Molecular Biology

cDNA of mutants and wild-type *AtClCa* was inserted in the *pfunct+TAG* vector. This allows to identify single transformed protoplasts under the microscope by fluorescence and to extract the vacuole from fluorescent protoplasts for patch-clamp analysis. The *pfunct+Tag* vector contains two independent cassettes (one for *GFP* and one for *AtClCa*). The two cassettes are under the control of two identical promoters *(1)*. Therefore this vector assures that fluorescent protoplasts are transformed with the gene of interest without tagging the resulting protein. The cloning of *pfunct+Tag* vector needs an entry fragment with XhoI and SmaI restriction sites at the edges. We amplified the cDNA by PCR with primers that added these restriction sites at the gene borders, which gave a fragment of 2322 kb. The PCR fragment was first cloned into *pGEMTeasy* vector by TA cloning and from this vector to *pfunct+Tag* by restriction-digestion. Site directed mutagenesis was done with Stratagene Quickchange Mutagenesis kit. Purification of the DNA for transient transformations was done with Macherey-Nagel Nucleobond Xtra Maxi kit.

The used primers were:

Cloning

XhoI F a: 5' AAT CTC GAG ATG GAT GAA GAT GGA AAC TTG CAG 3'

SmaI R a: 5' AAT CCC GGG TCA TCT AGC TTT TCC ACT TTT GTG 3'

Mutagenesis:

H620A/S: 5'AAG GAA CAC AAC GGC TAA GCG ATT CCC A 3'

H620A/AS: 5' TGG GAA TGC GTT AGC CGT TGT GTT CCT T 3'

D753A/S: 5' TCT TAA CAA GGC AAG CTC TCA GGG CTT ATA 3'

D753A/AS: 5' TAT AAG CCC TGA GAG CTT GCC TTG TTA AGA 3'

Computer simulations.

Minimisation of the models and molecular dynamics (MD) simulations were done with the program NAMD (2) running in computer cluster. To simulate explicitly the solvent, each system was hydrated with a shell of TIP3P water model (3) using the LEAP module of AMBER 7 (4). The SHAKE (5) procedure was employed to constraint all hydrogen atom bonds. Before to proceed with MDsimulations, each complex was minimized to reach a constant energy (variation less than 0.01 Kcal/mol). The integration time step was 2 fs, with a cut off of 12 Å for the non-bounded interactions. The non bounded pairs were updated every 20 steps.

After minimization, each construct was stepwise heated to 300 K, and successively equilibrated to this temperature for 200 ps. Data collection was then carried out every 1 ps during a 1.5 to 2 ns simulation. The production phase after equilibration was determined by the criteria that the standard

deviation of the energy of the system does not change significantly.

Free energy of binding calculation.

The calculation of the free energy of binding ($\Delta G_{binding}$) was performed using the LIE (Linear Interaction Energy) approximation method (6, 7). The basic idea in the LIE method is to consider the binding free energy $\Delta G_{binding}$ as the free energy change when the ligand is transferred from the aqueous solution to the solvated receptor binding site. The solvation energy of the ligand can be considered as formed by two terms: the non-polar interactions energies and the polar interactions energies. Therefore $\Delta G_{binding}$ can be approximated as:

$$\Delta G_{binding} = \Delta G^{(non\ polar)} + \Delta G^{(polar)}$$

The polar energy term can be deduced from the electrostatic potential energy of interaction of the ligand l and its surrounding solvent s ($\left\langle V_{l-s}^{(elec)} \right\rangle$) and the non-polar energy term from the Van der Waals potential energy of interaction of the ligand l and its surrounding s ($\left\langle V_{l-s}^{(vdw)} \right\rangle$).

The binding energy is then calculated with the equation (6, 7):

$$\Delta G_{binding} = \alpha \Delta \left\langle V_{l-s}^{(vdw)} \right\rangle + \beta \Delta \left\langle V_{l-s}^{(elec)} \right\rangle + \gamma$$

where α and β are weight parameter of the non-polar and polar binding energies contributions, respectively and γ is an additional parameter. $\Delta \left\langle V_{l-s}^{(x)} \right\rangle$ is the difference of electric (elec) and van der Waals (vdw) energies between the ligand bounded to the receptor and free in solution.

The α, β and γ parameters are semi-empirical scaling factors that have been determined for many different protein-ligand systems (avidin, neuroaminidase, endopeptidase and different values for each parameter have been derived in each system) (7-10).

Our strategy was to use the K_d of ATP, ADP and AMP in hClC-5 to derive the $\Delta G_{binding}$ and to determine the constants for our system with these values. However, hClC-5 has an experimental $\Delta G_{binding}$ = -5.6 Kcal/mol for all the three nucleotides (11) that this makes a statistical parameterization for our system impossible.

Hence, we used parameters derived for different proteins (7-9) to calculate the $\Delta G_{binding}$ for every nucleotide to hClC-5, and choose the parameter set that better reproduce experimental values. In order to remove the offset eventually due to the γ parameter, which is strongly system dependent (12), we preffer to express the results in terms of $\Delta\Delta G_{bindind}$ between the wild-type and mutant models (Tab S1) for each set of parameter used. Interestingly, with all the sets of parameters the

$\Delta\Delta G_{binding}$ between the models gave values within the same range (Tab S1). The set of parameter that better reproduce the experimental data for of hClC5 is: $\alpha = 0.476$; $\beta = 0.165$;$\gamma = 0$.

Table S1. Average MD difference of interaction energies between bound and free nucleotides and calculated and observed free energies of binding. Energies are in Kcal/mol. Data are mean ± standard error of the mean, from 1400 to 1900 configurations were used for the calculation of the mean. ΔG_{Exp} are the experimental values, calculated from the dissociation equilibrium constants. $\langle \Delta V_{l-s}^{(elec)} \rangle$ and $\langle \Delta V_{l-s}^{(vdw)} \rangle$ are the electrostatic and vad der Waals terms of the difference of the poptential energy between the ligand and its sourounding solvent. $\Delta G_{binding}$ was calculated with LIE, parameters were derived from the literature, as indicated. The variation of the binding free energy was defined as $\Delta\Delta G_{bind}$= ΔG_{bind}(D753A/H620A/ADP/AMP)-ΔG_{bind}(AtClCa).

Model	Ligand	ΔG_{Exp}	$\langle \Delta V_{l-s}^{(elec)} \rangle$	$\langle \Delta V_{l-s}^{(vdw)} \rangle$	$\Delta G_{binding}$			$\Delta\Delta G_{binding}$		
					$\alpha = 0.181$ $\beta = 0.5$ $\gamma = 0$	$\alpha = 0.476$ $\beta = 0.165$ $\gamma = 0$	$\alpha = 0.87$ $\beta = 0.5$ $\gamma = 0$			
(reference)					(6)	(7)	(8)	(6)	(7)	(8)
AtClCaa	ATP	-5.9	-24.4±1.0	-17.4±0.1	-40.7±0.5	-22.2±0.6	-55.2±0.6			
AtClCaa	ADP		-21.5±0.4	-10.1±0.5	-12.6±0.4	-8.4±0.4	-19.5±0.5	-28.1±0.6	-13.8±0.7	-35.6±0.8
AtClCaa	AMP		-149.8±0.4	-3.0±0.3	-75.4±0.3	-26.1±0.3	-77.5±0.4	34.1±0.6	3.9±0.5	22.3±0.8
H620Aa	ATP		-45.2±1.0	-18.7±0.3	-26.0±0.7	-16.4±0.3	-38.9±0.8	-14.7±0.9	-5.8±0.9	-16.3±0.9
D753Aa	ATP		23.6±1.0	-17.6±1.0	8.6±0.7	-4,5±0.2	-3.5±0.8	-49.3±0.9	-17.7±0.6	-51.7±1.0
hClC5b	ATP	-5.5	13.1±0.5	-16.4±0.1	11-4±1.4	-9.8±0.6	-21.3±1.4			
hClC5b	ADP	-5.5	16.6±0.4	-14.9±0.1	1.3±0.9	-6.2±0.5	-9.7±0.9	-12.7±1.0	-3.5±0.8	-11.6±1.3
hClC5b	AMP	-5.5	11.1±0.8	-23.7±0.2	1.3±0.6	-9.4±0.3	-15.1±0.6	-12.7±1.0	-0.3±0.6	-6.3±1.5

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