Mechanisms of tetraethylammonium ion block in the KcsA potassium channel

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Received 23 February 2001; accepted 26 March 2001

First published online 11 April 2001

Edited by Maurice Montal

Abstract We report results from automated docking and microscopic molecular dynamics simulations of the tetraethylammonium (TEA) complexes with KcsA. Binding modes and energies for TEA binding at the external and internal sides of the channel pore are examined utilising the linear interaction energy method. Effects of the channel ion occupancy (based on our previous results for the ion permeation mechanisms) on the binding energies are considered. Calculations show that TEA forms stable complexes at both the external and internal entrances of the selectivity filter. Furthermore, the effects of the Y82V mutation are evaluated and the results show, in agreement with experimental data, that the mutant has a significantly reduced binding affinity for TEA at the external binding site, which is attributed to stabilising hydrophobic interactions between the ligand and the tyrosines.

Key words: Potassium channel; KcsA; Block; Tetraethylammonium; Binding site; Linear interaction energy method

1. Introduction

Ion channels mediate passive ion transport across cell membranes and are in this way involved in the transduction of electric signals and the control of ionic concentrations and membrane potentials [1]. An important aspect of ion channels is the inhibition (block) of ion currents by external agents [2–5], where the most frequent mechanism of inhibition involves direct mechanistic block of ion permeation paths by small or medium sized molecules. Such blocker molecules presumably either compete with ions for binding sites within the channel pore or bind to sites near the pore. The structural factors underlying mechanisms of such blocking effects presents a challenging problem in molecular pharmacology and biophysics.

The experimental determination of the three-dimensional (3D) structure of the KcsA potassium channel [6] and subsequent computational studies of the underlying ion permeation mechanisms in KcsA [7–12] constitute important preliminary steps towards better understanding of the structural aspects of blocking effects in ion channels. The quaternary ammonium ions are quite often used as molecular probes in exploring the structural and functional features related to K^+ channel blocking. Tetraethylammonium (TEA) is among these a particularly well studied compound that binds relatively strongly to K^+ channels. Continuous experimental efforts have made it possible to classify two major binding regions for quaternary ammonium ions near the extracellular and intracellular entries of the K^+ channel pore (see, e.g. [5,13–20] and Fig. 1). An important observation is related to the crucial role of a set of four aromatic residues (Y82 in KcsA) in TEA binding to the external vestibule of potassium channels [14,15,19,20]. However, the structural characterisation of the binding modes of TEA to K^+ channels is still not available. The purpose of the present study is to explore computational models for the binding of TEA to the KcsA channel and its Y82V mutant, based on the experimental 3D structure of the protein and using microscopic docking and molecular dynamics (MD) approaches.

2. Materials and methods

The binding of TEA to KcsA is explored here in a two-step procedure. First, the structures of TEA–KcsA complexes are predicted using an automated docking approach (AutoDock, version 3.0) [21,22]. In AutoDock the energies of the ligand–protein interactions are calculated using probe atom energies evaluated over a cubic grid that covers the active region of the protein. The scoring function used for the binding free energy includes electrostatic, van der Waals, hydrogen bond, and solvation terms [22]. The protein is taken to be frozen at the experimental structure while torsional flexibility of the ligand is allowed, in addition to overall translation and rotation. The crystallographic structure of the KcsA channel [6] both with and without four explicit water molecules in the selectivity filter is used as the template for docking of TEA. Residues that are missing in the experimental structure are rebuild as was previously described in [7,11]. Amino acids E51, R64, D80, R89, R117, E118 are taken in their ionised forms, while the buried E71 is considered to be in the neutral form according to our former calculations of the pK_a value for this glutamic acid [11]. The total charge of the channel is taken to be neutral in the AutoDock calculations. The mutant channel structure is generated from the original KcsA structure by substituting the coordinates of the side chain atoms of Y82 to V82 in each of the four monomers. The grid of probe atom potentials is built with a step of 0.25 Å within a 30 Å×30 Å×52 Å box, where the axis of the KcsA pore coincides with the central axis of the grid box along its long side. The actual coordinates of the grid points along the pore axis range from −28.8 Å to 22.8 Å which effectively covers the region around the external pore entrance and the internal cavity volume in KcsA. The Lamarckian genetic algorithm of AutoDock 3.0 [22] is used in the search for local minima on the potential surface of generated complexes. 10–30 low energy complexes are determined for each of the two major binding regions, i.e. the external entrance to the selectivity filter and the internal water cavity including the intracellular channel pore. The docking procedure is thus mainly used to generate a set of putative binding conformations, based on protein–

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0014-5793/01/$20.00
PII: S0014-5793(01)02381-X

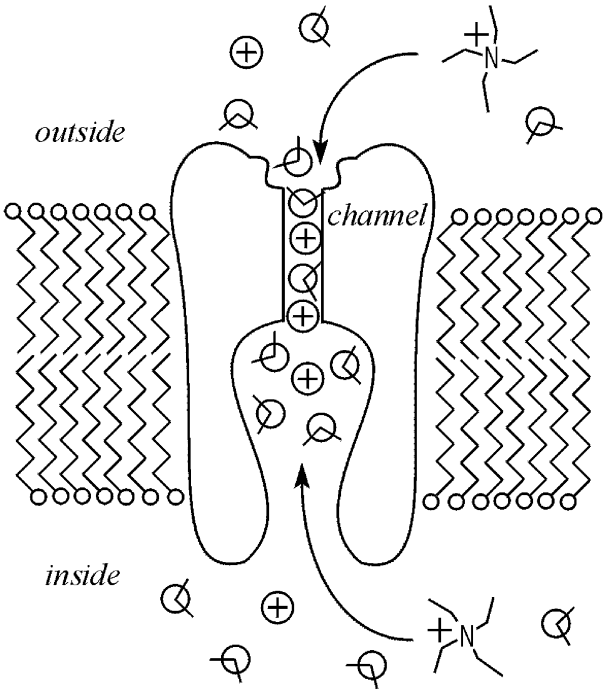

Fig. 1. Schematic picture of K^+ channel block by external and/or internal TEA.

ligand matching. The further refinement of such complexes regarding their dependence on ion occupancies is considered to be more consistently treated by an explicit microscopic model.

The second phase of the docking procedure thus involves MD calculations of average structures and energies for the ligand–protein complexes generated by the automated docking procedure. In the MD calculations the simulated system includes the channel tetramer with one–three K^+ ions inside, immersed in a model membrane of 34 Å width and solvated by a water sphere with a radius of 35 Å. The reference solvation energetics of TEA in water is similarly calculated in a 35 Å sphere. The full description of the system setup is given in our previous work [7,11]. The low energy ion occupancy states of the channel with one or two ions in the selectivity filter and one ion in the internal water cavity are considered explicitly. MD calculations are carried out with the programme Q using the GROMOS87 potential energy function [23] (see [11] for further details of the simulation procedure). The MD trajectories are calculated at a constant temperature of 300 K and a time step of 2 fs with 100 ps for equilibration and 200 ps for data collection. In MD simulations of the ligand–protein complexes the overall charge of the 'receptor' system, i.e. the channel plus K^+ ions, is made neutral by counterbalancing the total positive charge of K^+ ions with partial negative charges at four peripheral E51 residues. The TEA binding free energies are estimated with the linear interaction energy (LIE) approach [24,25]. The binding free energy is then obtained from scaled differences of the MD average intermolecular electrostatic and van der Waals (Lennard–Jones) energies of the TEA ligand in the complex (subscript 'bound') and in aqueous solution (subscript 'free')

$$\Delta G_{\text{bind}} = \alpha(\langle V^{\text{vdw}}_{l-s}\rangle_{\text{bound}} - \langle V^{\text{vdw}}_{l-s}\rangle_{\text{free}}) + \beta(\langle V^{\text{el}}_{l-s}\rangle_{\text{bound}} - \langle V^{\text{el}}_{l-s}\rangle_{\text{free}}) \quad (1)$$

where the earlier parametrisation with the coefficients $\alpha = 0.18$ and $\beta = 0.5$ is used [25]. This approach provides a simple and efficient way of estimating binding affinities from microscopic MD simulations of ligand–protein complexes in solution.

The atomic charges for TEA in aqueous solution are evaluated from ab initio calculations with the 6-31G* basis set coupled with the PCM continuum model [26] and are further used in the AutoDock and MD simulations. Electronic structure calculations for isolated TEA are performed with full geometry optimisation and are followed by single-point calculations in the aqueous medium using the frozen gas-phase geometry. Since the use of atomic charges estimated from molecular electrostatic potential is often expected to give better results in the framework of force field calculations (see e.g. [27–29]), we follow this approach in our study. The calculation procedure involves a two-step conformational averaging of charges (RESP charges [27,28]) with extra symmetry conditions being applied. In the first step the charges on the equivalent carbon atoms are set equal to each other, while the hydrogen atoms are varied independently. In the second step a complete symmetry of charges on the equivalent atoms in the $-CH_2$ and $-CH_3$ groups is enforced.

3. Results and discussion

3.1. AutoDock and MD studies of the TEA–KcsA complexes

TEA is a fairly small positively charged molecule with an estimated effective radius of around 4.5 Å. The molecule has two main types of conformers (Fig. 2). In the TEA1 conformation the end carbon atoms (CB) form an asymmetric pyramid, while in the TEA2 conformer the CB atoms form a quasi-planar configuration that includes also the central nitrogen atom. TEA2 is predicted to be slightly more stable than TEA1 (by 0.6–0.8 kcal/mol) both for the isolated molecule and when surrounded by high dielectric continuum. The Mulliken analysis of electron population predicts that in both conformers the central N atom carries a negative charge of around −0.6, while the overall positive charge is distributed over the ethyl groups. On the contrary, the electrostatic potential derived charges on the N atom in all cases are more positive and lie between −0.1 and −0.2. The estimated value for the RESP atomic charge on the N atom is −0.144 and for the united atoms CA and CB +0.220 and +0.066, respectively. These charges are used in both the AutoDock calculations and in the molecular dynamics simulations of channel–ligand complexes.

We consider here the binding of TEA to the extracellular vestibule of the channel pore and to the internal water cavity formed by membrane spanning protein helices. Available experimental data show that TEA does not penetrate through K^+ channels but blocks them both from the external and internal sides of the cell membrane. At the same time TEA, as many other blockers, easily enters the internal water cavity of the channel through the relatively non-rigid part of the channel pore that is open to the cell interior (see e.g. [5]). This observation clearly indicates that the intracellular binding sites for quaternary ammonium ions are located inside the internal water cavity of the channel. An important issue is related to the fact that channel blocking occurs in the presence of permeating ions and in competition with them. This makes it necessary to consider the dependence of TEA binding on the positions of permeating ion inside the pore, i.e. the chan-

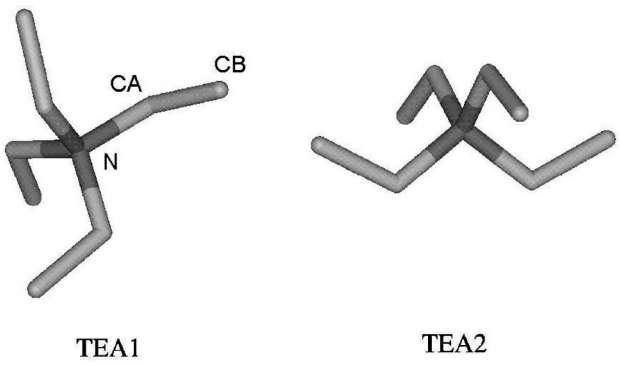

Fig. 2. The pyramidal and quasi-planar conformations of TEA.

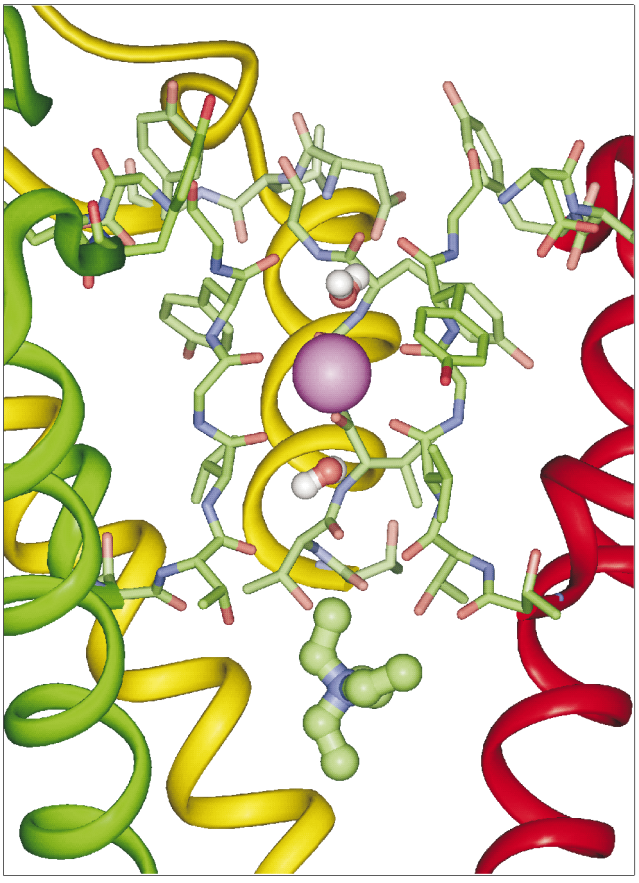

Fig. 3. Side view of the selectivity filter region for the internal docked structure of TEA after 300 ps of MD simulation. One of channel monomers is omitted for clarity. The simulations started from the lowest energy docked pyramidal configuration of TEA and the 010*(0) occupancy state of the channel.

nel loading state. The computational evaluation of the thermodynamics of possible ion occupancy states has been reported by us earlier in [7,11].

The automated docking of TEA to the *external region* of the KcsA channel produces structures, where the ligand is positioned precisely at the entrance to the selectivity filter. No other extracellular binding sites for TEA within a distance of ~15 Å from the pore axis are found. Docking calculations for the channel with an empty selectivity filter produce exclusively one type of structures. In this case TEA binds in pyramidal conformation to the entrance of the selectivity filter with partial insertion of one ethyl group into the filter pore. The corresponding CB carbon atom finds a position of about 0.5 Å away from the centre of the first ring of oxygen atoms formed by four carbonyl groups of Y78 and close to the first ion binding site. However, docking of TEA to the channel with four waters in the selectivity filter (the first binding site in the filter is occupied in this case) predicts that the most stable type of ligand–channel complex corresponds to the quasi-planar TEA2 conformer. In this case TEA is tightly positioned at the pore entrance with the central atom N lying on the pore axis and all four ethyl groups being in contact with the protein. A set of looser TEA–KcsA complexes where the ligand adopts a pyramidal conformation is produced in case of the water filled pore as well. Docking of TEA to the *internal cavity* of KcsA similarly shows the apparent prevalence of structures where TEA binds to the inner entrance of the selectivity filter. For the empty selectivity filter the most stable complexes correspond to the partial insertion of one ethyl group into the pore. The ligand in such complexes is in pyramidal configuration. The quasi-planar ligand configuration, with the central nitrogen atom located near the filter entrance and with four ethyl groups in contact with the protein, is also found among 10 most stable internal complexes. Besides, the docking procedure predicts the possibility of binding to the walls of the internal cavity. In this case interactions of TEA with the T107 side chain OH groups and backbone carbonyls of I100 and F103 in the long transmembrane helices apparently provide some stabilisation. Finally, it should be noted that the AutoDock binding energies have rather similar values for the external and internal binding modes, where the corresponding values lie between −3.3 and −3.9 kcal/mol, and −3.7 and −4.2 kcal/mol, respectively.

The AutoDock generated TEA–KcsA complexes are used as starting structures for further microscopic MD/LIE simulations. The docked structures show that the positively charged ethyl groups of TEA occupy positions inside the pore of the selectivity filter which partially overlap either with the first or with the fourth binding sites in the selectivity filter depending on the binding mode (Figs. 3 and 4). Consequently, this indicates that electrostatic repulsion within the selectivity filter between the blocker and the permeating ions can be important. Such structures also indicate that TEA should, in principle, not bind to all possible ion occupancy states of the channel. The influence of the permeating potassium ions and water molecules on binding of the blocker is therefore considered in microscopic MD simulations. We thus performed the LIE calculations of binding energetics for external and internal types of ligand–channel complexes in several of the low energy ion occupancy states [7,11], where TEA in most cases can be thought of as partially substituting for

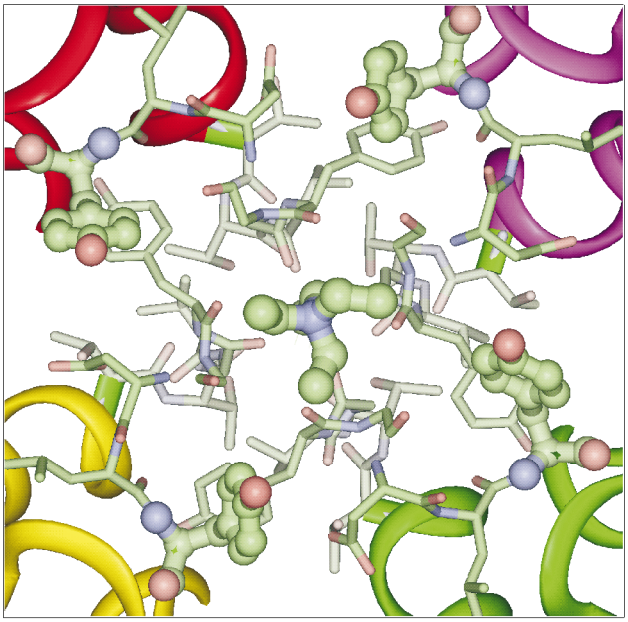

Fig. 4. Top view of the external docked structure with pyramidal TEA after 300 ps of MD simulation in the *001(1) occupancy state. The TEA molecule and the side chains of Y82 are shown in ball-and-stick mode.

one of the K^+ ions (Table 1). The resulting complexes are characterised by the presence of one–two K^+ ions in total within the selectivity filter and internal water cavity.

The MD simulations show that TEA effectively binds at the external side only in cases when the first binding site is vacant, i.e. not occupied by an ion or water molecule, so that the ethyl group can partially penetrate into the filter. The best affinity is attained to the state where K^+ ions are located in the third or fourth filter position and in the centre of the internal water cavity. Such occupancy states are designated as *010(1) or *001(1), respectively, in Table 1 in accordance with our previously used five-symbol code [7,11]. The '*' thus denotes that the outermost filter position is initially vacant, allowing partial insertion of the blocker into the site, and '0' and '1' denote a water molecule or ion, respectively, where the last digit enclosed in parentheses refers to the internal cavity. The influence of the particular starting structure of the complex turns out to be less important than the effect of the ion loading state (Table 1). This can, e.g. be seen from the very similar binding energies of −4.1 and −4.0 kcal/mol for the lowest energy complex and the average over four different initial complexes in the *001(1) occupancy state. Hence, several of the initial complexes that AutoDock considers as distinct conformations converge to the same ensemble of structures in the MD calculations which could, in fact, be expected if the initial complexes are not too dissimilar. As a comparison to the high affinity states *010(1) and *001(1) one finds significantly more positive binding energies of −0.1 and −1.3 kcal/mol for the lowest energy complexes in the *100(1) and *101(0) states, respectively. The destabilisation of the latter binding states is related to the increased electrostatic repulsion at closer distances between TEA and K^+ as can be seen from the average electrostatic energies, $\langle V^{el}_{l-s}\rangle_{bound}$, in Table 1. The low energy complex with TEA in the planar conformation, that was found when the first (outermost) filter position is occupied by water molecule, yields a small binding affinity in the LIE calculations of −0.4 kcal/mol. In this case the ligand does not either retain its starting quasi-planar configuration in the course of the MD simulation. These results clearly point towards the importance of (partial) insertion of the blocker into the selectivity filter for attaining a high affinity.

The MD simulations of TEA binding to the internal water cavity show that the blocker effectively binds in cases when one K^+ ion is present in the selectivity filter and, particularly, when the K^+ ion is located in position one or two (Table 1). For pyramidal blocker configurations, the ethyl group remains inserted into the region of the fourth selectivity filter site during the simulations (Fig. 3). For the planar blocker conformation better binding also occurs in the case when site four contains no water molecule. TEA then 'sticks' to fourth site entrance while retaining its planar structure during the simulations. Addition of a K^+ ion to the internal water cavity makes the TEA binding to internal entrance of the selectivity filter unfavourable. Interestingly, binding of the pyramidal and quasi-planar TEA conformers shows approximately the same LIE energies. In total the most favourable energy values for internal binding are calculated to be lower by about 2–3 kcal/mol than for the external binding site. However, the binding free energies on the external and internal sides should not be directly comparable since the total numbers of ions differ in two cases. That is, the internal cavity ion is also displaced for the most stable internal complexes and binding of that ion has been predicted to be exothermic by about 3 kcal/mol [7,11]. A better estimate of the overall internal binding affinity would thus be around −4 kcal/mol, but this should still be regarded as a rather uncertain value.

Another interesting aspect of TEA blocking is related to simultaneous binding of TEA from the external and internal sides of the channel which is observed experimentally for rKv1.1, rKv1.2, and *Shaker* B potassium channels [17]. The MD simulations show that such complexes of KcsA with two TEA molecules bound in the pyramidal conformations to the

Table 1
MD LIE calculations of binding energies for selected TEA–KcsA complexes

Ligand conformer[a]	Channel occupancy state[b]	$\langle V^{el}_{l-s}\rangle_{bound}$ (kcal/mol)	$\langle V^{vdw}_{l-s}\rangle_{bound}$ (kcal/mol)	ΔG_{bind} [c] (kcal/mol)
External vestibule sites				
Pyramid	*101(0)	−83.6 ± 0.4	−25.8 ± 0.1	−0.4 ± 0.3
Pyramid	*100(1)	−85.9 ± 0.1	−26.1 ± 0.8	−1.5 ± 0.3
Pyramid	*010(1)	−90.3 ± 0.2	−27.1 ± 0.1	−4.0 ± 0.2
Pyramid	*001(0)	−88.3 ± 0.8	−25.1 ± 0.1	−2.7 ± 0.5
Pyramid	*001(1)	−91.1 ± 0.1	−27.3 ± 0.3	−4.5 ± 0.2
Pyramid, mean	*001(1)	−90.8 ± 0.3	−27.5 ± 0.1	−4.3 ± 0.3
Planar	0001(1)	−85.5 ± 0.8	−22.1 ± 0.5	−0.7 ± 0.6
Pyramid	*00'TEA'(0)	−89.5 ± 0.4	−27.6 ± 0.3	−3.7 ± 0.4
Internal water cavity sites				
Pyramid	010*(1)	−79.3 ± 0.2	−28.0 ± 0.1	1.1 ± 0.3
Pyramid	100*(1)	−77.8 ± 0.5	−27.1 ± 0.1	2.2 ± 0.4
Pyramid	101*(0)	−90.4 ± 0.9	−28.0 ± 0.3	−4.2 ± 0.6
Pyramid	100*(0)	−95.8 ± 0.1	−27.5 ± 0.1	−6.9 ± 0.2
Pyramid	010*(0)	−96.4 ± 0.6	−27.4 ± 0.1	−7.1 ± 0.4
Pyramid, mean	010*(0)	−95.8 ± 0.6	−27.6 ± 0.1	−6.8 ± 0.4
Planar	010*(0)	−96.7 ± 0.7	−27.0 ± 0.1	−7.2 ± 0.5
Planar	0100(0)	−86.3 ± 8.0	−27.4 ± 0.2	−2.1 ± 0.5
Pyramid, cavity wall	0101(0)	−76.1 ± 1.3	−24.6 ± 0.1	3.5 ± 0.8
Pyramid	'TEA'00*(0)	−97.2 ± 0.3	−27.5 ± 0.2	−7.7 ± 0.3

[a]The lowest energy docked configuration (for a given site and conformer) is taken as the starting one. The mean values are calculated over the first four ligand–channel complexes of similar type having the highest rank from the automated docking procedure.
[b]The symbols '0', '1' and '*' in the description of the channel occupancy states indicate that the corresponding site is occupied by water, K^+, or taken as vacant in the starting structure, respectively. The notation 'TEA' indicates the presence of an additional TEA molecule with its ethyl group inserted into the given site.
[c]The calculated TEA reference energies in water are: $\langle V^{el}_{l-s}\rangle_{free} = -86.1 \pm 0.2$ kcal/mol, $\langle V^{vdw}_{l-s}\rangle_{free} = -16.4 \pm 0.1$ kcal/mol.

first and the fourth filter positions are stable in case when no K^+ ions are present within the selectivity filter. The calculated LIE binding energies are also found to be negative in both cases when an additional TEA molecule is present at the opposite end of the selectivity filter (Table 1).

3.2. Binding of the TEA to the Y82V mutant channel

Experimental data show that binding of TEA at the extracellular side of K^+ channels to a large extent depends on a ring of aromatic amino acid residues near the pore entry. Particularly for KcsA, mutation of such aromatic residues leads to a loss of the blocking effect for TEA [19,20]. To examine this issue we performed calculations of TEA binding to the Y82V mutant of KcsA. Results from these MD LIE calculations are given in Table 2. The structures of the TEA–Y82V complexes generated by automated docking are very similar to the structures described for the KcsA complexes. TEA adopts the pyramidal conformations in the generated set of complexes and partial insertion of the ethyl group into the channel pore is again observed. The AutoDock binding energy for the most stable TEA–Y82V complex is only $\sim$0.5 kcal/mol higher than the energy for the most stable TEA–KcsA complex. However, subsequent MD/LIE simulations for the TEA–Y82V complexes yield a reduction of the binding affinity at the external side of the pore (Table 2). The binding of TEA at the internal site is essentially unaffected by the mutation and neither the AutoDock nor the MD LIE binding energies change significantly.

The stabilising effect of tyrosines on external TEA binding has previously been rationalised in terms of stabilising cation–π interactions between the aromatic rings and the TEA positive charge and/or attractive electrostatic interactions between the tyrosine hydroxyls and the peripheral atoms of ammonium ions [30–32]. The characteristic distance for cation–π interactions between the centre of phenyl ring and the nitrogen atom (in case of NH_4^+) is around 2.9 Å [32]. For the TEA–KcsA complexes the distances from the centres of the Y82 rings to the closest carbon atoms in TEA exceed 5.8 Å both in the pyramidal and planar docked structures. The cation–π interactions are not explicitly treated in the GROMOS potential energy function. It is therefore not possible here to evaluate the energy contributions of these effects directly. However, the observed distances clearly indicate that cation–π interactions are not expected to be important in stabilising TEA. The distances between the hydroxyl groups of Y82 and the TEA ethyl groups are also too large (>5.3 Å) to provide any significant electrostatic stabilising interactions between them due to the screening effect of water molecules. But what is the origin of the stabilising effect then? Analysis of the structures that result from the MD simulations shows that the phenyl rings are oriented in a manner in which their edges are pointing in the direction of the TEA ethyl groups (Fig. 4). Accordingly, the phenyl moieties form a kind of cage for TEA where the corresponding contact distances between the terminal TEA carbon atoms and the phenyl rings can be as small as 4.2 Å. This type of cage might thus be responsible for the stabilising effect in terms of hydrophobic interactions. Thus, the influence of these four phenyl rings of Y82 would mainly be to provide a hydrophobic environment for the least polar part of the TEA ethyl groups. This result is, in fact, in agreement with the experimental observation that the Y82F mutants for potassium channels, where only the tyrosine hydroxyl groups are deleted, do not have a reduced affinity for TEA [14,15].

3.3. Discussion

The present study provides new structural information regarding the complexes of TEA with KcsA. The automated docking procedure suggests that the major binding sites for this quaternary ammonium ion are located at the external and internal entrances of the narrow selectivity filter, in agreement with what has been deduced from experiments (see, e.g. [5,15]). TEA binds favourably to these sites primarily with partial insertion of one of the ethyl groups into the filter pore. The ligand remains stable in these positions for most of the docked complexes during MD simulations. As expected, binding of the charged blocker apparently depends on the loading state of K^+ ions inside the channel. Here we estimated the TEA binding energies for several low energy configurations corresponding to possible functional ion occupancy states of the channel that have been determined earlier [7,11]. The calculations thus provide predictions regarding which channel loading states have a high affinity for TEA.

The comparison between binding free energies for the most stable internal complexes with the external ones is, however, complicated because the favoured internal binding also displaces the cavity ion. Nevertheless, a reasonable estimate for the internal binding free energy is around -4 kcal/mol, when the cost of removing the cavity ion [7,11] is taken into account. This would therefore suggest that the relative affinities of the outside and inside for TEA are approximately the same. One can also note that binding free energies on the order of -4 kcal/mol seem to be in reasonable agreement with experimentally measured inhibition constants [19] and IC_{50} values [20] for TEA towards the KcsA channel.

The effect of mutation of the four tyrosine residues (Y82V) located at the external entrance to the channel pore has also been examined. We find that this mutation has no significant effect on TEA binding to the internal site, as would be expected, but that the affinity of the outer site is reduced by several kcal/mol. Furthermore, the reason for this reduced affinity appears to be that the four tyrosine residues form a hydrophobic cage-like structure that interacts favourably with TEA.

Table 2
MD LIE calculations of binding energies for TEA–KcsA Y82V complexes

Ligand conformer	Channel occupancy state	$\langle V_{l-s}^{vdw}\rangle_{bound}$ (kcal/mol)	$\langle V_{l-s}^{vdw}\rangle_{bound}$ (kcal/mol)	ΔG_{bind} (kcal/mol)
External vestibule sites				
Pyramid	*001(1)	-86.8 ± 0.1	-25.8 ± 0.1	-2.0 ± 0.1
Pyramid, mean	*001(1)	-87.4 ± 0.6	-25.4 ± 1.2	-2.3 ± 0.7
Internal water cavity sites				
Pyramid	010*(0)	-98.0 ± 0.2	-27.1 ± 0.1	-7.8 ± 0.2
Pyramid, mean	010*(0)	-95.7 ± 1.3	-27.6 ± 0.3	-6.8 ± 0.9

Acknowledgements: Support from AstraZeneca and the Swedish Research Council is gratefully acknowledged.

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