the ion in going from conformation 1 to 2, and from conformation 2 to
770   3.

771   There is another major difference between the ions. We have already discussed
772   conformations 1 and 2. Consider the water molecule that separates the ions along
773   the pore (the water in the ion – water – ion sequence). We just saw that in
774   conformation 2, with K$^+$, the upper ion – pore water oxygen distance is 2.81Å, lower
775   ion to the same oxygen, 3.08 Å. For Na$^+$ the corresponding distances are 2.24 Å for
776   the upper ion, and a fairly huge 5.04 Å for the lower ion. It is possible that the
777   energy required to separate this water from the ion is responsible for much of the
778   large barrier shown in Table 3 for Na$^+$ in passing from conformation 1 to 2. Once
779   one gets to conformation 2, the upper Na$^+$ ion is tightly hydrated, while the other
780   ion is completely separated, and held by waters below, approximately in the basket
781   location. These four conformation 2 waters have Na$^+$ ion-water distances (d) 2.30
782    $\leq$ d $\leq$ 2.44 Å, so that they hydrate the ion well (recall the 2.36 Å distances in
783   conformation 1), but, being also hydrogen bonded to neighboring protein groups,
784   also anchor it in conformation 2. Since our conformation 3 assumed the K$^+$ ion in
785   the upper position, S3, had moved up, the inability of the lower Na$^+$ ion to move
786   might not show up clearly in the energy computation. K$^+$ has transferred from
787   hydration below to solvation by the T374 carbonyls above, and with distances to
788   neighboring oxygens that suggest that the ion is able to continue to transfer almost
789   exactly on the pore axis, while Na$^+$ is trapped. In other words, moving to
790   conformation 3 for Na$^+$ is not progress.

791   This result can be looked at in a slightly different manner that reinforces the point.
792   There are hydrogen bonds between the T373 carbonyl (this is the threonine below
793   the beginning of the filter proper at T374) and the T374 hydroxyl. The asymmetry
794   noted for Na$^+$, but not K$^+$, can also be understood from ion to hydrating water
795   distances, especially in conformation 3.

796   Conformation 2: For K$^+$ the hydrating water distances are 2.72±0.07 Å, for Na$^+$ 2.80
797   ± 0.20 Å, with a range from 2.68 to 3.00 Å; the Na$^+$ appears to twist the pore slightly
798   (see Fig. 3).

799   Conformation 3: the distance for K$^+$ is 2.78 ± 0.03 Å, while for Na$^+$, the
800   corresponding distances are 2.79 ± 0.29, ranging up to 3.08 Å. K$^+$ hydration has the
801   water molecules an order of magnitude more tightly grouped (i.e., symmetric) than
802   does Na$^+$. The overall structure of the pore is not greatly changed, but the
803   symmetry is somewhat broken when Na$^+$ is in the pore. Nimigean and Miller

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804 showed that it takes about 200 mV to drive the Na^+ ion through the KcsA potassium
805 selective SF (62), much more than physiological potentials. The Na^+ ion is also
806 solvated differently from K^+ in bulk solution, which may be expressed through the
807 stronger electric field for the smaller ion, in addition to steric consequences of the
808 small size difference; the distances above suggest that both effects play a role. The
809 differences between Na^+ and K^+ can be seen by comparing the conformation 2
810 figures.

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| CONFORMATION 1 | CONFORMATION 2 |

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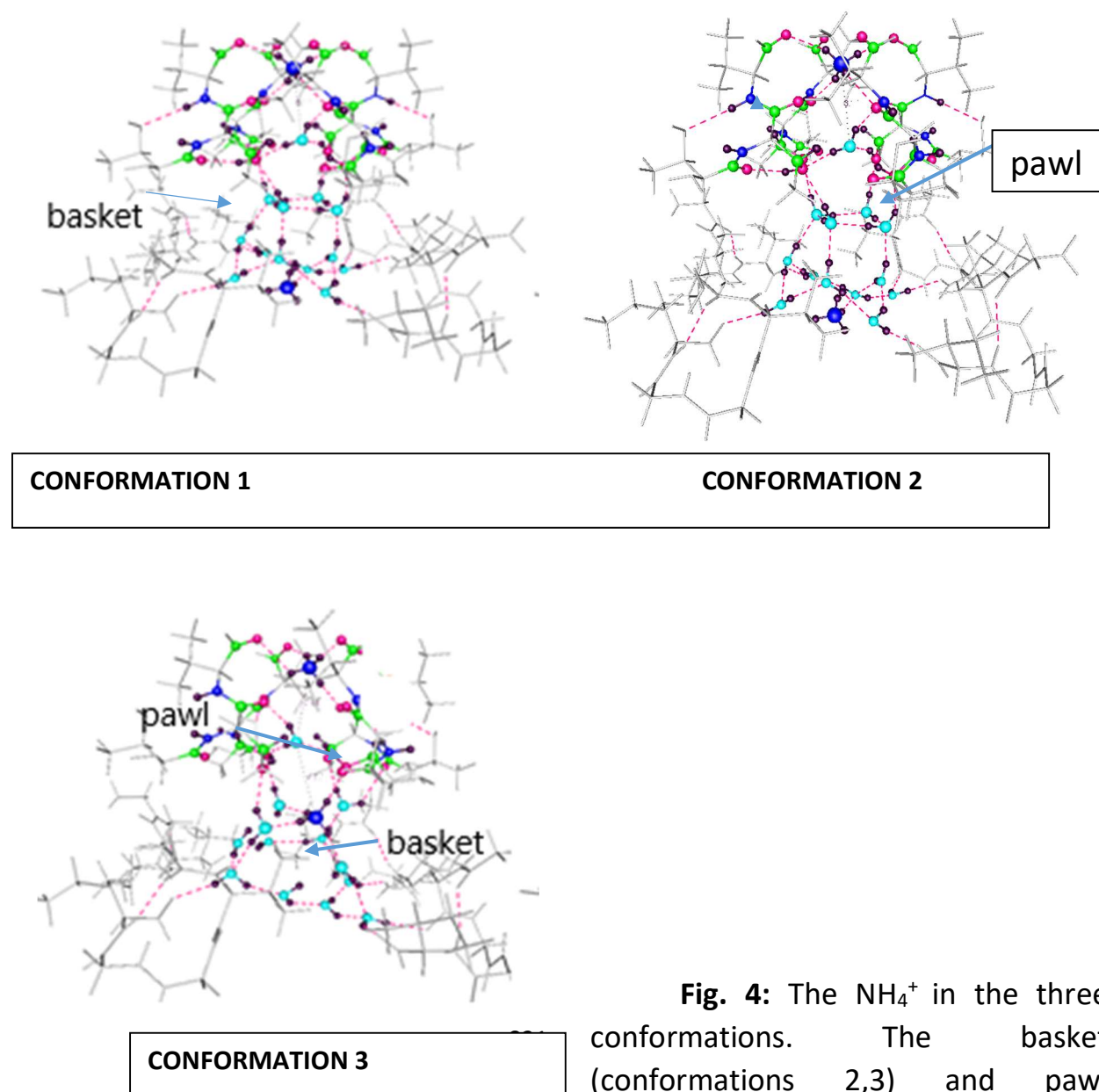
CONFORMATION 3

Fig. 4: The NH_4^+ in the three conformations. The basket (conformations 2,3) and pawl

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823   (conformations 1,3) are indicated on the figures. The basket begins to reform in
824   conformation 3. The NH_4^+ remains essentially on the axis, avoiding the asymmetry
825   of the Na^+.

826   **2) NH_4^+**: *Conformation 1:* The most remarkable thing about conformation 1 is that
827   it is unremarkable; the ions take positions similar to those taken by K^+.
828   Conformations 2 and 3 are different.

829   *Conformations 2 and 3:* The NH_4^+ ion differs from the other two ions in a rather
830   obvious way, as it has five atoms, and stretches therefore over a distance which
831   allows different hydrogen bonding partners. While this ion is less well able to
832   traverse the pore than K^+ (about 1/8 the rate of K^+), it is much better able to do so
833   than Na^+. Also, there is a difference in charge transfer (net charge on the entire ion
834   is about 0.84 in most cases, larger than for the metal ions) as well as the fact that
835   it does form hydrogen bonds, rather than only electrostatic and charge transfer
836   interactions as with the K^+ and Na^+ ions. The angles in Table 5 do not look promising,
837   as three are large, and thus obstruct the path in conformation 2. However, NH_4^+ can
838   rotate, so that its relation to the progress of the ion is not as direct as with the
839   metal ions. While it appears that the decrease in the number of unfavorable
840   hydrogen bonds (Table 5) in going from conformation 2 to conformation 3 should
841   help, the barrier gets larger (Table 3). We can suggest that the rearrangement
842   requires more energy, so that a larger barrier results. The figures help in
843   understanding how this ion differs from Na^+ and K^+; the comparison turns out to be
844   complex. In conformation 2, the lower NH_4^+ has inserted itself into what remains
845   of the now asymmetric basket, replacing one water, and forming hydrogen bonds
846   in its place. By replacing water, it appears that it can work its way through the
847   selectivity filter, going off axis, but not in the way Na^+ does, so it can produce a
848   reasonable ionic current, albeit not as large as K^+.

849   **3) K^+ (T374S mutation)** *All conformations:* Here the difference with WT is a
850   consequence of the difficulty the ion has in charge transfer and electrostatic
851   interactions with the serine hydroxyls, which are a little more than 1 Å more distant
852   in each direction than the threonine hydroxyls.

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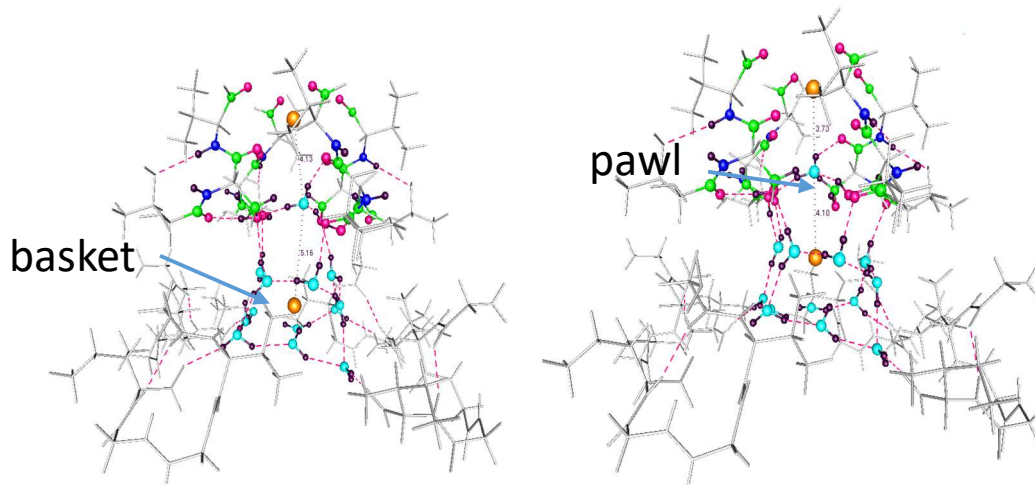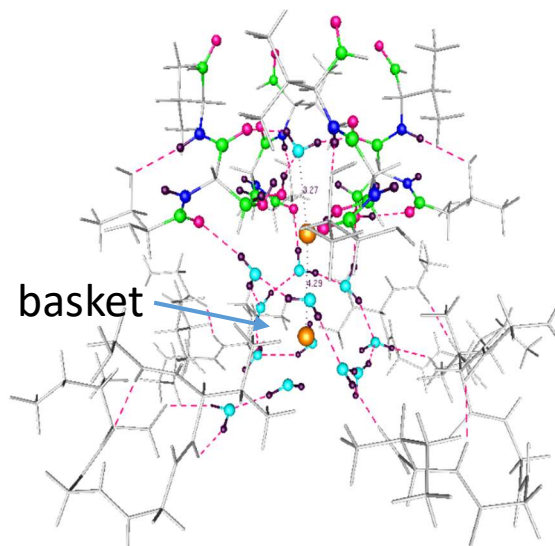
| CONFORMATION 1 | CONFORMATION 2 |

Fig. 5: The three conformations for the T374S mutant. All color conventions as in Fig 2 and 3. The ions stay on axis; unlike Na^+, symmetry is almost maintained. However, progress is impeded by the ratchet and pawl mechanism; some hydroxyls are folded into the ion path; see Table 5, and discussion.

| CONFORMATION 3 |

868

869 However, again the same $O - O - H$ angles turn out to be the main issue. Two out
870 the four hydrogens are bent back, but two are in the path of the ion. This is true in
871 all three conformations. The key $O - O - H$ angles do not allow free passage. The
872 consequence is a major blockage in transferring from conformation 1 to 2. By the
873 time the ion reaches conformation 3, it has broken through the barrier and reached
874 a state somewhat similar to the ion in the WT channel.

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875 Going back to conformation 1, we find that the H-bonds are only slightly
876 asymmetric: two are similar to those in the WT, but one is with an –OH from T374,
877 one with a –C=O from T373. As a consequence, the basket is distorted, impeding
878 the ion's progress. The distance from the lower ion to the S4 water is 5.16 Å, from
879 the S4 water to the upper ion 4.13 Å, so that the solvation of the ion by these waters
880 is weak to non-existent; the occupancy of the SF is distorted, and, with two O – O -
881 H hydrogens blocking the ion, the path is difficult. As shown in Table 1, the lower
882 ion is solvated by three basket waters (the basket is now also distorted).

883 *Summary:* The results with the T374S mutant support the basic lesson of the WT,
884 that the position of hydroxyl groups, especially those on T374, and their hydrogen
885 bonding, determines whether the ion has a clear path to move forward in the SF.

886 *Electrostatics of the lower transition:* We saw earlier that the sum of the charges
887 on the atoms in the plane above the S4 plane is more negative than those in the
888 plane below, the difference being 876 mV. Therefore, this requirement is fulfilled.
889 We can only postulate at this point that the charges above this remain
890 approximately the same all the way to S0, the final position in the SF, so that the
891 ion can exit to the extracellular medium with no gradient needed; that computation
892 has not yet been completed. However, we note that the carbonyls are very similar
893 to those in the upper S4 plane, so it is reasonable to assign roughly the same
894 charges to them. If so, this, combined with the driving force and the ratchet and
895 pawl in the lower section of the SF, would explain how the knock-on mechanism
896 works in a forward only direction. From this, one might guess a mechanism by
897 which K_{ir} channels have opposite rectification, although it is not clear where in the
898 SF this would be; it is not necessary that it be the first or last position in the SF. It
899 does suggest that a different, or differently oriented, mechanism, must be involved.
900 This remains for future work.

901 **DISCUSSION:** The transition of the ion from the cavity to the selectivity filter is
902 complex. It requires that the ion be properly solvated in both the cavity and the SF,
903 but quite differently. The exchange of solvation, with preservation of symmetry,
904 works for K^+ but not for Na^+, and rather differently for NH_4^+, which is itself not
905 spherically symmetrical, so that its orientation matters. The cavity → SF ion
906 transfer appears to be responsible for much of selectivity. Each ion is solvated
907 differently, both in the cavity and in the selectivity filter, and the transfer between

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908 the two locations requires that the ion be able to remain on the pore axis in order
909 to continue through the filter. We can draw four lessons from our results: 1) not all
910 ions are solvated equally in the transfer into the SF. 2) Threonine is the right size to
911 insure the proper solvation of K^+ but not Na^+. 3) by examining the charges on the
912 solvating groups, we understand the importance of electrostatic forces in moving
913 the ions into the first position of the SF. 4) the orientation of water with respect to
914 the pore axis and the protein is important in producing rectification. This is not a
915 direct knock-on transition; such a mechanism would in any case require a trap for
916 the lower ion, so that it did not become a knock-back mechanism, in which the
917 upper ion pushes the lower ion back down. The ratchet and pawl part of the
918 mechanism requires water between the ions. 5) Finally, there is a role of symmetry
919 in the transition into the SF from the cavity, with part of the selectivity against
920 Na^+ coming from the slightly asymmetric placement of the ion, which increases
921 local electrostatic interaction. The shorter serine side chain in the mutant disrupts
922 the proper hydration, and produces some asymmetry, even for K^+.

923 It appears that the ion transfer mechanisms cannot be so easily classified as direct,
924 or hard, or indirect or soft, but require consideration of the side chains of the amino
925 acids that are thoroughly conserved. Water is present near the ions, but can be
926 pushed somewhat to the side (albeit possibly not normally so, with K^+ In WT). We
927 have seen how the ion may be coordinated to multiple water molecules. Because
928 of the role of the charges on the oxygens that solvate the ions, including K^+ in WT,
929 it may not be entirely fair to describe the mechanism as simply "knock-on". KWK
930 configurations are not all the same, as the water may sometimes be more strongly
931 hydrogen bonded to the amino acid side chains than connected to the ion. A
932 detailed examination of the orientation of water, charges on the atoms with
933 consequent electrostatic forces, and symmetry, is required to understand
934 rectification and selectivity in the SF. It would be interesting to calculate a Kir
935 channel to see whether the electrostatic gradient is reversed, and whether or not
936 the ratchet and pawl remain.

937 **CONCLUSIONS:**

938 1) The transfer of an ion from the cavity of the $K_v1.2$ channel to the SF is a complex
939 problem in re-solvation, involving transfer of hydrogen bonds and switching of

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940 electrostatic interactions. The co-solvation of the ion by threonines and water is
941 necessary for the ion to move through the SF.

942 2) The problem is solved for K^+ by the pore structure, with a consequent
943 electrostatic gradient driving the ion forward, and the orientation of a water acting
944 as the pawl in a ratchet and pawl that prevents a return of the ion (see (6) below).

945 3) Na^+ goes slightly off the axis by enough to be more strongly bound
946 electrostatically by the oxygens on one side of the pore than the other, as well as
947 by tightly bound hydrating water molecules at the top of the cavity when the ion is
948 in the S4 SF position. This makes it more difficult to move up, as there is a
949 substantial barrier at the entrance to the SF.

950 4) It is a rather different problem for NH_4^+ which itself is not spherically symmetric.
951 It is not as tightly held as Na^+, but does not move as easily as K^+, in part because the
952 ion can form hydrogen bonds, unlike either Na^+ or K^+. It acts almost like a charged
953 water.

954 5) The threonines are the right size to solvate the K^+ on the pore axis; substituting
955 serines leads to another form of asymmetry, as the ion can no longer be fully
956 solvated on the pore axis.

957 6) The O(T373 –C=O) – O(T374 –OH) – H(T374 –OH) angle may be as important as
958 electrostatics in making the "knock-on" mechanism possible, by acting as a pawl in
959 a ratchet and pawl mechanism, allowing the knock on to be unidirectional, with no
960 back flow (except for thermal fluctuations, which are necessary to comply with the
961 Second Law of Thermodynamics (65)).

962 7) Taken together, these considerations suggest that the SF, and the pore as a
963 whole, is a finely tuned structure. The requirement for fine tuning suggests in turn
964 the reason for the conservation of the SF amino acid sequence through perhaps a
965 billion years of evolutionary time. How much of this is related to C-type inactivation
966 will require a study of the complete SF, which remains for future work; most
967 probably, this too will require consideration of a combination of subtle effects.

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