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Soft–Soft Interactions in the Protein–Protein Recognition Process: The K^+ Channel-Charybdotoxin Case

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Molecular recognition between peptide blockers and ionic channels is a complex process that involves many effects. To determine if the short-range charge transfer effects play a significant role in this interaction, a chemical reactivity analysis of charybdotoxin (ChTX) and six of its mutants was carried out using global and local reactivity indices. The results show that global softness correlates with the affinity of ChTX, and its mutants to the channel indicating that soft–soft interactions play a role in the recognition process between ChTX and a potassium channel. The analysis of the local reactivity indicates that the toxin as a whole can be seen as a complex polydentate ligand with several places to coordinate with the external vestibule of the pore of the potassium channels. The successful treatment of point mutations supports the idea of using this tool in the study of chemical reactivity in proteins, in a similar way as substituent effects in organic chemistry. © 2012 Wiley Periodicals, Inc.

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Introduction

The protein–protein recognition process is complex and involves many effects. The most common are the steric and electrostatic interactions, hydrogen bond formation, and hydrophobicity.[1] In some cases, the information about key residues involved in the recognition process is available from site directed mutagenesis experiments. In a more restricted number of cases, structural data of the interacting proteins are at hand. For some of the later cases, it may be even possible to pursue computational studies at an atomistic level including the evaluation of the electronic structure of such proteins. In such context, this work establishes a structure–function relationship between short-range charge transfer effects and kinetic constants for the dissociation process in the interaction of a peptide type toxin and a potassium channel. To reach this goal, the electronic structure of charybdotoxin (ChTX)[2] and six of its mutants was obtained; and the reactivity analysis was performed using local and global density functional theory (DFT) concepts that have been widely used to rationalize the chemical behavior of small molecules.[3] In small molecules, the analysis is performed by changing atoms or functional groups, however, the experimental information available for ChTX is obtained by site directed mutagenesis, that is, the exchange of an amino acid for a different one at a particular position in the peptide chain. Consequently, it is desirable to identify global and local changes in the electronic structure of the peptide induced by such mutations and correlate them to changes in the reactivity of the protein. The present work tests if such analysis is feasible because that could open a new procedure for the chemical reactivity analysis in proteins.

Scorpion toxins inhibit ion conduction through potassium channels by occluding the pore at the extracellular vestibule. The stoichiometry of the process indicates that one toxin molecule binds to a single channel.[4] These toxins comprise peptides of 35–40 amino acids in length, are rigid structures held by three disulfide bridges,[2] and according to their amino acid sequence homology, they have been classified in 18 subfamilies.[5] Electrophysiological or binding experiments have shown that the affinity of these peptides toward a variety of potassium channels varies from picomolar to micromolar range.[6]

Before the determination of the first high-resolution crystal structure of the pore of a potassium channel,[7] mutagenesis studies involving the toxin and the channel were the experimental approaches applied to get a qualitative description of the external surface of the vestibule of a K^+ channel pore.[8–11] Several residues in the toxins were found to be crucial for binding to a potassium channel.[8,12] One conserved lysine residue located in the β-strand of all of these toxins interacts directly with a tyrosine residue in the selectivity filter of the "Shaker"-K^+ channel.[11] Based on the observation that several channel-specific toxins, with unrelated folds and different origins, possess a common dyad component of a lysine and an aromatic residue,

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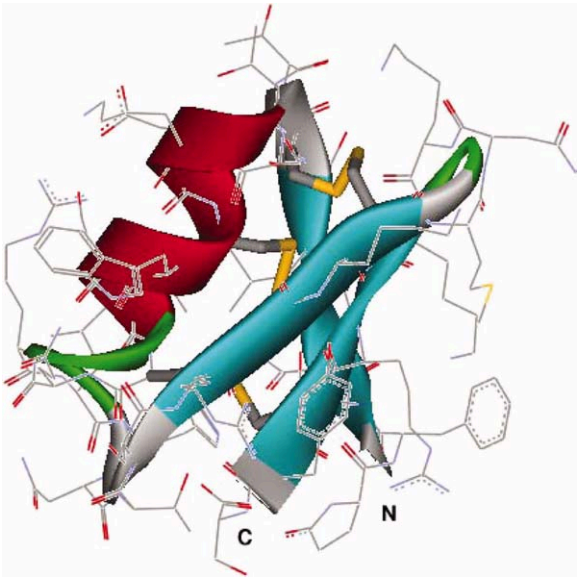

Figure 1. Three-dimensional structure of ChTX. The geometry of the toxin was taken from reported NMR experiments.[16] α-helix (red) and β-sheet (blue) regions are shown. The disulfide bridges are rendered with yellow sticks. For a better visualization of the structure, the hydrogen atoms have been hidden. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

it has been proposed that this structural motif represents the result of convergent evolution and may be the most relevant feature of a toxin-Kv channel interaction.[13] However, there is a toxin without this dyad that blocks a Kv channel,[14] and some others with the dyad are unable to inhibit Kv channels.[15] These facts support the hypothesis that additional residues are also involved in the toxin-K^+ channel interaction.

ChTX, a 37 amino acid residue protein isolated from the venom of the scorpion *Leiurus quinquestriatus*, is a powerful inhibitor of some types of K^+ channels.[2] This toxin adopts a three-dimensional globular compact structure composed of a short α-helix interacting with a β-sheet and three disulfide bridges (Fig. 1).[16]

ChTX is a highly basic protein, with eight positively charged residues (four lysines, three arginines, and one histidine), two negatively charged groups (a single glutamate and the C-terminus), and a net charge of $+6$ at neutral pH.[17] The mechanism by which the ChTX blocks Ca^{2+}-activated K^+ channel has been studied in depth[18]; and one of the main conclusions indicates that a single ChTX molecule physically occludes the K^+ conduction pathway by binding to a site located in the external mouth of the channel protein. Because the peptide's inhibitory mechanism is simple and its receptor site is located in a mechanistically important region of the ion channel, ChTX has been a useful functional and structural probe of K^+ channels. Site directed mutagenesis of ChTX and the channel provided a useful mapping of the outer vestibule structure before the elucidation of the crystal structure of a K^+ channel.[19]

Kinetic studies of the reaction

$$\text{ChTX} + \text{Channel} \underset{k_{\text{off}}}{\overset{k_{\text{on}}}{\rightleftharpoons}} \text{ChTX} - \text{Channel}, \qquad (1)$$

using mutants of ChTX, have shown that crucial amino acids for the activity of the toxin alter the kinetics of the ChTX–channel interactions, mainly through increasing the dissociation constant, k_{off}.[12] So far, all the above studies have only been focused on the amino acid residues that comprise the interaction surface between the toxin and the potassium channel, but no atomistic model exists that can address the complete rationalization of the kinetics of this interaction. Recently, the importance of the electrostatic forces in the interaction of ChTX and other toxins with the Kv1.3 potassium channel has been discussed using the correlation of the electrostatic energies and the quotient $k_{\text{on}}/k_{\text{off}}$.[20] The fact that the channel vestibule is negatively charged and the ChTX is positively charged supports the idea that charge transfer, from the channel toward the toxin, could take place at the boundary surface between the two macromolecules. The results of this work support such conjecture. Along this line, it has been determined that the crucial amino acids on the interaction surface of the toxin are characterized by a high charge acceptance quality.[21] This property seems to be related to the intriguing fact that mutations mainly affect the dissociation constant, k_{off}, because dissociation involves the rupture of short range interaction forces whereas the electrostatic interactions are present in both, association and dissociation.

Chemical Reactivity Approach

Two types of properties are computed to investigate the chemical reactivity of the mutated proteins (mutants) and compared to those of the "wild-type" toxin: a global parameter and a local quantity. The global parameter measures the charge transfer capabilities of the macromolecule as a whole and the local quantity indicates the reactive regions. The properties used in this study have been successfully applied to rationalize the reactivity of small molecules.[3] They are obtained from the electronic structure of the systems and are defined as follows. The chemical potential, μ, has been identify as the negative of the electronegativity[22] and its derivative with respect to the number of electrons is the chemical hardness, η,[23] which is related to the hard and soft acids and bases (HSAB) principle of wide applicability in reactive processes.[24] The local softness, $s(r)$,[25] is related to the derivative of the charge density with respect to the number of electrons and measures local charge transfer capabilities; this quantity can be estimated by scanning tunneling microscopy experiments in surfaces[26] and gives a qualitative estimation of local chemical bonding forces as determined by the atomic force microscope.[27] The integral over the whole space of the local softness is called global softness, S, and it is equal to the inverse of hardness: $\eta = 1/S$.[25]

Using the chemical potential and the global hardness, electrophilicity, ω, is defined as[28]

$$\omega = \frac{\mu^2}{2\eta}, \qquad (2)$$

This property measures the ability of a system to stabilize the electron charge, and it has a local companion quantity, the local electrophilicity index $\omega(r)$,[29] which, for a given molecule, is proportional to local softness:

$$\omega(r) = \mu^2 s(r). \quad (3)$$

Using the local density of states, $g(E,r)$, the local electrophilicity index can be estimated as

$$\omega(r) \approx \frac{\mu^2}{\Delta\mu} \int_{\mu}^{\mu+\Delta\mu} g(E,r)dE. \quad (4)$$

The approximation represented by the integral is a finite differences scheme that estimates local softness using the "frontier bands" of the system.[30] In this work, $\Delta\mu$ is related to the width of the frontier lowest unoccupied molecular orbital (LUMO) band (conduction band), because the process of interest is the acceptance of electron charge by the macromolecules (nucleophilic attack). As has been shown in many cases involving small molecules, the chemical reactivity approach outlined above is useful to rationalize reactivity trends in families of compounds.[3]

For the purposes of this work, it is also important to recall the idea of substituent effect that is a key concept in structure–activity relationships.[31] One of the most appealing features of such concept, is the possibility of establishing correlations between equilibrium and rate constants and changes in the electronic structure induced by substituents. In addition, and important for this work, the substituent effect analysis is performed using properties of the isolated species rather than properties of the interacting complexes; this is crucial for the systems involved in this study because to obtain such information for protein–protein interaction is particularly difficult. In this context, this work will show results that support the idea that the substituent effect concept can be extended to study mutation effects in protein–protein interactions.

Computational Details

The electronic structure of "wild type" and six mutants of ChTX was obtained by total energy pseudopotential calculations (TEPC)[32] within the DFT++ program.[33] The plane wave expansion was set using an energy cutoff of 6 Ry, and the local density approximation[34] was used. It is worth mentioning that plane wave basis set is inherently free of the so called basis set superposition error (BSSE). Contrary to atomic orbital basis sets that are centered on the atomic nuclei and have a rather large BSSE, plane waves are not centered thus describing any point in the periodic supercell with the same quality. For ChTX, a 19.1 × 24.6 × 28.6 Å^3 supercell was used to fulfill the condition that the minimum distance between molecules in neighbor cells must be 5 Å.[35] The last condition was satisfied by all the supercells used in this work. TEPC only considers the valence electrons; the corresponding pseudopotentials were produced following the prescription developed by Rappe et al.,[36] using a 40 Ry cutoff. The electronic structures of the macromolecules were obtained as single point calculations of geometries determined by classical molecular dynamics simulations as described later.

To test the calculation procedure for the reactivity parameters used in this work, ω was evaluated for a set of molecules containing the same set of atoms as the toxin. As this property depends on several global parameters, it is a good way for calibrating the approximations used to evaluate them. In addition, for the molecules used, the electrophilicity calculated with standard localized basis sets is known and gives a good correlation with the experimental reactivity.[37,38] The results for these molecules are displayed in Table 1. One can see that

Table 1. Global electrophilicity (ω) for a set of small molecules in eV.

	Plane waves			
Molecule	6 Ry	20 Ry	40 Ry	B3LYP/6-311G**
CH_3^+	12.40	17.81	18.33	9.98[a]
$CH_2NHCH_3^+$	3.66	5.92	6.62	8.97[b]
SO_3	3.19	3.47	4.37	1.57[a]
CH_2CHCHO	1.49	3.21	3.73	1.84[b]
HCOH	1.10	2.32	2.88	0.81[a]
C_2H_4	1.06	1.30	1.37	0.73[b]
$CH_2CHN(CH_3)_2$	1.00	0.84	0.82	0.27[b]

[a] Values taken from Ref. [37]. [b] Values taken from Ref. [38].

the tendency observed with localized standard basis sets is preserved at the three levels of truncation for the plane wave expansion, 6, 20, and 40 Ry. It has been recently reported that a truncation of 40 Ry, combined with a proper unit cell size such as those used in this work, provides eigenvalues of quality equivalent to those obtained with a localized basis set close to the limit of an infinite expansion.[39] The evaluation of μ, η, and ω, only needs the eigenvalues of the highest occupied molecular orbital (HOMO) and LUMO states,[23] and results obtained at 6 and 40 Ry agree qualitatively, thus estimations of reactivity indices at 6 Ry may provide similar trends as those obtained with very large localized basis sets (see Table 1 and Ref. [39]).

The mutants studied in this work are those in which the tyrosine 36, of the wild-type ChTX, is replaced by phenylalanine, asparagine, leucine, alanine, histidine, and proline; these mutants correspond to those experimentally characterized in Ref. [12]. The rationale for selecting these mutants is that they are made in the amino acid site producing a wide range of changes in the kinetic constant; furthermore, mutations conserve the total charge respect to that in the wild ChTX and maintain the geometry relaxation localized at the same region of the macromolecule. Mutant structures were constructed from the NMR geometry of wild-type toxin[16] (PDB file 2CRD) replacing TYR36 for each of the residues studied. The structures were obtained in two steps: first hydrogen atom geometries on the new residue were optimized and the side chain adjusted by hand to avoid any atomic clashes, then minimized and later subjected to a short molecular dynamics simulation (30 ps) and minimized again. In a second step, the final structure was obtained by a molecular dynamics (100 ps after energy equilibration) followed by a geometry optimization. The wild-type structure was also subjected to the same modeling process obtaining a structure that only differs noticeably, from the original NMR structure, at the position of an alpha carbon by a root mean square (RMS) of 0.6 Å. Energy minimizations and molecular dynamic simulations used the Dreiding

II force field[40] and the BIOGRAF package. Geometry regularizations were performed until a 4 kJ mol^{-1} nm^{-1} or lower forces were reached. All molecular dynamics simulations were calculated by numeric integration of Newton's equations of motion for all atoms using the Verlet algorithm,[41] a step size of 1 fs, at 300 K in isothermal conditions, and updating velocities every 0.1 ps. Nonbonded interactions were considered within a distance of 0.9 nm with a smooth switch-off function from 0.80 to 0.85 nm to avoid discontinuous forces. Modeling procedures were done *in vacuo* with all hydrogen atoms explicitly included. A distance-dependent dielectric constant was used to simulate water screening of electrostatic interactions.

Results and Discussion

The global reactivity of ChTX and its mutants is presented in Table 2. Results show that the toxin with greater affinity for the channel, that with the smaller k_{off} (the wild type, Y36Y), is the system of greatest softness. According to the HSAB principle,[24] this result implies that the vestibule of the channel must behave like a soft species. The results also show that mutants with less affinity for the channel, that is, larger k_{off}, have a large value of η. Thus, mutants lose affinity for the channel if its global hardness has increased. The behavior of global electrophilicity shows that, in the case of electron charge-transfer toward the toxin, the wild-type ChTX has the maximum capacity for stabilizing it. As the mutants tested are charged conserved, the electrostatic effects due to the presence of charged aminoacids are avoided. This fact strengthens the importance of charge transfer processes to understand the trends observed in k_{off} variations.

Table 2. Global reactivity indexes and dissociation rate constant (k_{off}).

Toxin	μ	η	S	ω	k_{off}[a]
Y36Y	−2.504	0.0004	2605.08	8164.78	62 ± 3
Y36F	−2.778	0.0007	1493.29	5763.96	72 ± 5
Y36N	−2.563	0.0019	537.83	1766.70	920 ± 220
Y36L	−2.653	0.0028	363.32	1278.43	56,000 ± 2000
Y36A	−2.855	0.0033	301.48	1228.28	91,000 ± 3000
Y36H	−2.555	0.0071	139.87	456.65	89,000 ± 1000
Y36P	−2.525	0.0159	62.96	200.62	120,000 ± 3000

All quantities are expressed in eV, except k_{off} which is expressed in ($\times 10^{-3}$ s^{-1}). $\mu = 1/2(\varepsilon_{HOMO} + \varepsilon_{LUMO})$, $\eta = \varepsilon_{LUMO} - \varepsilon_{HOMO}$, $S = 1/\eta$, and ω is calculated by Eq. (2).
[a] Values taken from Ref. [12].

In addition, one can use the logarithm of the kinetic constant and the logarithm of global softness as basic variables to perform a "substituent effect" type of analysis.[42] It is important to recall that, in this type of approach the rationale is to compare the kinetic constants with respect to a point of reference that in our case is the kinetic constant of the wild-type toxin. Figure 2 shows a Hammett type[43] relationship between the dissociation kinetic constants and global softness: $\ln(k/k_o) = \rho\sigma$. In our case, ρ is the slope of the adjusted line and the Hammett parameter, σ, for each mutant is equal to $\ln(S/S_o)$. This empirical approach, which is standard for the treatment of substituent effects, gives a good qualitative description of the affinity of ChTX and its mutants toward the channel if global softness is used as the property to correlate with.

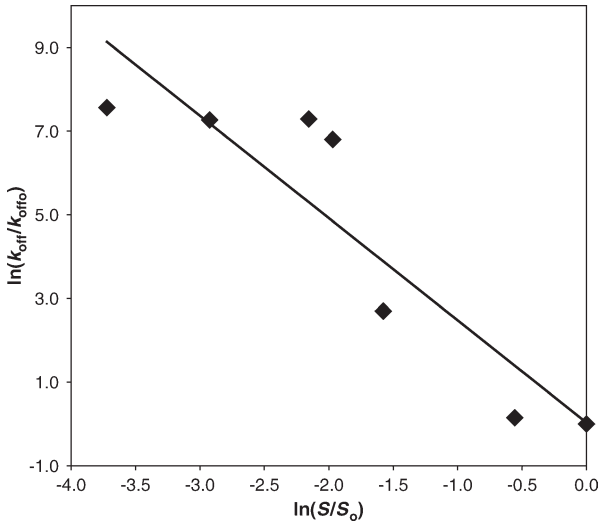

Figure 2. Structure–activity relationship between kinetic dissociation constants, k_{off}, and the global softness (S) of the different species studied. Points come from our calculations and the reported experimental constants, whereas the solid line indicates the general trend. The corresponding equation is $\ln(k_{off}/k_{off,o}) = -2.44\ \ln(S/S_o) + 0.03$ with a correlation coefficient of −0.904.

To look for correlation between k_{off} and geometrical or experimental parameters, we tested a set of 55 quantities based on different chemical and physical properties of the amino acids, such as hydrophobicity, flexibility, antigenicity, bulkiness, high performance liquid chromatography (HPLC) retention in different conditions, and secondary structure conformational potential, as included in the ProtScale server of the Expert Protein Analysis System (ExPASy, Swiss Institute of Bioinformatics, http://au.expasy.org/tools/protscale.html). To account for steric effects on the k_{off} values, we measured on our toxin models geometric properties of the side chain in position 36, such as accessible surface area, molecular volume, and lateral chain length. We found that the strongest correlation to k_{off} is obtained with the lateral chain length, when the latter is measured as the projection of the vector connecting the alpha carbon and the farthest atom in the side chain, into the unit vector along the bond between the alpha and beta carbons. The correlation coefficient between these two quantities is ∼−0.7 (data not shown). The lateral chain length can thus be seen as a measure of steric effects, because the contact surface between the toxin and the channel involves the site where the mutation takes place. The correlation between the kinetic constants and two variables, one of electronic structure origin, S, and one of structural origin, the length of the lateral chain, is displayed in Figure 3. A small quantitative enhancement is seen, but the qualitative pattern attained by global softness alone in Figure 2 is remarkable, even better than the attained by lateral chain length alone. The latter strongly suggests that global softness is an important property for understanding the dissociation of the toxin–channel complex.

Finally, the local electrophilicity index was calculated using Eq. (4), with $\Delta\mu = 2.1$ eV. The results are shown in Figure 4. It is interesting to note that the region defined by Lys27, Met29,

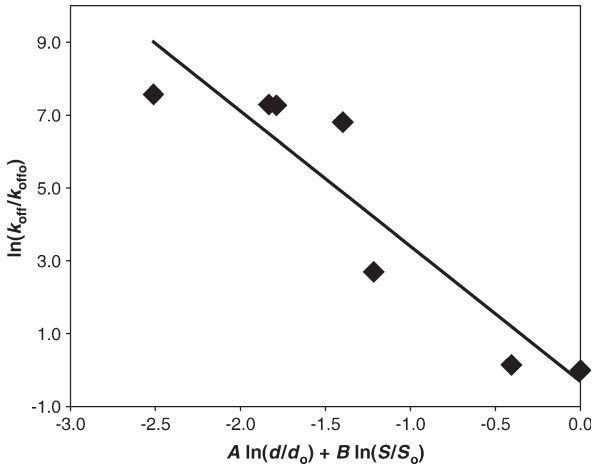

Figure 3. Structure–activity relationship between kinetic dissociation constants and an expanded structural parameter including the length of side chain in residue 36 (d) and global softness according to $A * \ln(d/d_o) + B * \ln(S/S_o)$, where A and B were optimized as 0.44 and 0.56, respectively. Again, the solid line sketches the general trend. In this case, the correlation coefficient is −0.923.

Arg34, and residue 36 is the only one exhibiting a high capacity to stabilize charge. Experimentally, it is known that this region, which is close to the channel mouth and is the most active in reducing k_{off} values upon mutations, plays an important role in the affinity of ChTX for a K^+ channel.[2] It is worth to point out that according to the local analysis, the wild-type toxin and the mutants have several points on the active region with charge transfer capabilities. However, at the level of analysis of the local quantity used in this work, it is not possible to distinguish if one of these points is more important than the others.

As was mentioned before, the interaction between the channel and the toxin involves a negative charged surface of the channel and a positively charged surface of the toxin. Consequently, if a charge transfer process occurs, this would be toward the toxin. All the electronic structure parameters estimated in this work indicate that such charge transfer is related to the stabilization of the toxin–channel complex. It is clearly seen from Figure 2 that a global softness increase is related to a reduction of k_{off} and therefore an increase of the equilibrium constant for the formation of the toxin–channel adduct. Also, the analysis of Figure 3 reveals that the most important steric effect has a small impact on the correlation coefficient, reinforcing the idea that the stabilization involves a soft–soft interaction. An attempt to visualize the docking maneuver of the toxin in its way to sit on the channel mouth could be as follows: long-range electrostatic interactions put the docking partners into an aligned orientation; and the short-range charge transfer effects are added when the two protein interfaces are close enough, so that these quantum mechanical properties are able to emerge. One may point out that, the effect described in our work is additional to the hydrogen bonds formed and to the classical electrostatic interactions between the channel and toxin surfaces. The solvent effect is also important, but as we are focusing on the behavior of the k_{off} related to the dissociation process, the solvent is not present in the contact surface between the macromolecules. As concern to the local analysis, one may say that it points out the presence of more than one site with charge transfer capabilities; if one sees the toxin as a polydentate ligand one may expect that charge transfer is present in more than one site on the interface between the channel and the toxin. This qualitative picture is consistent with the fact that the channel mouth has also several possible electron donor sites. In addition, we can see in Figure 4 that the mutations analyzed do not change the shape of the electron acceptor surface of the toxin (green-blue region); indeed, the right bottom area (blue

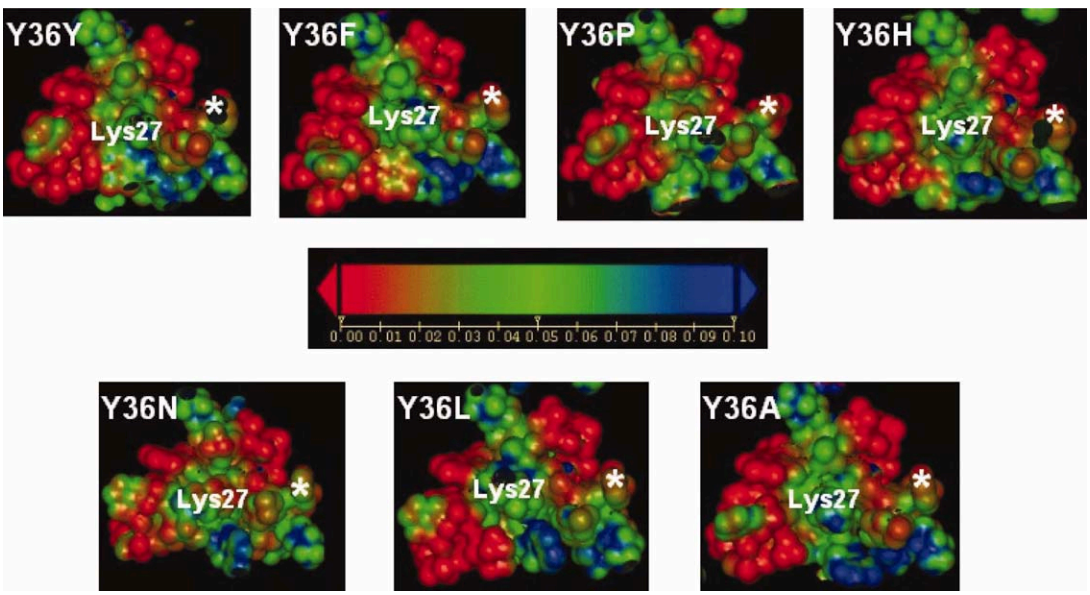

Figure 4. Local electrophilicity is shown as a projection onto a surface of overlapping spheres centered in the nuclei positions. The radius of the spheres is 1.5 Å. In order to have reference points, Lys27 is labeled and Met29 residues are identified by the symbol *. All systems are displayed with the same orientation that ChTX in Figure 1. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

regions) remains as the preferred site for accepting electrons for all mutants. This is in agreement with the fact that the key quantity to rationalize the changes in k_{off} is the global charge transfer capacity.

Concluding Remarks

Global softness shows an acceptable correlation to describe the affinity of the toxin to the channel, indicating that the acceptance of electronic charge by the toxin is an effect that plays a central role in the toxin–channel interaction. The local analysis reveals that the toxin has several possible sites for charge transfer; indeed, it may be seen as a generalized ligand that has several branches to interact with the channel. It is important to notice that, the impact of mutations on the reactivity of this particular system can be treated with empirical approaches, like those used to address the substituent effects in organic chemistry. Also, the combination of electronic derived concepts and geometrical parameters allows us to combine steric and electronic effects to enhance the quantitative description of the dissociation kinetic constant in this complex interaction. One may notice that, according to our results the dissociation process is strongly related to a soft–soft interaction. Besides the facts mentioned earlier, the main contribution of our work is to show that there are interactions between macromolecules in which electronic charge transfer processes are relevant, in addition to previously determined effects[20] such as hydrogen bonds and classical electrostatic interactions. Also, it is important to notice that the approach used in this work establishes a correlation between site directed mutagenesis experiments and electronic structure quantities.

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