

## The Ionization State and the Conformation of Glu-71 in the KcsA K<sup>+</sup> Channel

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**ABSTRACT** The side chain of Glu-71 of the KcsA K<sup>+</sup> channel, an important residue in the vicinity of the selectivity filter, was not resolved in the crystallographic structure of Doyle et al. (Doyle, D. A., J. M. Cabral, R. A. Pfuetzner, A. Kuo, J. M. Gulbis, S. L. Cohen, B. T. Chait, and R. MacKinnon. 1998. *Science*. 280:69–77). Its atomic coordinates are undetermined and its ionization state is unknown. For meaningful theoretical and computational studies of the KcsA K<sup>+</sup> channel, it is essential to address questions about the conformation and the ionization state of this residue in detail. In previous MD simulations in which the side chain of Glu-71 is protonated and forming a strong hydrogen bond with Asp-80 it was observed that the channel did not deviate significantly from the crystallographic structure (Bernèche, S., and B. Roux. 2000. *Biophys. J.* 78:2900–2917). In contrast, we show here that the structure of the selectivity filter of the KcsA channel is significantly disrupted when these side chains are fully ionized on each of the four monomers. To further resolve questions about the ionization state of Glu-71 we calculated the pK<sub>a</sub> value of this residue using molecular dynamics free energy simulations (MD/FES) with a fully flexible system including explicit solvent and membrane and finite-difference Poisson-Boltzmann (PB) continuum electrostatics. It is found that the pK<sub>a</sub> of Glu-71 is shifted by ~+10 pK<sub>a</sub> units. These results strongly suggest that Glu-71 is protonated under normal conditions.

### INTRODUCTION

The three-dimensional structure of the KcsA K<sup>+</sup> channel of *Streptomyces lividans* determined by x-ray crystallography (Doyle et al., 1998) provides the essential information to begin to understand the fundamental principles governing ion permeation through biological membrane channels. On the basis of the crystallographic structure, further insight can be gained by constructing detailed atomic models of KcsA, including ions, water and lipid molecules, and simulating the motions of all the atoms in the system using molecular dynamics (MD) calculations (Allen et al., 1999; Åqvist and Luzhkov, 2000; Bernèche and Roux, 2000; Biggin et al., 2001; Crouzy et al., 2001; Guidoni et al., 1999, 2000; Luzhkov and Åqvist, 2000, 2001; Shrivastava and Sansom, 2000). The calculated classical trajectory, though an approximation to the real world, provides ultimate detailed information about the time course of the atomic motions, including the transient configurations of the ions, channel, and water molecules occurring during the conduction process, thus extending the static picture corresponding to the crystallographic structure to the time domain, which is difficult to observe experimentally. However, some details in the present three-dimensional crystallographic structure remain uncertain because of the moderate resolution of the x-ray data. In particular, the side chain of Glu-71 could not be completely resolved in the electron density map and

its atomic coordinates were not determined (Doyle et al., 1998). Furthermore, the ionization state of Glu-71 can have a very critical influence on the energy of ions in the pore. Continuum electrostatic calculations indicate that the free energy of the K<sup>+</sup> ion in the outer site is further stabilized by at least −6 kcal/mol when the Glu-71 side chains of the four monomer are fully charged (Roux et al., 2000). This influence on the inner ion binding sites is even larger.

We adopted, following discussions with R. MacKinnon and co-workers (private communication), an atomic model in which the side chain of Glu-71 is constructed in a protonated state to form a di-acid hydrogen bond with the carboxylate group of Asp-80 near the extracellular surface (Bernèche and Roux, 2000). The model, which is shown in Fig. 1, was subsequently used in MD simulations of the KcsA channel embedded in a lipid bilayer (Bernèche and Roux, 2000). For these simulations, the root-mean-square (RMS) deviation of the tetramer backbone relative to the crystallographic structure was on the order of 1.9 Å, while the deviation for all the main secondary elements was <0.9 Å, including the selectivity filter. Such a stability is a strong indication that the protonated state for Glu-71 is correct. However, the results from previous simulations with unprotonated Glu-71 are somewhat at odds with this conclusion. Guidoni et al. (1999) simulated KcsA with the Glu-71 of each of the four monomers in the ionized state and observed a widening of the pore on the extracellular side sufficient to allow a fully solvated Na<sup>+</sup> surrounded by six water molecules to enter the channel. In contrast, Sansom and co-workers (Shrivastava and Sansom, 2000; Biggin et al., 2001) simulated a similar system and did not report any significant structural distortion of the channel. It is difficult to ascertain the significance of those results because of

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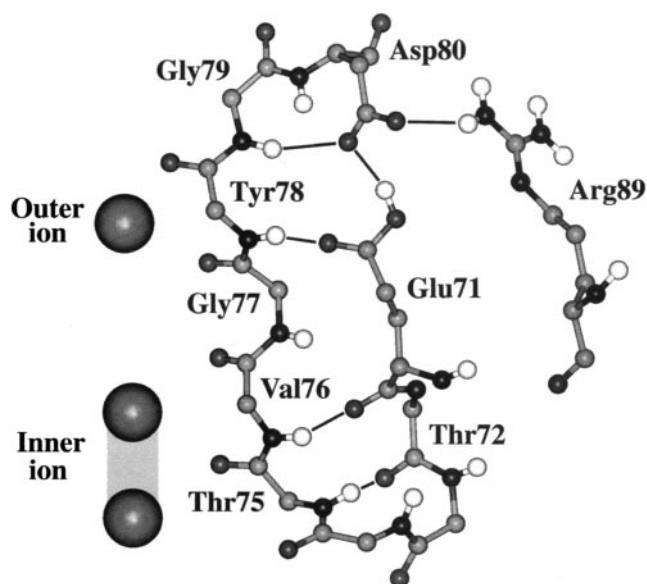


FIGURE 1 Detail of the selectivity filter from a single monomer of the KcsA K<sup>+</sup> channel. The side chain of Glu-71 is constructed in a protonated (neutral) state, forming a di-acid hydrogen bond with the carboxylate group of Asp-80 near the extracellular surface.

differences in computational methodologies, force fields, and treatment of long-range electrostatic interactions.

The configuration with protonated Glu-71 forming a strong hydrogen bond with Asp-80 is supported by recent x-ray diffraction data, which indicate that Glu-71 is very close to Asp-80 (pdb entry code 1J95) (Zhou et al., 2001). Because it would be unlikely to have two ionized side chains at such short distance, the observation is strongly suggestive that Glu-71 and Asp-80 are sharing a proton. Furthermore, substitution of Glu-71 by an aspartic acid dramatically disrupts the stability of the tetrameric channel, while substitution by an asparagin does not (E. Perozo, personal communication). Such a destabilization of the channel is consistent with the existence of a strong hydrogen bond between Glu-71 and Asp-80: molecular modelization indicates that the strong hydrogen bond cannot form when an aspartic acid, which has a shorter side chain than a glutamic acid, is substituted at position 71 (Bernèche and Roux, unpublished data). Interestingly, the residue at the corresponding position in *Shaker* is nonpolar (Val-438) (Doyle et al., 1998), which suggests that a substitution by a neutral residue maintains the structural integrity of the channel.

Questions about the ionization state of Glu-71 have been addressed using computational methods. Results from finite-difference Poisson-Boltzmann (PB) continuum electrostatic calculations indicate that the pK<sub>a</sub> of the Glu-71 sidechain is shifted by nearly +10 pK<sub>a</sub> units (Roux et al., 2000). The results from similar PB calculations by Ranatunga et al. (2001) and from molecular dynamics free energy simulations (MD/FES) on a reduced model system by Luzhkov and Åqvist (2000) also indicate that the pK<sub>a</sub> of

Glu-71 is shifted. The results from all those calculations strongly suggest that Glu-71 is protonated under normal conditions at pH 7. However, significant approximations were involved. In particular, all the previous calculations were based on fixed (Roux et al., 2000; Ranatunga et al., 2001) or restricted (Luzhkov and Åqvist, 2000) channel conformations. Therefore, the currently available results are not conclusive. Calculations incorporating the full flexibility of the side chain are needed to resolve questions about the ionization state of Glu-71.

For meaningful theoretical and computational studies of the KcsA K<sup>+</sup> channel, it is essential to resolve the current issues about the conformation and the ionization state of Glu-71 in detail. The goal of the present article is twofold: first, quantify the impact of unprotonated Glu-71 on the structure of the selectivity filter of KcsA, and second, obtain as robust an estimate as possible of the pK<sub>a</sub> of Glu-71 using MD/FES with a fully flexible system including explicit solvent and membrane. The main conclusions of these calculations are that the selectivity filter of the KcsA channel deviates unrealistically from the crystallographic structure during simulations in which the Glu-71 side chains of each of the four monomers are fully ionized, and the pK<sub>a</sub> of Glu-71 is shifted by ~+10 pK<sub>a</sub> units and should be protonated at pH 7.

## THEORY AND METHODS

### Calculation of ΔpK<sub>a</sub>

The ionization state of a residue is determined by the relative free energy of its protonated and unprotonated forms. The problem can be expressed in terms of ΔpK<sub>a</sub> relative to the intrinsic pK<sub>a</sub> of the amino acid isolated in solution by considering the free energy of transfer ΔΔG that corresponds to the reversible work needed to protonate the side chain in the protein compared to the work needed to protonate the same side chain in an isolated peptide in bulk water (Bashford and Karplus, 1990). This free energy difference can be converted in terms of ΔpK<sub>a</sub> relative to the intrinsic pK<sub>a</sub> of the amino acid isolated in solution by

$$\Delta pK_a = -\frac{\Delta\Delta G}{2.3k_B T} \quad (1)$$

where ΔΔG is the free energy difference between the protonated (p) and the unprotonated (u) states of an amino acid embedded in the protein in reference to the difference for the isolated amino acid in solution

$$\begin{aligned} \Delta\Delta G = & [\Delta G_{\text{protein}}(u) - \Delta G_{\text{protein}}(p)] \\ & - [\Delta G_{\text{isolated}}(u) - \Delta G_{\text{isolated}}(p)] \end{aligned} \quad (2)$$

The free energy difference ΔΔG can be calculated using continuum electrostatic (Bashford and Karplus, 1990; Sharp and Honig, 1990; Yang et al., 1993), in which the solvent is represented as a dielectric medium, or using MD/FES (Kollman, 1993) with explicit solvent molecules.

### Continuum electrostatic

To obtain the ΔpK<sub>a</sub> of Glu-71 on the basis of continuum electrostatic, one must calculate the various ΔG values, corresponding to the electrostatic

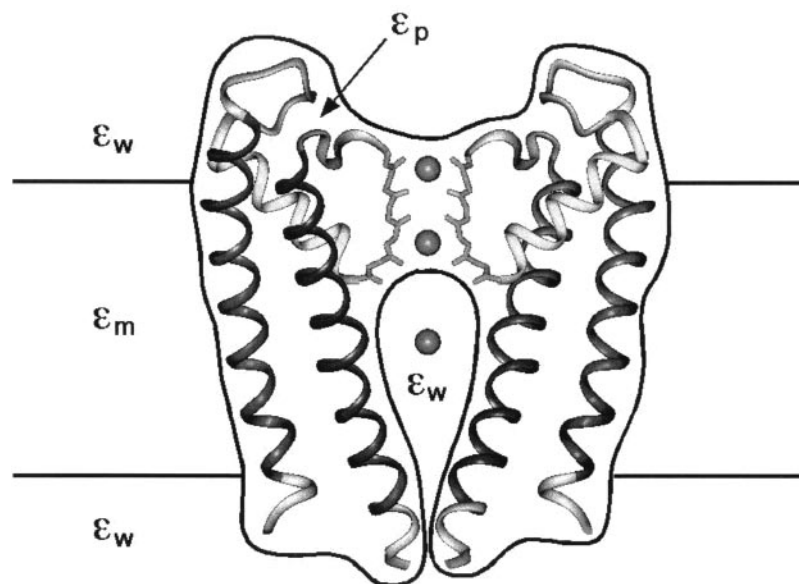


FIGURE 2 Schematic representation of the different dielectric regions used in the finite-difference PB calculations. The membrane is 30 Å thick. Periodic boundary conditions are applied in the direction of the membrane plane. Debye-Hückel potentials are assumed at the boundary of the grid perpendicular to the membrane plane.

free energy of the protonated and unprotonated states of ionizable side chains in the complex dielectric environment (Bashford and Karplus, 1990; Sharp and Honig, 1990; Yang et al., 1993; Roux et al., 2000),

$$\Delta G = \frac{1}{2} \sum_{\alpha} q_{\alpha} \phi(\mathbf{r}_{\alpha}) \quad (3)$$

where  $\phi(\mathbf{r}_{\alpha})$  is the electrostatic potential at the position of the atomic charge  $\alpha$ . The electrostatic potential  $\phi(\mathbf{r})$  is calculated by solving the PB equation

$$\nabla \cdot [\epsilon(\mathbf{r}) \nabla \phi(\mathbf{r})] - \bar{\kappa}^2(\mathbf{r}) [\phi(\mathbf{r})] = -4\pi \rho_{\text{prot}}(\mathbf{r}) \quad (4)$$

for both the protonated (p) and unprotonated (u) state of a residue when it is in the protein environment and when it is isolated in solution (Roux et al., 2000). Here,  $\epsilon(\mathbf{r})$  is the position-dependent dielectric constant,  $\bar{\kappa}^2(\mathbf{r})$  is the position-dependent ionic screening constant, and  $\rho_{\text{prot}}(\mathbf{r}) = \sum_{\alpha} q_{\alpha} \delta(\mathbf{r} - \mathbf{r}_{\alpha})$  is the total charge density of the protein.

### Free energy simulations

To obtain the  $\Delta pK_a$  of Glu-71 on the basis of MD/FES, one must calculate the reversible thermodynamic work for alchemically transferring the proton from the residue in the protein to a reference residue isolated in solution (Kollman, 1993; Luzhkov and Åqvist, 2000). We use a (*N*)-acetyl-Glu-(*N'*)-methyl-amide dipeptide (one glutamic acid side chain blocked by neutral termini) as a reference to incorporate the influence of the backbone charges while avoiding spurious electrostatic interactions caused by the zwitterionic form of the residue. A convenient formal device for calculating such reversible work consists of introducing the thermodynamic parameter  $\lambda$  (Kirkwood, 1935) to control the alchemical process of transforming a residue from one state to another in a continuous manner

$$U(\lambda) = U_{\text{rest}} + (1 - \lambda)[U_{\text{protein}}(\text{p}) + U_{\text{peptide}}(\text{u}^*)] + (\lambda)[U_{\text{protein}}(\text{u}^*) + U_{\text{peptide}}(\text{p})] \quad (5)$$

where  $U(\text{p})$  and  $U(\text{u}^*)$  correspond to the potential energy of the protonated and unprotonated species, respectively. The asterisk denotes that a dummy

(non-interacting) hydrogen is attached to the carboxylate group in the unprotonated form (Shobana et al., 2000).  $U_{\text{rest}}$  is the potential energy of the remaining part of the system, including the solvent and the membrane. The free energy difference needed to determine the protonation state of Glu-71 is then given by the thermodynamic integration (Kirkwood, 1935; Kollman, 1993)

$$\Delta \Delta G = \int_0^1 d\lambda \left\langle \frac{\partial U(\lambda)}{\partial \lambda} \right\rangle_{(\lambda)} \quad (6)$$

where  $\langle \dots \rangle_{(\lambda)}$  represents a Boltzmann average calculated with the energy  $U(\lambda)$  as defined in Eq. (5).

### Atomic system and computational details

For the PB calculations, a detailed atomic model of the KcsA channel is embedded in a low dielectric membrane surrounded by a high dielectric aqueous solution. The various dielectric regions in the system are illustrated schematically in Fig. 2. The thickness of the membrane is 30 Å and its dielectric constant is  $\epsilon_m = 2$ . The pore and the cavity region of the KcsA are filled by water with  $\epsilon_w = 80$ . No electrolyte was included in the calculations for the sake of simplicity ( $\bar{\kappa}^2(\mathbf{r}) = 0$ ). The atomic radii optimized for PB calculations (Nina et al., 1997) were used to set up the dielectric boundary between the protein and the surrounding area. The protein-solvent dielectric boundary was constructed according to two different methods: as the surface formed by overlapping spheres with effective atomic Born radii (atomic surface) (Nina et al., 1997), and as the molecular surface sensed by a probe of 1.4 Å radius corresponding to the size of a water molecule (Richards, 1977). It is assumed that the protein has a uniform dielectric constant of  $\epsilon_p$ . Values of  $\epsilon_p$  ranging from 1 to 20 were considered to examine the sensitivity to this parameter. The total electrostatic potential is calculated at each point of a discrete grid by solving the finite-difference PB equation. The calculation is performed in two steps, first using a grid spacing of 1.0 Å ( $130^3$  points, with periodic boundary conditions in the membrane plane), followed by a focusing around the main region with a grid spacing of 0.5 Å. The reference calculations with the



isolated Glu-71 surrounded by a high dielectric medium were performed using the same side-chain conformation as in the protein. All the backbone atoms of the residue were kept. The same discrete grid was used with a focusing protocol. All the continuum electrostatic calculations were performed using the PBEQ module (Im et al., 1998) of the biomolecular simulation program CHARMM (Brooks et al., 1983).

The MD/FES were performed on the basis of an atomic model of the KcsA channel embedded in dipalmitoyl phosphatidylcholine (DPPC) surrounded by a 150 mM KCl aqueous salt solution that was previously equilibrated and simulated for over 3 ns (Bernèche and Roux, 2000). Briefly, the model comprises the KcsA channel (four subunits of 97 amino acids for a total of 5292 atoms), 112 DPPC, 6532 water molecules, 3 K<sup>+</sup> in the pore, and 12 K<sup>+</sup> and 23 Cl<sup>-</sup> in the bulk solution. The total number of atoms was slightly above 40,000. A Cl<sup>-</sup> ion in bulk water was replaced by an unprotonated glutamate dipeptide, and water molecules that were within 2.6 Å from the dipeptide were removed. The system was then further equilibrated for 50 ps with a harmonic restraint on the center of mass of the dipeptide to keep it in the bulk region. A harmonic restraint of 10 kcal/mol · Å<sup>2</sup> was applied to the three K<sup>+</sup> ions in the pore to preserve their relative position to the selectivity filter along the pore axis. The MD/FES were generated in the forward direction, from  $\lambda = 0$  to  $\lambda = 1$  by steps of 0.1, giving 11 equally separated simulations with intermediate values of  $\lambda$  using the PERT module of CHARMM (Brooks et al., 1983). Each of those “windows” was simulated for 20 ps, yielding a total simulation time of 220 ps. The MD/FES were also run backward following the same protocol. For each window, the first two picoseconds of simulation are considered as equilibration. The weighted histogram analysis method (WHAM) was used to calculate the free energy difference between the initial and final state of the system (Kumar et al., 1992; Shobana et al., 2000). The forward and backward MD/FES calculations were repeated four times, once for the residue Glu-71 of each of the four monomers. Periodic orthorhombic boundary conditions were applied in all directions to simulate a multilayer system of membranes extending in the XY plane. The Z dimension of the unitary cell was allowed to vary according to the constant pressure and temperature thermodynamic ensemble with fixed surface area (CPTA) (Feller et al., 1995, 1997). The dimensions of the periodic system are 72 × 72 × 78 Å<sup>3</sup>. The electrostatic interactions were computed with no truncation using PME (Essmann et al., 1995). All the calculations were performed using the academic version c28a1 of the biomolecular simulation program CHARMM (Brooks et al., 1983). As a control, the MD/FES protocol was applied to a system containing two glutamate dipeptides with blocked C- and N-termini in a box of 216 water molecules. By symmetry, a free energy difference of zero is expected for transferring a proton from one dipeptide to the other. The calculated free energy difference was on the order of 1 kcal/mol, which is indicative of the statistical uncertainty caused by the finite sampling for this system.

## RESULTS AND DISCUSSION

### Stability of KcsA with unprotonated Glu-71

What is the impact of four ionized Glu-71 on the channel structure? Results from MD simulations of the KcsA K<sup>+</sup> channel with protonated (Bernèche and Roux, 2000; Guidoni et al., 2000) and unprotonated Glu-71 (Guidoni et al., 1999; Allen et al., 1999; Shrivastava and Sansom, 2000; Biggin et al., 2001) have been described previously. However, it is difficult to compare the results from these previous simulations because of significant differences in computational methodologies. In our simulations (Bernèche and Roux, 2000), we used the all-atom CHARMM (Brooks et al., 1983) force field version PARAM 22 for proteins (MacKerell et al., 1998) and lipids (Schlenkrich et al.,

1996). Guidoni et al. (1999) used the all-atom AMBER force field (Cornell et al., 1995), while Shrivastava and Sansom (2000) and Biggin et al. (2001) used the extended-atom GROMOS force field (van Gunsteren et al., 1999). All electrostatic interactions were truncated at a cutoff distance of 17 Å in the MD of Shrivastava and Sansom (2000). Truncation of the electrostatic interactions can have important consequences on the results of MD simulations with charged species, even if a relatively large cutoff distance is used. For example, the distance between the Glu-71 side chain of the two subunits on opposite sides of the tetrameric channel is on the order of 17 Å and the distance between the K<sup>+</sup> in the central cavity of KcsA and the outer site in the selectivity filter is >17 Å. To avoid such a truncation Guidoni et al. (1999, 2000) and Bernèche and Roux (2000), calculated the electrostatic interactions using a particle mesh Ewald (PME) technique (Essmann et al., 1995). The treatment of the solvent and membrane environment may also be critical. Guidoni et al. (1999) simulated KcsA embedded in an octanol phase surrounded by bulk water. Shrivastava and Sansom (2000) and Bernèche and Roux (2000) simulated KcsA embedded in a phospholipid bilayer.

To examine the stability of the channel under those conditions and to compare with our previous simulation results with protonated Glu-71, we simulated the KcsA embedded in a solvated DPPC membrane with fully ionized Glu-71 in all four monomers. The system was constructed from the structure obtained with protonated Glu-71 that was equilibrated for 300 ps (configuration C1 in Bernèche and Roux, 2000). The conformation of the selectivity filter from this initial configuration is shown in Figure 3. The four residues Glu-71 were unprotonated and the system was further equilibrated for another 300 ps with progressively decreasing harmonic restraints on the hydrogen bonds involving Glu-71. The equilibration procedure was as described previously. No distance restraint was applied between residues Glu-71 and Asp-80. After equilibration, trajectories were generated for 500 ps at 315 K and 330 K.

Two types of conformation, represented in Fig. 4, are observed for the unprotonated Glu-71. In the monomer shown on the right of Fig. 4, the side chain retains an upright orientation analogous to the conformation with the protonated Glu-71 (see Figs. 1 and 3), while two to three water molecules form a network of hydrogen bonds between the carboxylate groups of Glu-71 and Asp-80. In the other monomer, shown on the left of Fig. 4, the side chain adopts a bent conformation to allow hydrogen bonding interactions between the carboxyl group and the amide hydrogens of residues Val-76 and Gly-77 of the selectivity filter. Through both the 315 K and the 330 K trajectories, the bent conformation of Glu-71 was observed on one or two monomers at a time, with a few transitions to the upright. Similar results were obtained in other simulations in which residues Glu-71 were first equilibrated in a bent conformation (results not shown), i.e., the carboxylate oxygens formed hydrogen bonds with the amide hydrogen of the selectivity filter. As a consequence, the structure of the selectivity

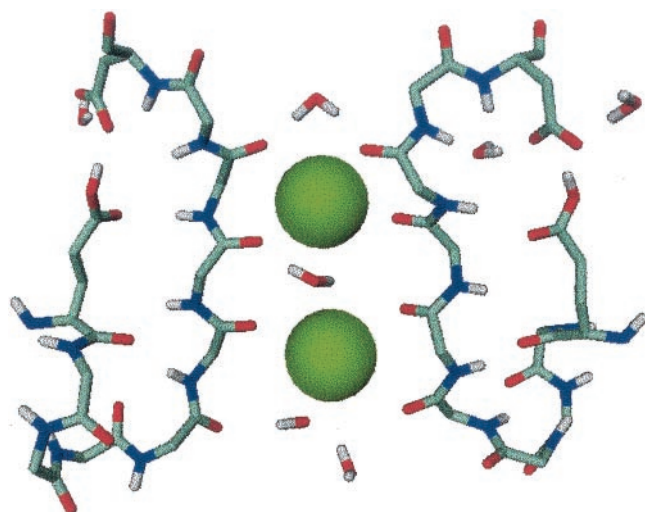


FIGURE 3 Configuration of the selectivity filter taken from the simulation of Bernèche and Roux (2000) after equilibration of the system. Two of the four monomers are shown. This configuration was used as the initial structure for all MD/FES.

filter is significantly distorted and the pore is widened, allowing a few more water molecules to enter in this region. Interestingly, a similar behavior was observed in the simulations of Guidoni et al. (1999) with four ionized Glu-71. In this case, the widening of the pore occurring on the extracellular side was sufficient to allow a fully solvated  $\text{Na}^+$  surrounded by six water molecules to enter the channel.

In presence of ionized Glu-71, the average RMS deviation of the selectivity filter backbone relative to the crystallographic structure is  $\sim 1.3$  Å and  $1.6$  Å for the 315 K and

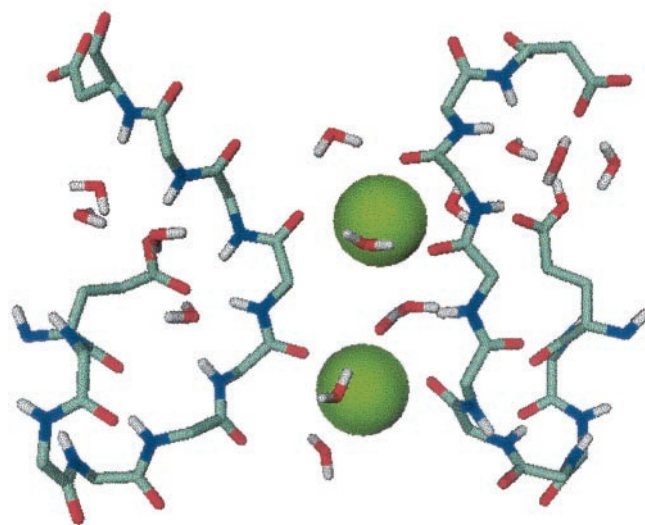


FIGURE 4 Result from a simulation including four unprotonated Glu-71: structure of the selectivity filter and neighboring residues after 500 ps of MD at 330 K. Only two monomers are shown; the conformation of the left monomer is particularly affected by the charged Glu-71.

330 K simulations, respectively, with some atoms having deviations as large as  $2.4$  Å. In contrast, the structure remained very stable through the MD trajectories with protonated Glu-71, and the corresponding RMS deviations were  $<0.9$  Å (Bernèche and Roux, 2000). These observations are a strong indication that the crystallographic structure is not stable if the Glu-71 side chains of the four monomers are ionized.

### pK<sub>a</sub> of Glu-71

Using calculations based on the PB equation, it has been estimated that the pK<sub>a</sub> of Glu-71 of KcsA is shifted by  $\sim +10$  pK<sub>a</sub> units (Roux et al., 2000; Ranatunga et al., 2001) (n.b., the estimate of Ranatunga et al. was obtained with no  $\text{K}^+$  ions in the pore). Because the intrinsic pK<sub>a</sub> of glutamic acid isolated in solution is around 4.4 (Stryer, 1988), this implies that side chain of Glu-71 should be protonated at neutral pH. However, the approach has several limitations (Antosiewicz et al., 1996; Alexov and Gunner, 1997; Havranek and Harbury, 1999; Dwyer et al., 2000). In particular, the electrostatic free energies calculated with the PB equation depend sensitively on the dielectric constant assigned to the protein interior (Antosiewicz et al., 1996; Simonson and Brooks, 1996). A dielectric constant of 1 for the interior of the protein provides a direct correspondence with a frozen protein configuration with a nonpolarizable force field in which the atomic partial charges are present explicitly. Values larger than 1 incorporate approximately the influence of induced electronic polarization and small-amplitude vibrational atomic fluctuations (Simonson and Brooks, 1996). Calculations and experiments on biological systems indicate that a value ranging from 8 to 20 should be used for the dielectric constant of the protein to account for different effects such as water penetration, protein flexibility, and conformational rearrangements in response to the ionization state (Antosiewicz et al., 1996; Dwyer et al., 2000). In contrast, the dielectric constant of protein measured from dry powders ranges from 2 to 4 (Takashima and Schawn, 1965). Because these effects can hardly be parametrized in a general way and remain specific to each system, the optimal value for the dielectric constant of the interior of the protein is usually treated as an empirical parameter in pK<sub>a</sub> calculations (Antosiewicz et al., 1996). Furthermore, the results are also sensitive to the method for constructing the dielectric boundary between the protein and the surrounding environment (see Nina et al. (1997)). For example, the boundary can be constructed as a surface formed by overlapping spheres with effective atomic Born radii (atomic surface) (Nina et al., 1997). A different method is to construct the dielectric boundary based on the molecular surface sensed by a probe of  $1.4$  Å radius corresponding to the size of a water molecule (Sharp and Honig (1990)). The latter definition includes the contact surface and the voids between the solute atoms forming the reentrant surface and

**TABLE 1** Free energy of ionization of Glu-71 from continuum electrostatic PB calculations (kcal/mol)

$\epsilon_p$	Atomic Surface	Molecular Surface
1	28.33	88.27
2	14.64	41.87
4	7.40	19.15
8	3.24	7.88
12	1.59	4.22
20	0.11	1.27

is equivalent to the solvent-inaccessible volume (Richards, 1977). Lastly, in the case of a membrane protein the results may also depend on the treatment of the membrane, although these should be lesser effects in the case of a buried residue such as Glu-71.

To examine the variation of the results on the calculated  $pK_a$  shifts we performed PB calculations using values for the dielectric constant of the protein interior ranging from 1 to 20, and two different approaches for constructing the protein-solvent boundary. The x-ray structure of KcsA (Doyle et al., 1998) in which the Glu-71 residues were constructed and equilibrated in their protonated form was used for these calculations (Bernèche and Roux, 2000). The free energy of transfer of a proton from Glu-71 to a solvated glutamate for different combinations of protein dielectric constant and probe radius are given in Table 1. The free energies vary from 0.11 to 88.27 kcal/mol, showing that the choice of the parameters has direct consequences on the conclusion that may be drawn from those calculations. The use of overlapping atomic spheres has the consequence of leaving small high dielectric regions in the interior of the protein. Thus, similar results can be obtained as well with a dielectric boundary constructed with overlapping atomic spheres and a low dielectric constant for the protein, or with a dielectric boundary constructed from the molecular surface and a higher dielectric constant for the protein.

Although the results from the PB calculations described above are suggestive, they are critically limited by the fact that a single frozen configuration equilibrated with a protonated Glu-71 was used. The influence of the protein flexibility is incorporated in a very approximate way via the dielectric constant assigned to the protein interior. Significant structural changes take place and a few water molecules penetrate the cavity between Glu-71 and Asp-80 when both side chains are ionized (see above). To accurately take these effects into account it is necessary to compute the free energy difference between the protonated and unprotonated forms using MD/FES with explicit solvent (Kollman, 1993). The results from the MD/FES are given in Table 2. Combining the data accumulated for the forward and backward run for all the MD/FES runs (for each of the four monomers) yields a free energy of ionization of +12.5 kcal/mol, which corresponds to a  $\Delta pK_a$  of +9.2 units. Although there is a significant hysteresis between the forward and the

**TABLE 2** Free energy of ionization of Glu-71 from MD/FES (kcal/mol)

Monomer	Forward	Backward
A	19.83	-2.85
B	18.84	3.30
C	31.21	11.28
D	23.13	-3.06

The MD/FES calculations correspond to the free energy for transferring a proton from residue Glu-71 to a solvated glutamate dipeptide. All accumulated data from the forward and backward runs yield a free energy of ionization of +12.5 kcal/mol.

backward perturbations, and variations for the different monomers, the results clearly show that Glu-71 should be protonated at normal pH.

The hysteresis in the MD/FES is due to the structural differences between the endpoints. The initial structure was equilibrated with all Glu-71 residues in their protonated form, making a hydrogen bond with the carboxylate group of Asp-80 (see Fig. 3). Fig. 5 shows the structure of the selectivity filter after the Glu-71 residues were perturbed from their protonated state ( $\lambda = 0$ ) to their unprotonated state ( $\lambda = 1$ ). When the perturbation simulations are run backward, the system does not return to its initial structure, i.e., that the hydrogen bond between the protonated Glu-71 and the carboxylate group of Asp-80 is not formed. For three of the four monomers the lateral chain of Glu-71 did not stabilize in any specific conformation at the end of the simulation. For one of the monomers the Glu-71 took an upright conformation and a network of water molecules was formed between Glu-71 and Asp-80. In this network, one water oxygen interacts directly with the proton on the Glu-71 carboxyl acid group (see Fig. 6). This is the closest that the system gets to its initial structure (i.e., the starting configuration of the forward perturbation), although it should be noted that the selectivity filter backbone was distorted in the process. During other simulations, it has been observed that the hydrogen bond between a neutral Glu-71 and a charged Asp-80 could spontaneously break and form again on a time scale of a few hundred picoseconds, but these events are rare. Even though the residue Glu-71 is buried in the protein core, it is still accessible to water molecules and the presence of the amide hydrogens of the selectivity filter can provide favorable interactions. In these distorted conformations, the calculated  $\Delta\Delta G$  values are close to zero according to the backward MD/FES and the protonation state of Glu-71 is dominated by its intrinsic  $pK_a$ . The structure of the channel is essentially denatured in the neighborhood of the selectivity filter when the Glu-71 is unprotonated. Test calculations with longer MD/FES did not decrease the hysteresis.

To have total reversibility of the MD/FES, the channel would have to return to its initial conformational state within the duration of the simulation. This process, which corresponds



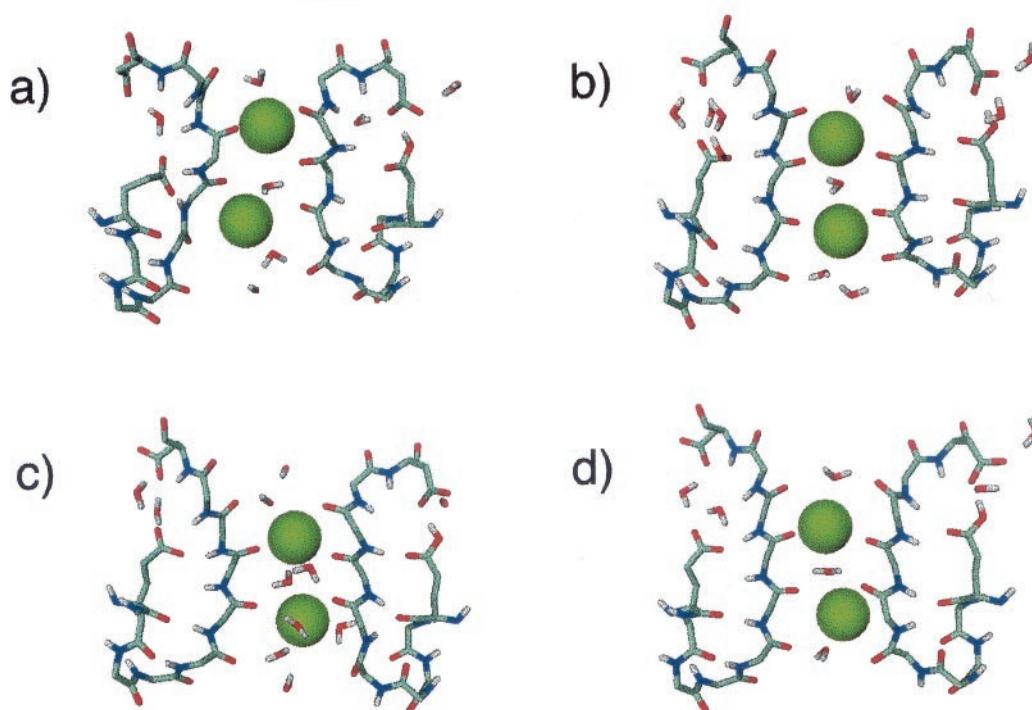


FIGURE 5 Structure of each of the four monomers at the end of the forward perturbations ( $\lambda = 1$ ). The unprotonated Glu-71 is shown on the left side of each figure. Bent and upright side-chain conformations are observed with different hydrogen bond networks involving either water molecules or amide hydrogens of the selectivity filter.

to the local re-folding of a partially denatured protein, is likely to occur in a much longer time scale than the one accessible here. To clarify this, MD/FES were generated with harmonic restraints applied to the KcsA channel. Table 3 gives the results for unrestrained and restrained perturbations for monomer B. These results illustrate that the perturbation is reversible when the protein is restricted to a small ensemble of conformations. Therefore, the fact that the forward and backward MD/FES do not converge to similar values is not a flaw of the methodology, but rather due to the dynamical properties of the system. The MD/FES calculations with harmonic restraints highlight the importance of the protein flexibility in the determination of its response to electrostatic perturbation. Although ionization free energy of Glu-71 is  $\sim +57$  kcal/mol in the most restrained simulation, it is only  $\sim +11$  kcal/mol on average in the unrestrained simulations. The ionization free energy is much lower in the unrestrained simulation because the structure has the freedom to adjust in response to the electrostatic perturbation arising from the charge of the side chain, an effect that is somewhat similar to having a high protein dielectric constant. By comparison with the continuum electrostatic calculations given in Table 1, the results for the unrestrained protein are reproduced by a single configuration for the protein using a dielectric constant  $\epsilon_p$  of 4 and the molecular surface with a probe radius of 1.4 Å. In contrast, the highly constrained protein corresponds to  $\epsilon_p \approx 1$  or 2.

Luzhkov and Åqvist (2000) calculated the relative free energy of the protonated and unprotonated forms of Glu-71 using similar MD/FES calculations with explicit water molecules. They estimated that the  $pK_a$  of Glu-71 is shifted by  $\sim +10$   $pK_a$  units. To account for the absence of an explicit bilayer membrane, they applied harmonic energy restraints varying from 0.5 to 100 kcal/mol/Å<sup>2</sup> to the channel, ions, and water molecules. The results in Table 3 show that the presence of artificial harmonic restraints has a significant impact on the results of the calculations. Interestingly, the present calculations suggest that the electrostatic environment of the residue Glu-71 varies depending on its conformation. In contrast, the results obtained by Luzhkov and Åqvist (2000) suggest that the ionization state of Glu-71 does not depend on its conformation (a difference of only a few kcal/mol was found between alternative conformations). Differences in force field and atomic charge distribution for the protein are probably the cause of the discrepancies. They used an extended-atom GROMOS force field (van Gunsteren et al., 1999), while we used the all-atom PARAM 22 force field (MacKerell et al., 1998).

The results from the MD/FES strongly suggest that the protonated state of Glu-71 is the most stable. It is satisfactory that this result is in general accord with previous calculations (Roux et al., 2000; Ranatunga et al., 2001; Luzhkov and Åqvist, 2000). Because the protonated Glu-71

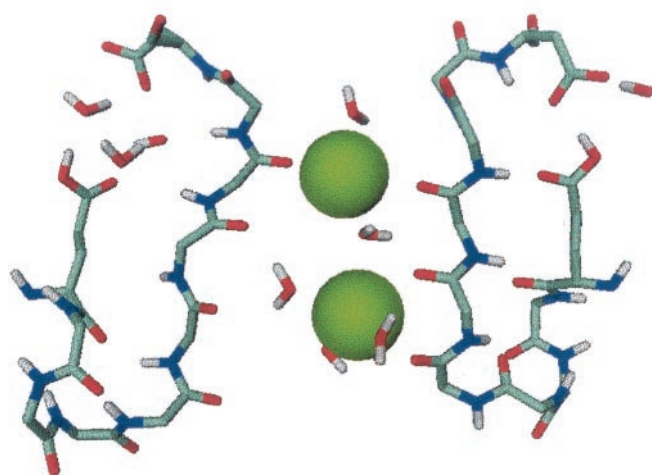


FIGURE 6 Structure of monomer C after the backward perturbation ( $\lambda = 0$ ). The side chain did not return to its initial conformation (shown in Fig. 3). Three water molecules have entered the small cavity between the side chain of Glu-71 and Asp-80.

side chain is involved in a strong di-acid hydrogen bond with Asp-80, the question of which residue should be protonated arise. On the basis of PB calculations, Ranatunga et al. (2001) proposed that protonation of Asp-80 is actually favored by  $\sim 1.5$ – $2$  kcal/mol relative to Glu-71 when two  $K^+$  ions are in the selectivity filter. Small  $pK_a$  shifts between Glu-71 and Asp-80 depending on the position of  $K^+$  ions in the pore were described. In contrast, the MD/FES calculations of Luzhkov and Åqvist (2000) indicated that protonation of Asp-80 is less favorable by  $\sim 2$ – $3$  kcal/mol under the same conditions. The ionization free energies of Glu-71 and Asp-80 were calculated for 80 configurations extracted from the MD trajectory (generated with a protonated Glu-71) using PB. The results indicate that protonated Asp-80 is more stable by  $\sim 2$  kcal/mol on average, with RMS deviations on the order of 2 to 3 kcal/mol, i.e., the difference is not statistically significant. More fundamentally, a detailed analysis of how the proton is shared between Glu-71 and Asp-80 based on the current atomic models may not be meaningful. In particular, the results are likely to be very sensitive to the set of fixed atomic partial charges assigned by the force field to the protonated and

ionized acidic side chains. For this reason, the significance of a small free energy difference based on such a model may be limited. It must be stressed that those charges were not developed for a highly polarized Glu-Asp di-acid pair involved in a strong hydrogen bond (MacKerell et al., 1998). For example, ab initio calculations at the Hartree-Fock 6-31g\*\* level suggest that the partial charges of the proton and of the neighboring carboxylate oxygens may vary by  $\sim 0.05$ – $0.10$  unit charges if the two fragments are considered separately or together (data not shown). Such small variations in partial charges would be sufficient to affect the outcome of  $pK_a$  calculations, based on PB or MD/FES. Lastly, other factors, such as the coulombic interaction with Arg-89, which is only 4 Å away from the carboxylate oxygens of Asp-80 (Zhou et al., 2001), may also have some influence on the protonated pair. Therefore, it is likely that more sophisticated treatments combining ab initio and MD simulations would be required to examine in detail how a proton is shared between Glu-71 and Asp-80.

## CONCLUSIONS

Results from MD, MD/FES, and PB calculations were used to address specific detailed questions about the conformation and protonation state of Glu-71 in the KcsA  $K^+$  channel. The results from the MD/FES calculations show that the protonated state of Glu-71 is stabilized by  $\sim +12.5$  kcal/mol, which corresponds to a  $\Delta pK_a$  of 9.2  $pK_a$  units. This value is in general accord with previous results based on PB (Roux et al., 2000; Ranatunga et al., 2001) and MD/FES with restricted conformers (Luzhkov and Åqvist, 2000). Furthermore, we observed that the selectivity filter of the KcsA channel is significantly distorted during MD simulations with unprotonated Glu-71 on each of the four monomers, deviating significantly from the crystallographic structure. These results strongly suggest that computational studies based on detailed atomic models of KcsA should include Glu-71 in a protonated state (Guidoni et al., 2000; Bernèche and Roux, 2000; Luzhkov and Åqvist, 2000, 2001; Crouzy et al., 2001), rather than unprotonated (Guidoni et al., 1999; Allen et al., 1999; Shrivastava and Sansom, 2000; Biggin et al., 2001).

Figs. 3–6 were produced with DINO, a freely distributed visualization program available at <http://www.dino3d.org>.

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**TABLE 3** Free energy of ionization of Glu-71 from MD/FES with harmonic restraints on the heavy atoms of the KcsA channel (kcal/mol)

Force Constant (kcal/mol $\cdot$ Å <sup>2</sup> )	Free Energy (kcal/mol)	
	Forward	Backward
0.0	18.84	3.30
0.5	46.56	33.17
2.0	57.65	57.24

The calculations correspond to the free energy for transferring a proton from residue Glu-71 of monomer B to a solvated glutamate dipeptide.

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