Characterization of the AmtR signal Transduction-protein

Caracterización de la proteína transductora de señal AmtR

COLCIENCIAS TIPO 1. ARTÍCULO ORIGINAL
RECIBIDO: FEBRERO 12, 2013; ACEPTADO: MARZO 17, 2013

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Abstract

Each year, two million tons of amino acids are produced, most of them by application of *Corynebacterium glutamicum* in bioprocesses. Nowadays the amino acids industry is developing methods to increase the production yield. This development has integrated understanding of the elaborated mechanisms to provide an optimal nitrogen supply for metabolism and to overcome and survive situations of nitrogen limitation (Muhl, 2009; Hasselt, Sevvana, Burkovski, & Muller, 2009; Beckers et al., 2005). In this way the master regulator of nitrogen control called AmtR is responsible to block transcription of various genes when nitrogen is present in surplus. This work analyses the fascinating repression complex, in the sophisticated network of interaction that correspond to the relation of AmtR with the DNA in a homology model in 100 ns of molecular dynamics. Where the results revealed six amino acids located in a correct position in the binding zone that they permit to interact strongly with the DNA and the spectacular role of amino acid tyrosine in the protein binding.

Keywords

Amino acids industry; C. glutamicum; signal transduction-protein; nitrogen control; AmtR-DNA; TetR repressor; atomic fluctuations; regulated transcription.

Resumen

Cada año dos millones de toneladas de aminoácidos se producen, la mayoría de ellas por aplicación de Corynebacterium glutamicum en bioprocesos. La industria de aminoácidos está desarrollando métodos para aumentar el rendimiento de la producción. Este desarrollo ha integrado el entendimiento de los mecanismos elaborados para proporcionar un suministro óptimo de nitrógeno para el metabolismo y para superar y sobrevivir a situaciones de limitación de nitrógeno (Muhl, 2009; Hasselt, Sevvana, Burkovski, & Muller, 2009; Beckers et al., 2005). De esta manera, el regulador maestro de control de nitrógeno llamado AmtR es responsable de bloquear la transcripción de diversos genes cuando el nitrógeno está presente en exceso. Este trabajo analiza el fascinante complejo de represión, en la red sofisticada de interacciones, que corresponden a la relación de AmtR con el ADN en un modelo homólogo en 100 ns de dinámica molecular. Donde los resultados revelaron, seis aminoácidos situados en posición correcta en la zona de unión que permiten que interaccionen fuertemente con el ADN y el papel espectacular de aminoácido tirosina en la proteína de unión.

Palabras Clave

Industria de aminoácidos; C. glutamicum; proteína transductora de señales; control de nitrógeno; AmtR-ADN; represor TetR; fluctuaciones atómicas; transcripción regulada.

I. INTRODUCTION

During the last forty years, numerous scientists and research groups from Japan, the USA, South Korea, China and Europe, began to conduct researches in systems biology in several fields such as: Biological application in medicine, identification of novel drugs (Hood & Perlmutter, 2004) studying microbial systems and quantitative understanding of the total cellular system as well as its regulation, signal transduction (Takors et al., 2007), and industrial amino acid production that, is a billion dollar enterprise. Today, there seems to be no common definition of systems biology. Nevertheless, the concerted application of genome, transcriptome, proteome, fluxome and metabolome analysis for the quantitative, model-based analysis of microbial strains doubtlessly is a core element of all systems biology activities (Takors et al., 2007). The central dogma of Systems Biology in this assumption is that system dynamics and organizing principles of complex biological phenomena give rise to the functioning and function of cells (Wolkenhauer & Mesarovic, 2005). In this context simulations can provide the ultimate detail information as they can describe individual particle motions as a function of time (Karplus & McCammon, 2007).

In this work we employ comparative modeling and computer simulations of physical movements of atoms and molecules. The theoretical methods and computational techniques used are Homology Modeling and Molecular Dynamics (see next section). The aim of this work is creating three-dimensional protein models and predicts the trajectories of molecules and atoms in biological systems. In other words, in this work biological comportment of the AmtR signal transduction protein in complex with a specific DNA sequence are simulated.

A. Corynebacteria

Corynebacteria are immobile, non-spore-forming bacteria with a characteristic rod-shaped or sometimes curved or club-shaped with a tapered end; *they* are aerobically living, are Gram-positive organisms of the family of *Corynebacteriaceae*, they are containing mycolic acids. *Corynebacterium diphtheriae* is the oldest and best known representative of the family described as *Corynebacteriaceae.*

Its genome was sequenced in a 2003 research project on diphtheria a contagious disease spread by direct physical contact or inhalation of aerosolized secretions of infected individuals.

Corynebacterium glutamicum is a non-pathogenic, easily manageable and very well-studied model organism for the *actinomycetes. C. glutamicum* was discovered around 1957 by S. Kinoshita at Kyowa Hakko Kogyo Co., in Japan. Kinoshita isolated soil bacteria that produce large amounts of glutamic acid. Producing strains were found by inoculating soil isolates in a grid pattern on duplicate Petri plates. The colonies were permitted to grow and one set of duplicates was killed by UV irradiation. The deadened plate was coated with soft agar containing a Leuconostoc mesenteroides which require glutamic acid for growth; it only grew in the area of colonies that had excreted glutamate. Possible glutamate producers were then selected from the duplicate plate.

At present, the amino acid industry has occupied an important role in world chemical industries. More than 2 million tons of amino acids are produced per year, most of them by application of *coryneform* bacteria in bioprocesses. The annual market growth for most amino acids is 10% and higher (Hermann, 2003). In China where the industry production of Lysine amino acid is working at 98.5% of capacity, it is producing 510,000 ton/year (Table 1) *Corynebacterium crenatum* and *Corynebacterim pekinese* as well as *Corynebacterium glutamicum* is used in amino acid production process. Still the mechanism of amino acids accumulation of those mutants has not been widely investigated. Until now the strain improvement has mostly been carried out by an iterative process of mutagenesis and screening. Today is difficult to increase the production yield by these methods, genetic - and metabolic engineering offer a promising alternative.

Table 1. China's Major Feed Amino Acid Producers and Capacities, 2010 (Unit: ton/year) (Research in China, 2011)

Type	Producer	Capacity
Methionine	Chongging Unis Chemical	10,000[1].
Lysine (98.5% of capacity)	Changchun Dacheng Group	245,000
	Ningxia Eppen Biotech	116,000
	CJ (Liaocheng)	60,000
	Anhui BBCA Biochemical	46,000
	Shandong Shouguang Juneng Group Golden Corn	43,000
Threonine	Star Lake Bioscience. Zhaoqing Guangdong	30,000
	Changchun Dacheng Group	40,000
	Meihua Group	35,000
	Ningxia Eppen Biotech	15,000
	Shandong Fufeng	15,000
	Zhejiang Guoguang	12,000
	NB Group	10,000
Tryptophan	Zhejiang Shenghua Biok Biology	200
	Henan Julong Starch Industrial	500
	Shandong Lukang Pharmaceutical Group	2,000[2]
	Anhui BBCA Biochemical	1,000[3]
	Changchun Dacheng Group	1,000[3]

1. Methionine project phase I was successfully put into production in April 2010, and the rest 40,000 tons are under construction; 2. he actual capacity is 500 tons at present; 3. put into production in 2010)

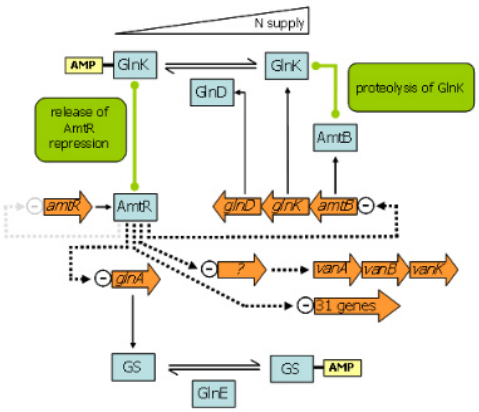
B. Nitrogen control in Corynebacteria glutamicum

Practically all macromolecules in a bacterial cell, e.g., proteins, nucleic acids and cell wall components, incorporate nitrogen. Therefore, prokaryotes have developed sophisticated mechanisms to provide an optimal nitrogen supply for metabolism and to surpass and survive situations of nitrogen limitation (Muhl, 2009; Hasselt et al., 2009; Beckers et al., 2005). In the case of nitrogen control in *Corynebacterium glutamicum*, a Gram-Positive soil bacterium used for the industrial production of amino acids (Takors et al., 2007) (see above), the transcription of genes in answer to nitrogen limitation is governed by the master regulator of nitrogen control, called AmtR, which blocks transcription of diverse genes during growth in nitrogen-rich medium (Muhl, 2009).

However the refined network of protein-protein interaction, modification and protein degradation shows that, when nitrogen is actual in surplus, nitrogen regulated genes are repressed by binding of protein to the promoter regions. Under nitrogen limitation, the relations of AmtR with adenylylated GlnK leads to the liberation of the repressor from the DNA and the expression of the protein AmtR-controlled genes. When the nitrogen levels are sufficiently elevated, the signal is turned off by deadenylyation of GlnK.

In this manner GlnK can't interact with AmtR but instead with AmtB which results after in the degradation of the signal transduction protein (see the network process of protein-protein in Figure 1).

Figure 1. Schematic representation of nitrogen control mechanisms in *C. glutamicum*. (Burkovski, 2007)



In this figure, crucial components of nitrogen regulation are shown as green squares. Orange arrows, connected with AmtR by black dotted lines, represent AmtR-controlled genes. A putative autoregulation of AmtR based on the presence of an AmtR binding motif could not be shown (grey dotted line and question mark). The vanABK operon is regulated indirectly by AmtR, the direct regulator (arrow with question mark) has not been identified. Interaction (grey lines with circles) of GlnK~AMP and AmtR is crucial for release of AmtR repression, while interaction of GlnK with AmtB leads to its degradation. Adenylylation/deadenylylation of GlnK and glutamine synthetase (GS) by GlnD and GlnE depends on nitrogen supply. The signals for modification/demodification are unknown.

C. AmtR signal transduction-protein as the master regulator of nitrogen control in Corynebacteria glutamicum

AmtR is a member of the Tetracycline repressors family of transcriptional regulators. Typically, these proteins control transcription basing on the binding of small effector molecules, example: antibiotics like tetracycline. Interestingly, AmtR binding upstream of nitrogen-regulated genes is administered by protein complex formation (Figure1).

The first fascinating repression complex, in the sophisticated network of interaction, corresponds to the relation of AmtR with the DNA, where the physiological function has been very well studied, but the characterization of the mechanistic aspects of AmtR-regulated transcription has yet to be accomplished (Hasselt et al., 2009). An attempt to explain this interaction by the analysis of a static three-dimensional model of AmtR-DNA binding domain revealed several amino acids, which are directly implicated in DNA binding, while others have more structural function. Still the mechanism, by which the protein stabilization or destabilization will reduce or increase DNA binding affinity, cannot unambiguously be determined from the static model structure (Muhl, 2009).

In this work a homology model of the complex AmtR-DNA in 100 ns of molecular dynamics was analyzed, and confirmed the existence of DNA binding site CTAT predicted by bioinformatics analyses. Such a complex was already verified by RNA hybridization analyses, real-time RT PCR and gel retardation tests (Beckers et al., 2005). This work revealed twenty amino acids in the binding zone AmtR protein-DNA, in the model integrated by AmtR dimer protein in complex with a DNA duplex, modeled using homology modeling and predictive techniques, four amino acids with strong affinity, directly responsible of the

interaction in the DNA binding site CTAT (Beckers et al., 2005) in the first monomer, and TGAT in the second, and also two amino acids with potentials characteristics for it cause stability or instability in the complex formation. The last ones are structurally located in the alpha helixes three and adopt positions in the space depending on a special role by virtue of the phenol functionality.

Based on simulations of the mutated models of free and complex protein a profound analysis of atomic fluctuation, distances between atoms and electron donor and acceptor was carried out. This analysis revealed the spectacular role of amino acid Tyrosine in the protein binding. When the phenol adopts a horizontal position respect to DNA, the high electrostatic surface causes repulsion and triggers protein destabilization. On the other hand, when phenol adopts a vertical position, the hydrogen in the alcohol group is attracted by the oxygen in the DNA phosphate group causing stabilization.

This analysis could be the first key for understanding how the AmtR signal transduction protein lost affinity with the DNA under nitrogen limitation. It provides information how to obtain the correct attraction or repulsion contributions that will be necessary to simulate in the near future with the induction mechanism, using the homotrimer complex Adenosine monophosphate (AMP) with the GlnK protein signal (Nolden et al., 2001).

II. METHODS

A. Basic local alignment search tool [BLAST]

Blast locates regions of local similarity between sequences. The program compares nucleotide or protein sequences to sequence databases and evaluates the statistical significance of matches. *Blast* can be used to deduce functional and evolutionary relationships between sequences as well as help recognize members of gene families.

Specifically, in Amino Acid similarity, the Blast program represents the similarity between amino acids as a $\log_2$ odds ratio, also known as a *lod score*. To derive the lod score of an amino acid, take the $\log_2$ of the ratio of a pairing's observed frequency divided by the pairing's random expected frequency. If the observed and expected frequencies are equal, the lod score is zero. A positive score indicates that a pair of letters is common, while a negative score indicates an unlikely pairing. The universal formula for any pair of amino acids is:

$$S_{ij} = log\left(\frac{q_{ij}}{p_i p_j}\right)$$

Where the score of two amino acids i and j, is S_{ij}, their individual probabilities are p_i and p_j, and their frequency of pairing is q_{ij} (Korf, Yandell, & Bedell, 2003).

B. Blocks Substitution Matrix [BLOSUM]

Blosum matrix is an amino acid substitution matrix. For its calculation only blocks of amino acid sequences with tiny change between them are considered. These blocks are called conserved blocks.

The first important reason for this is that one needs to find a multiple alignment between all these sequences and it is easier to create such an alignment with more similar sequences.

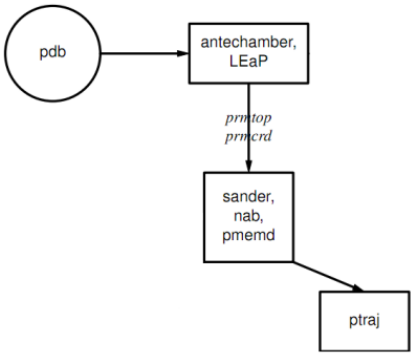
The *Blosum* matrices were constructed by extracting ungapped segments, or blocks, from a set of multiply aligned protein families, and then other clustering these blocks on the basis of their percent identity. The blocks normally used to derive the *Blosum62* matrix.

C. Assisted Model Building with Energy Refinement [AMBER] simulations Package

Amber is the collective name for a suite of programs that allow users to carry out molecular dynamics simulations, predominantly on biomolecules. None of the individual programs carries this name, but the variety of parts work reasonably well together, and provide a powerful framework for many common calculations (Pearlman et al., 1995; Case et al., 2005). Amber refers to two things: a set of molecular mechanical force fields for the simulation of molecules and biomolecules (which are in the public domain, and are used in a variety of simulation programs); and a package of molecular simulation programs which integrates source code and demos.

In the reality the basic work flow in *Amber* use Cartesian coordinates for each atom in the system. These usually derive from X-ray crystallography, NMR spectroscopy, or model-building. Topology: connectivity, atom names, atom types, residue names and charges. Force field: parameters for all of the bonds, angles, dihedrals. And atom types in the system and commands: the user specifies the procedural options and state parameters desired (see Figure 2).

Figure 2. Basic information flow in Amber (Case et al., 2010)



In the figure, the flow is showing the Information that all the simulation programs need. The first step is load the *pdf file* from the Protein Data Bank in LEaP with the purpose of create two files *.top* and *.crd* called topology and coordinates, then standard parameters for several force fields are used and several states parameters are loaded, in order to analyze the information using Ptraj program in the end.

1) Force field ff99

The ff99 force field points toward a common force field for proteins for *general* organic and bio-organic systems. The atom types are mainly those of Cornell et al, but changes have been completed in several torsional parameters. The ff99 force field uses these parameters, along with the topologies and charges from the Cornell et al. force field, to create an all-atom nonpolarizable force field for proteins and nucleic acids.

2) LEaP

LEaP is a module from the AMBER suite of programs, which can be used to generate force field files compatible with NAB (Nucleic Acid Builder). The name LEaP is an acronym of stands for Link, Edit, and Parameters. An enhanced version of the program called xLEaP is also available that supports an X-windows graphical interface.

To continue with the mentioned above, the parameter file, which comprises all information about bonds, angles, torsions, and the non-bonded parameters, is usually loaded from an appropriate leaprc file, which loads the information needed for a specific force field.

In the case of Amino Acids Residues for each one found in the LEaP libraries, there has been created an N-terminal and a C-terminal analog. The N-terminal amino acid UNIT/RESIDUE names and nicknames are preceded by the letter N and the C-terminal amino acids by the letter C. And in the case of Nucleic Acids Residues *D* or *R.* prefixes can be used to differentiate between deoxyribose and ribose units; with the default leaprc file, vague residues are supposed to be deoxy. Residue names like *DA* can be followed by a *5* or *3* (e.g., *DA5*, *DA3*) for residues at the ends of chains (Macke, et al, 2011).

3) Sander

Sander is a molecular dynamics program and the basic energy minimizer. This program relaxes the structure by iteratively moving the atoms down the energy gradient until a sufficiently low average gradient is obtained. The name Sander is an acronym of stands for *Simulated Annealing with NMR-Defined Energy Restraints*, and its molecular dynamics fraction generates configurations of the system by integrating Newtonian equations of motion.

The principal function is allows for NMR refinement based on NOE-derived torsion angle restraints, distance restraints and penalty functions based on chemical shifts and NOESY volumes.

4) Ptraj

Ptraj is a general intention utility for processing and analyzing trajectory or coordinate files created from MD simulations (or from various other wherewithal), carrying out extractions of coordinates, superpositions, calculation of bond/angle/dihedral values, atomic positional fluctuations, correlation functions, analysis of hydrogen bonds, etc. (Macke et al., 2011).

Ptraj is considered the best tool used to analyze MD trajectories, computing a variety of things, like RMS deviation from a reference structure, hydrogen bonding analysis, time-correlation functions, diffusional behaviour, etc. So its multiple analyses are used and explained deeply in the next section: Results.

D. Sampling

As I had written in previous section, Homology Modelling is currently the most popular method for predicting the three-dimensional structure of a protein from its sequence. In this way, when doesn't exist the experimental data the *sequence alignment* and *model building* are the necessaries steps for predict an initial protein model.

The next subsection are looking explain where the models of protein, DNA and complex DNA-protein come from.

1) Protein Modelling

The structure of AmtR signal transduction protein from *Corynebacterium glutamicum* was modelled based on the crystal structure of TetR repressor/operator complex from E. coli. (Muhl, 2009). (Protein Data Bank code: 1QPI). Where in the case of TetR each monomer contains 203 Amino acids and 1551 atoms in the follow sequence (FASTA):

LNRESVIDAALELLNETGIDGLTTRKLAQKLGIE
QPTLYWHVKNKRALLDALAVEILARHHDYSLPAA
GESWQSFLRNNAMSFRRALLRYRDGAKVHLGTRP
DEKQYDTVETQLRFMTENGFSLRDGLYAISAVSHF
TLGAVLEQQEHTAALENLPPLLREALQIMDSDDG
EQAFLHGLESLIRGFEVQLTALLQ.

On the other hand, each monomer of AmtR protein contains 201 Amino acids and 1562 atoms organized in 10 alpha helices with the follow sequence (FASTA):

NPREEILDASAELFTRQGFATTSTHQIADAVGIR
QASLYYHFPSKTEIFLTLLKSTVEPSTVLAEDLSTLD
AGPEMRLWAIVASEVRLLLSTKWNVGRLYQLPIVG
SEEFAEYHSQREALTNVFRDLATEIVGDDPRAELP
FHITMSVIEMRRNDGKIPSPLSADSLPETAIMLADAS
LAVLGAPLPADRVEKTLELIKQA.

The alignment of TetR with AmtR using the algorithm blastp (protein-protein BLAST) in a matrix BLOSUM62, it confirmed that Both proteins share a sequence identity of 37% in the DNA binding domain (Muhl, 2009) (Figure 3).

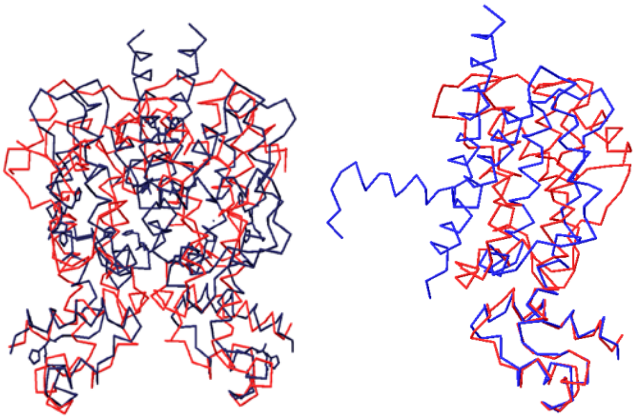
Figure 3. Align sequences protein Blast applied to TetR and AmtR proteins

```
>lcl|64171 unnamed protein product
Length=201

 Score = 38.1 bits (87),  Expect = 9e-08, Method: Compositional matrix adjust.
 Identities = 16/43 (37%), Positives = 26/43 (60%), Gaps = 0/43 (0%)

Query  3   RESVIDAALELLNETGIDGLTTRKLAQKLGIEQPTLYWHVKNK  45
           RE ++DA+ EL    G    +T ++A  +GI Q +LY+H  +K
Sbjct  3   REEILDASAELFTRQGFATTSTHQIADAVGIRQASLYYHFPSK  45
```

The figure shows the alignment result of two proteins (TetR and AmtR), where the algorithm could identify 37% of identities in the amino acids: Arginine (R), Glutamic acid (E), Aspartic acid (D), Alanine (A), Leucine (L), Glycine (G), Threonine (T), Isoleucine (I), Glutamine (Q), Tyrosine (Y), Histidine (H) and Lysine (K).

The sequence of this amino acids was located in the DNA binding domain (Figure 4).

Figure 4. Schematic view of the fit of TetR-AmtR proteins

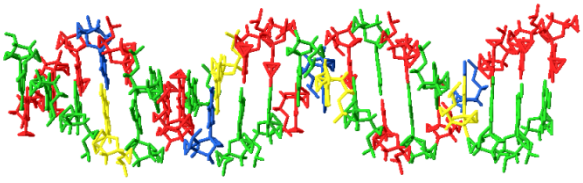


The TetR repressor/operator is represented in blue and the AmtR signal transduction in red. The model on the right side shows both proteins in monomers, and we can see the important similarity identified in 37% in the bottom of this model. The model on the left site shows the natural conformation in protein dimers.

2) DNA Modelling

The DNA (Watson-Crick duplexes based on fibre-diffraction data), was generated using fd_helix() routine in Nucleic Acid Builder (NAB) included as part of the AmberTools package in a helix type *abdna* (Right Handed B-DNA (Arnott). The DNA model contain: 40 nucleotides, 20 base pairs and 1276 atoms organized in two helical chains, with the sequence: TTTCTATAGATTGATAGAAA (Figure 5).

Figure 5. Schematic view of DNA duplex



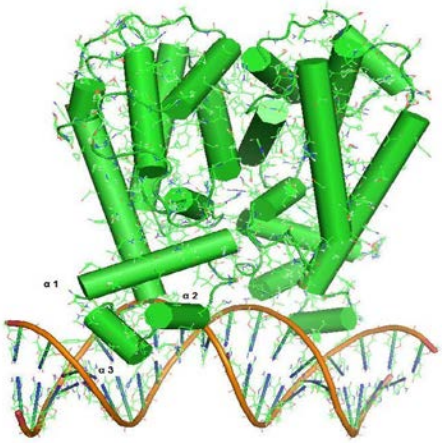
The nucleotides are represented in this model by a different colors, i.e. Adenine is green, Thymine-red, Guanine-yellow and Cytosine-blue. Each base pair has an X-offset of 2.25 Å, an inclination of -4.96 Å and a helical step of 3.38 Å rise and 36.0o twist. The first character of sequence is the 5' base of the strand *sense* of the molecule returned by bdna(). The other strand in the sequence is called *anti*. The phosphates of the two 5' bases have been replaced by hydrogens and hydrogens have been extra to the two O3' atoms of the three prime bases (Macke, 2011).

The DNA sequence was copied from the maxima score value in an experimental procedure with the AmtR binding sequence of 17 gene/operon and from the AmtR binding consensus motif represented by the highly conserved positions in the region CTET (see: Beckers et al., (2005); Burkovski (2007); and Hasselt, Rankl, Worsch, & Burkovski (2011), for more details).

3) Complex modelling

The complex construction was achieved, orienting the alpha helices 1, 2 and 3, from the each dimer, with the DNA sequence CTAT and the corresponding base pairs ATAG in concordance with the Hydrogen bonds theory (Kollman & Allen, 1971) and the Van der Waals forces (Tang & Toennies, 2010) (Figure 6).

Figure 6. Schematic view of the AmtR dimer as α-carbon trace



The ten α-helices of each monomer are displayed as green cylinders. The orientation of α-helices 1, 2 and 3 respect to the CTAT sequence was in a maximum distance of 2 Å.

The quality of each structure, including the complex, was visualized and explored using Swiss-Pdb Viewer V4.0.1 form the Swiss Institute of Bioinformatics, VMD 1.9 from Beckman Institute for Advanced Science and Technology University of Illinois and PyMOL Molecular Graphics System Version 1.3 from Schrodinger LLC, with the purpose of detect some steric clashes or unfavourable geometries.

E. Computational Details

All molecular dynamics simulations were conducted using the AMBER 10 (Pearlman et al., 1995) suite with the force field parameters (parm99) of Cornell et al. The calculations were performed in three groups: the AmtR protein, the DNA and the Complex DNA-AmtR.

The AmtR protein and the complex were solvated with ~10190 TIP3PEW water molecules (H_2O) in a truncated octahedron box 8.0, (8 angstrom buffer of TIP3P water around the molecule in each direction) also was add ~52 Sodium ions (Na+) located around the complex to obtain electrostatic neutrality and Ions parameters for TIP3P water: frcmod.ionsjc_tip3p. The specific force fields were integrated with the Amber 10 command leaprc.ff99bsc0 that contains the force fields parameters: parm99.dat, frcmod.ff99SB, and frcmod.parmbsc03

On the other hand the DNA free was exposed at the same parameters, but for this case, as DNA is a cylindrical molecule, it was necessary to solvate in 8 angstrom buffer of TIP4PEW water around the DNA, and Particle Mesh Ewald were used too. In terms of reducing the problem of solute rotation, the addition of frcmod.tip4pew solvent parameter model and the ions parameters frcmod.ionsjc_tip4pew were configured.

All groups were subsequently refined independently in three files by 2000 steps of energy minimization, full conjugate gradient minimization in 500 cycles. (The first four cycles are steepest fall at the start of the run and after every nonbonded pairlist update), periodic boundary at constant volume, NO Flag for restraining specified atoms in Cartesian space using a harmonic potential and the default value of 8.0 used to specify nonbonded cutoff, in Angstroms.

The AmtR protein was configured with generic Molecular Dynamics (MD) heat up without any restraints, moreover, the DNA and Complex system were configured for restraining specified atoms in Cartesian space using a harmonic potential, and equilibrates on the first molecular dynamics simulation (1000 ps) with temperature of 300 K and pressure of 1 bar. The inclusion of distress applicably at the base pairs into the DNA sequence and the monomers structures were also configured.

III. RESULTS

The results described here, are used to determine macroscopic thermodynamic properties in three groups of specifics system (see above) and it can predict the future by animating nature's forces (Schlick, 1996).

A. Dynamic Trajectory in Root-Mean-Square Deviation (RMSD)

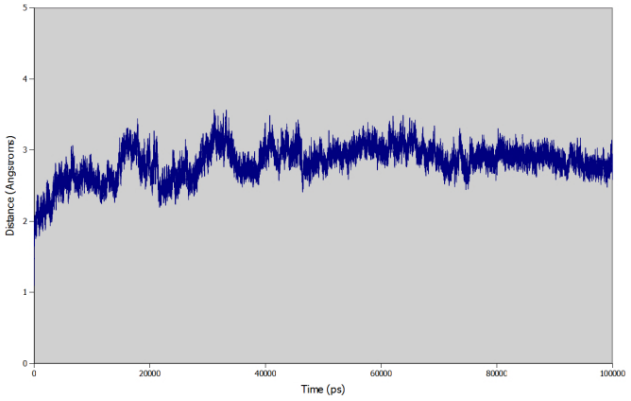
The root-mean-square deviation (RMSD) is used to quantify the geometrical difference of two structures by comparing pairs of atoms (Seidel, 2007). To achieve a minimum RMSD requires superimposition of the structures first, for which several methods are available e.g. one common algorithm has been described by Kabsch (1978). Subsequently the RMSD can be calculated using the equation:

$$RMSD = \sqrt{\frac{1}{n}\sum_{i=1}^{n} d_i^2}$$

Where n is the number of atoms involved in the RMSD measurement and d_i is the remaining distance resulting from the differences in the coordinates between the atom pair *i* after fitting (Seidel, 2007).

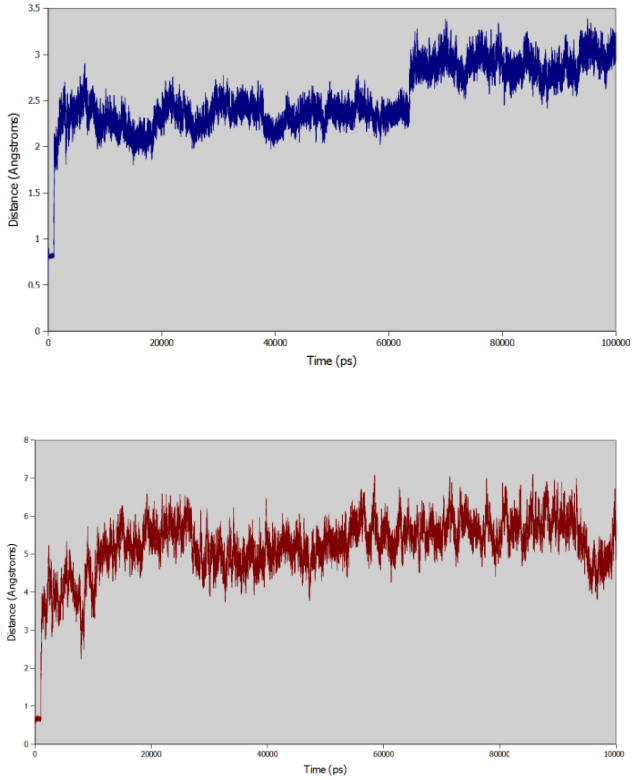
The Molecular Dynamic data concerning to the AmtR protein in independent truncated octahedron box was analyzed calculating the dynamic trajectory expressed in Rot-Mean-Square Deviation (RMSD) fit (Figure 7).

Figure 7. RMSD plot relative to the AmtR protein in independent water box



The plot shows the dynamic trajectory of AmtR protein free in a total time of 100 ns. At the beginning of the simulation (8 ns) the RMSD curve increases slowly, and after there, begins a fluctuation between 1.3 Å up 45 ns, then the simulation continuous very stable fluctuating less than 1 Å up the termination of the simulation. In another way the Molecular Dynamic data concerning to the complex (AmtR protein-DNA) in a truncated octahedron box, also was analyzed calculating the dynamic trajectory expressed in Rot-Mean-Square Deviation (RMSD) fit to the Protein (Figure 8, above), and non fit to the DNA (Figure 8, below).

Figure 8. RMSD plot relative to the AmtR protein and DNA in a complex



The plots are showing the dynamic trajectory of the complex in total time of 100 ns. The start of the system is indicated by restraints applied to the firsts 2.5 ns in booths plots. For the case of AmtR protein in complex (above), the beginning of the simulation (20 ns), is marked by the fluctuation in 1.5 Å, but after there up 65 ns, this distance is less (0.8 Å). The analysis also shows a jump in 65 ns that causes a significant variation in the trajectory without affecting the stability; this particular case is discussed in the next section.

The second plot (below) shows very high atomic variations in the DNA in complex, where in the beginning the simulation start in 4.5 Å, but it can achieve more stabilization (2.0 Å) after 25 ns. At this point is important to note that all simulations are stable, therefore, these are important for the subsequent next analyses, but some analysis showed here, obey to sections at time where the AmtR protein, DNA or Complex have more stability.

B. Atomic fluctuations

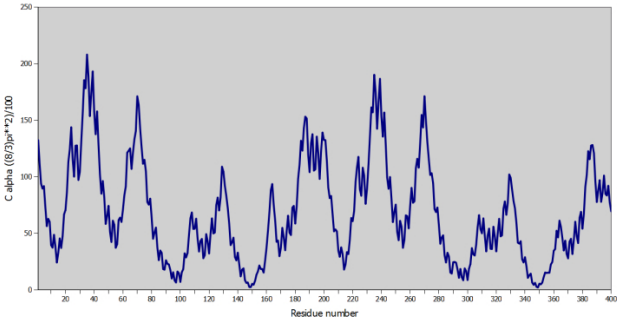
To compute the atomic positional fluctuations for alpha carbons, is relative to Debye-Waller factor or B-factor determined by X-ray crystallography. The isotropic atomic B-factor is given by:

$$B_i = \left(8^2/_3\right) < -R_i^2 >$$

Where, in the context of protein structures, the expression $< -R_i^2 >$ is the atomic mean square displacement of the i[th] atom.

The next plots (Figures 9 and 10) are indicating the relative vibrational motion of different parts of the AmtR protein in two types: free and in complex. Normally the analysis of this plots give information about molecular flexibility and can be a good start point to explore binding sites in a complex systems.

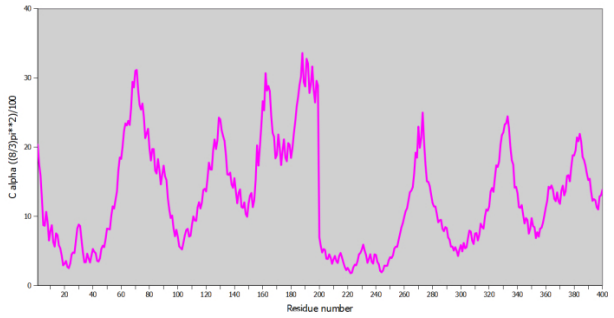
Figure 9. Atomic fluctuations to alpha carbon atoms in an AmtR protein free



The Figure 9 shows the atomic positional fluctuations for alpha carbons applied to an AmtR protein dimer of 400 residues, where in general booths monomers of 200 residues are demonstrating high flexibility. The maximums values are attributed to the residues 35 in the first monomer and 235 in the second one, wherever also shows some regions located between residues 90 up 102 and 140 up 160 for the first monomer and 290 up 302 and 345 up 355 for the second with low or very low flexibility.

The next plot (Figure 10) shows the same AmtR protein dimer, but with the DNA influence, where the 400 residues are demonstrating more low flexibility. The minimums values between the amino acids residues 18 up to 24 for the first monomer and 218 up to 224 and 243 up to 245 for the second correspond to the affinity with the binding site CTAT predicted by bioinformatics analyses (Beckers et al., 2005).

Figure 10. Atomic fluctuations to alpha carbon atoms in an AmtR protein complex



The charts also show the flexibility that is completely different in the first monomer with respect to the second, but is very similar in the alpha carbon that has direct relationship with the binding zone; this is the best start point to calculate the distances between atoms, because during all molecular dynamics, the maximum affinity residue-nucleotide is present there.

C. Electron donors and acceptors

In *Ptraj* the definition of donors and acceptors is reversed with respect to standard conventions; the *acceptor* is bonded to the hydrogen and the *donor* is the atom the hydrogen bond is formed (i.e. in ptraj a *donor* can be thought of as *donating* electrons to the hydrogen atom). This has not been changed in order to preserve backwards-compatibility (Macke, 2011).

In this way the system was configured using the donors and acceptors list from the standard amino acids and standard nucleotides, and then organized depending of the bond stability in each distance, during 50 ns of simulation time in the stable zone.

The Table 2 shows the results of the best marks of hydrogen bonds in the binding zone between AmtR protein and DNA.

All interactions correspond in its most, to hydrogen bond between any electronegative atom (in this case oxygen) located in the phosphate group, with any hydrogen atom.

The analysis ran each 50 sets, because this process consume high Random-Access Memory (RAM), also intra-residue interactions were not included and distance cutoff was 3.00 angstroms.

Table 2. List of electron donors and acceptors, organized by % occupied

Donor		Hydrogen Acceptor		% Occupied
Atom	res@atom	Atom	res@atom	
6637	413@O1P	3827	245@HG1	97.90
6927	422@O2P	3724	239@HH	83.10
7305	434@O2P	574	38@HH	70.10
6606	412@O2P	3477	223@HG1	57.20
7243	432@O2P	348	23@HG1	56.60
6638	413@O2P	3703	238@HH	42.10
6605	412@O1P	3813	244@HZ3	38.60
6991	424@O2P	3629	233@HH22	37.90
6991	424@O2P	3626	233@HH12	31.40
6605	412@O1P	3811	244@HZ1	29.50
6381	405@O2P	500	33@HH22	28.60
6605	412@O1P	3812	244@HZ2	26.70
7273	433@O2P	683	44@HZ2	26.20
6959	423@O2P	3744	240@HE2	24.10
6381	405@O2P	497	33@HH12	22.30
6319	403@O2P	615	40@HE2	22.30
7273	433@O2P	684	44@HZ3	20.60
7273	433@O2P	682	44@HZ1	20.30

D. Distances analyses

The distances were calculated using the distance command as implemented in the ptraj tool of the AMBER suite as a function of time, where the total time of 50 ns corresponds to the stable region in the RMSD fit to alpha carbons in AmtR protein complex (see Figures 7 and 8).

Only the affinity zone in a distance less to 3 angstroms between protein-DNA was considered, some others protein-protein or base pairs interactions are not included.

The first group of hydrogen bonds corresponds to the interaction of nucleotide Guanine 413, with the amino acids Threonine 245 (THR 245) and Tyrosine 238 (TYR 238).

The first plot (see Figure 11, above) is showing a constant signal in a distance ~1.7 Å that represents the distance between the oxygen atom 1P in Guanine with the hydrogen atom G1 in THR.

Different situation is shown in the next plot (see Figure 11, below) where in the beginning, the interaction Oxygen atom 2P with hydrogen atom H in TYR is ~4.5Å, but in the value of 27.5 ns can equilibrate in ~2.7Å. Both interactions are also shown in the average view of 50 ns located above in the Figure 11.

Moreover, the next amino acid TYR 239 goes in other direction and its interaction is in the opposite site of TYR 238, is for this that both amino acids could be considered responsible of the binding on or binding off between Protein and DNA (see next section).

Figure 11: Distances between the first and second oxygen, located in the phosphate group in Guanine 413, with the Hydrogen G1 in THR 245 (above) and Hydrogen H in TYR 238 (below)

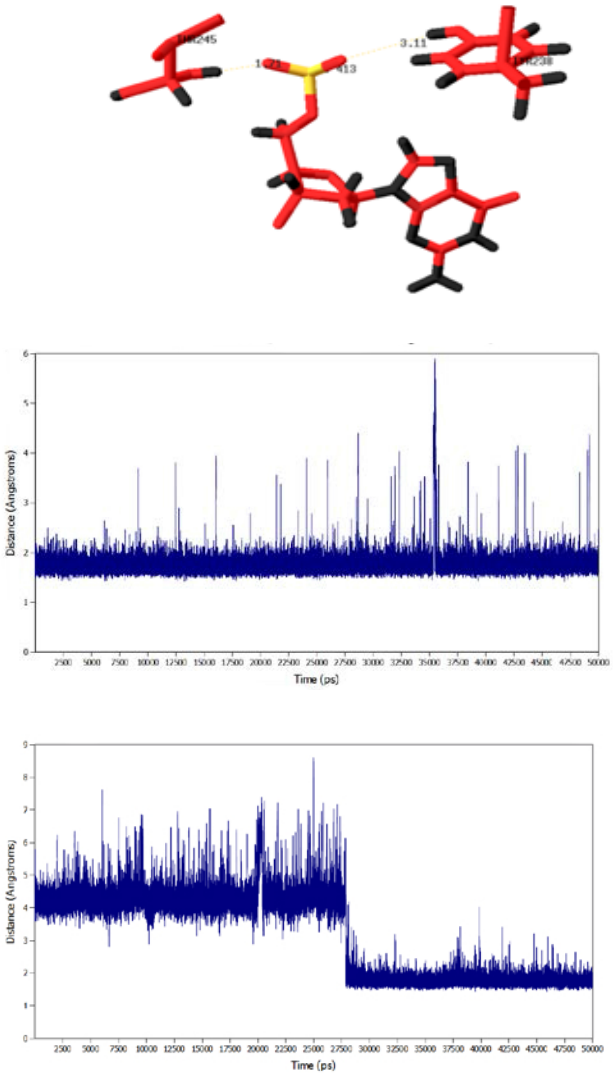
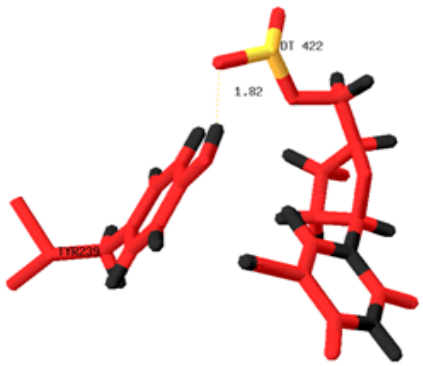


Figure 12. Distance between the second oxygen, located in the phosphate group in Thymine 422, and the Hydrogen H in TYR 239



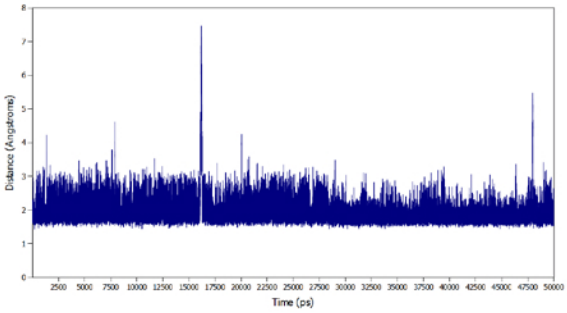
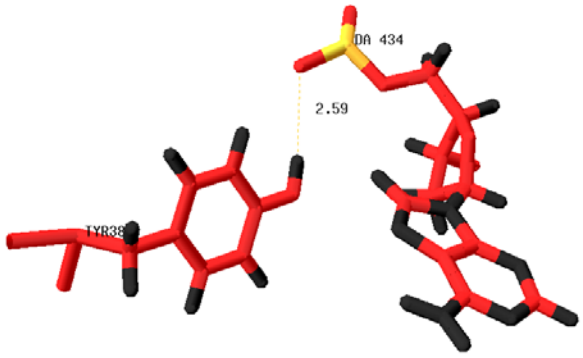
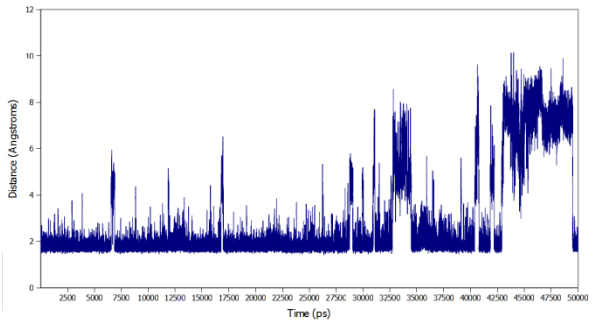


Figure 12 shows a constant signal in a distance ~1.8 Å that represent the distance between the oxygen atom 2P in Thymine 422, with the hydrogen atom H in TYR 239. Also compared with the beginning of the plot, in the range between 27.5 and 50 ns the atomic variations are less.

The schematic view taken from the PDB average in the last 50 ns (see Figure 12 above) shows at TYR 239 in a horizontal position in respect of base pairs in Thymine, achieving an interaction distance of 1.82 Å.

Figure 13. Distance between the second oxygen, located in the phosphate group in Adenine 434 and the Hydrogen H in TYR 38

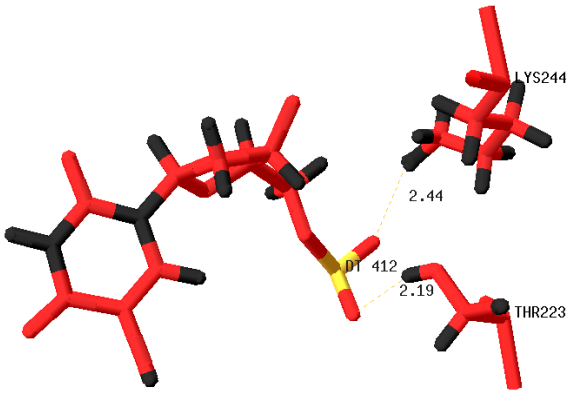


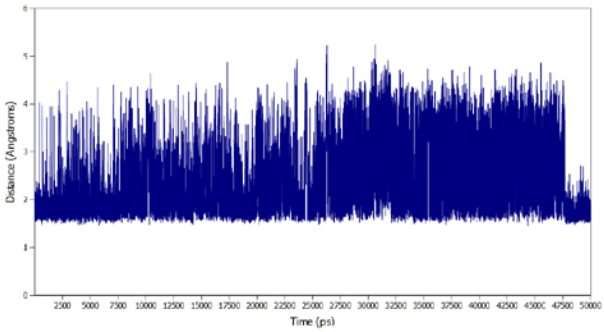


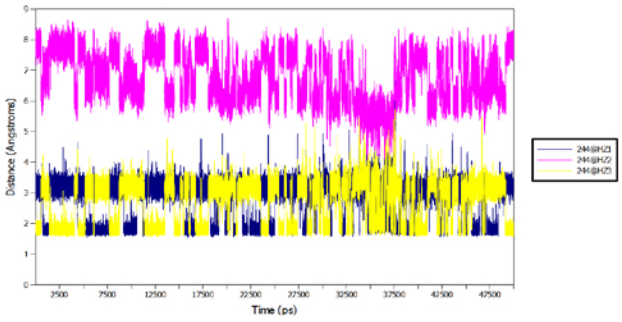
On the other monomer, the Hydrogen atom H in TYR 38 is attracted in the beginning (0 up ~32.5 ns) by the Oxygen atom 2P in Adenine 434 in a constant distance approximately of 2 Å, but from here, the affinity is lost in ~6 Å by ~2 ns. The distances returns to 2 Å during others ~6 ns, but after here, the amino acid can't stabilize and again shows lost affinity between ~43 and ~49 ns.

The schematic view (Figure 13 above) shows TYR 38 quasi perpendicular to the base pairs in Adenine 434 in an average of distance of 2.59 Å, influenced by the high values showed in the end of the plot.

Figure 14. Distances between the first and the second oxygen, located in the phosphate group in Thymine 412, with the Hydrogen G1 in THR 223 (above) and Hydrogen's Z1, Z2, Z3 in LYS 244 (below)







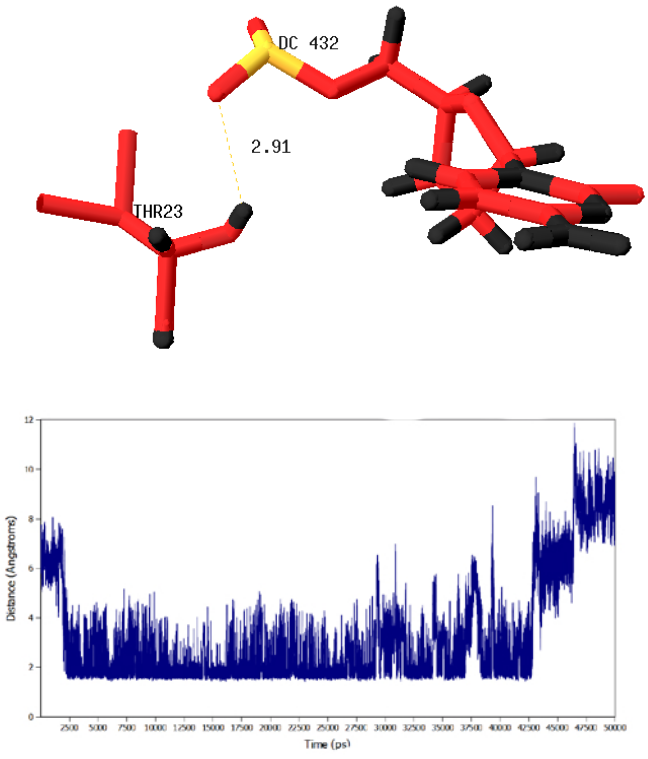
Sometimes only one phosphate group located in a nucleotide can interact with two amino acids using both oxygen atoms in different ways. This example is the case of the Figure 14, where the oxygen atom 2P in Thymine 412 is showing affinity with the hydrogen atom G1 in

THR 223 in a value of ~2.19 Å (see schematic view above Figure 14). However, this atomic interaction could be not very stable, because it shows large discontinuity in the signal represented in the plot (Figure 14).

The other oxygen atom, called 1P in Thymine, is interacting with three hydrogen atoms located in the amino acid Lysine 244 (see below Figure 14) where the best values are assigned to the hydrogen atoms HZ1 and HZ3 which are fluctuating between 2.5 Å.

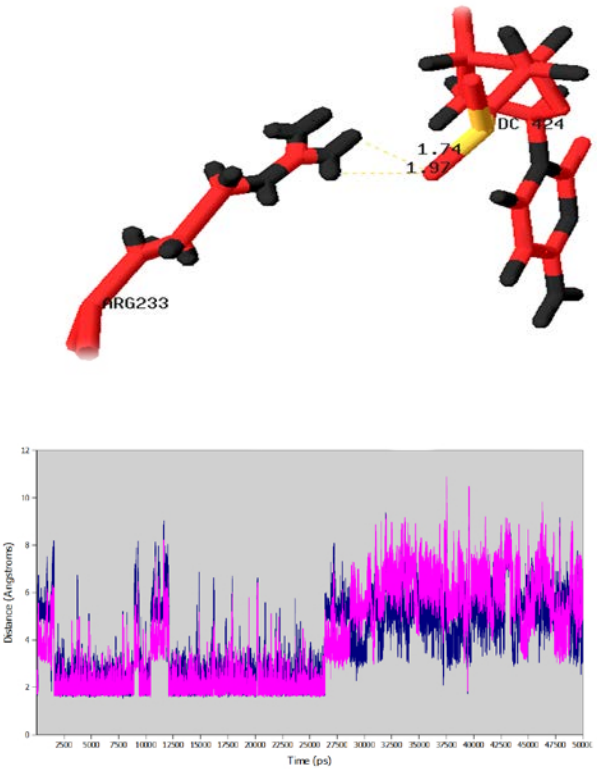
The hydrogen HZ1 (blue signal) frequently loses affinity during the analysis, after the hydrogen HZ3 (yellow signal) automatically occupies the place and so on. A perfect example is observed between ~1.2 and ~5.5 ns, where the best affinity is attributed to hydrogen HZ3, but after that the affinity is lost and retaken by hydrogen HZ1 up to ~7.5 ns. Otherwise is the hydrogen atom HZ2 (pink signal) that shows a very large distance demonstrating no interaction.

Figure 15. Distance between the second oxygen, located in the phosphate group in Cytosine 432 with the Hydrogen G1 in THR 23



The Figure 15 shows a schematic view taken from the average of 50 ns (see above), where the hydrogen atom HG1 belonging to THR 23 is attracted from the oxygen atom 2P belonging to a phosphate group in cytosine 432 in a average distance of 2.91 Å. Also the view is accompanied of a plot that represents the atomic variation in a same time (see below). The signal in this case is observed to be very high in the firsts ~2 ns, but it goes down during the next ~43 ns and afterwards goes up again.

Figure 16. Distance between the second oxygen, located in the phosphate group in Cytosine 424 with the Hydrogen's H22, H12 in ARG 233



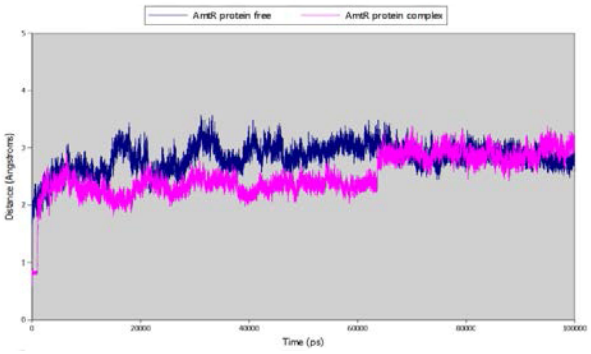
Particular importance is observed in the comparison of two hydrogen atoms located in Arginine 233 (ARG 233) (Figure 16), where the atomic signal shows a very similar comportment in the region between ~2 and ~27 ns, that reflect a distance of ~2 Å, afterwards the hydrogen bond disappears.

IV. Discussion

As was shown above in the beginning of results, the dynamic trajectory analysis applied to AmtR protein in complex (Figure 8) is showing a signal jump, in a value time of 65 ns. This variation could be attributed to problems with boundary effects caused by long calculations, large proteins or multiples restart files. But after exhaustive analysis it could be established that the initial periodic boundary conditions were configured centred in image origin box, using as reference the first

image after energy minimization. Therefore the jump in the signal corresponds to system stabilization, where the AmtR protein in complex goes extremely fast to the primary box image, simulating free comportment (see Figure 17).

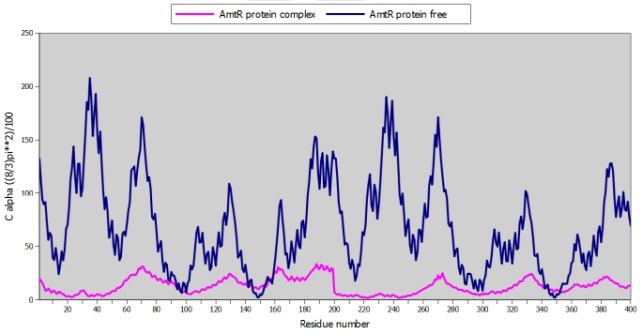
Figure 17. Comparative plot of Dynamic Trajectory expressed in RMSD for AmtR protein complex (pink) and free (blue)



However, is important to note that the jump and the similar dynamic trajectory after 65 ns, between AmtR protein in complex and free, it has not affected the atomic interactions DNA-Protein and also a profound visual exploration of trajectory files using principally VMD software, it has not revealed significant changes in the affinity zone.

The majority of the analyzed interactions that represented atomic distances less to 3 Å were considered with a strong effect to the protein atomic fluctuation, because the AmtR protein free showed high values in very flexible amino acid sequences in both monomers.

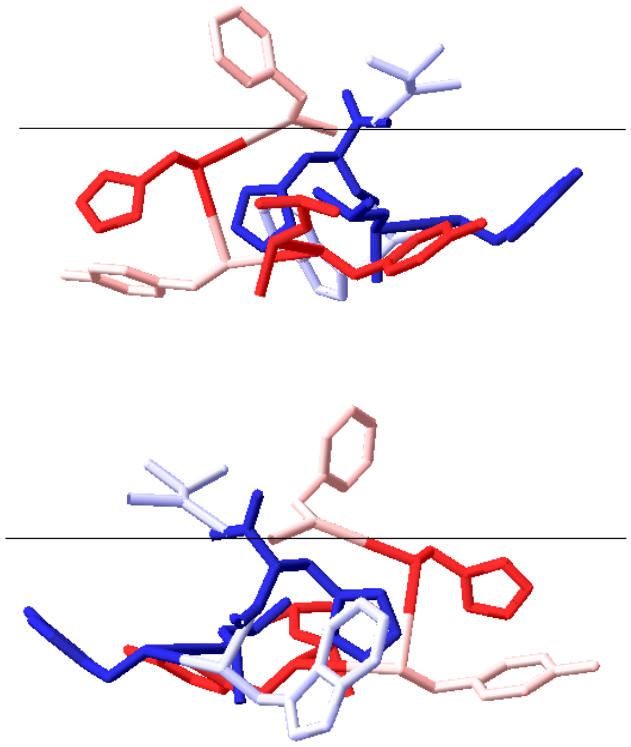
Figure 18. Comparative plot of Atomic Fluctuations in alpha carbons for AmtR protein complex (pink) and free (blue)



The analysis of a comparative plot of atomic fluctuations between protein in complex and free (Figure 18), also revealed two affinity zones between the amino acids number 20 up to 40 for the first monomer and ~220 up to 250 for the second, but not all amino acids located in the previous zones are responsible for the protein stabilization in a low value of atomic fluctuations.

Some structural amino acids are close to the affinity zone and shows low flexibility, but its atomic position in the space during all simulation is not adequate to create hydrogen bond. This could be a problem if we considered that in the model building 37% of amino acids were copied directly from the template (TetR) and 63% were predicted (see Table 2) and located in the same atomic position like its predecessor, all this without compare the side chain conformations *rotamers* (Figure 19).

Figure 19. Comparative schematic view in 180°, between TetR (blue) and AmtR (red) in a protein sequences LYWHV (TetR) and LYYHF (AmtR)



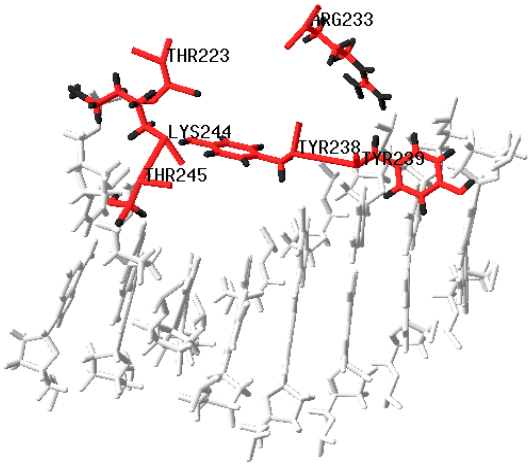
The Figure 19 shows in light colors the amino acids predicted after sequences align of TetR protein (blue) versus AmtR protein (red). In the case of AmtR the amino acids below the line demonstrated high affinity with the DNA during the whole simulation, however the amino acid phenylalanine above the line conserved the same dihedral angle and was near to the binding zone, but it couldn't interact, because the side chain conformation was not adequate.

An analysis and characterization of the binding site

discovered that not all amino acids studied showed only electrostatic attraction. Some amino acids in special tyrosine's, experimented electrostatic repulsion located in its special role by virtue of the phenol functionality, also some amino acid are in a specific structural position in the binding site that permits to interact with only one phosphate group located in only one nucleotide (Figure 20).

Therefore all written above maybe reaffirms the existence of a specific DNA sequence where the AmtR protein binds (Muhl, 2009; Beckers et al., 2005; Burkovski, 2007; Walter, Hänssler, Kalinowski, & Burkovski, 2007).

Figure 20. Schematic view of all amino acids with strong affinity in the DNA binding zone

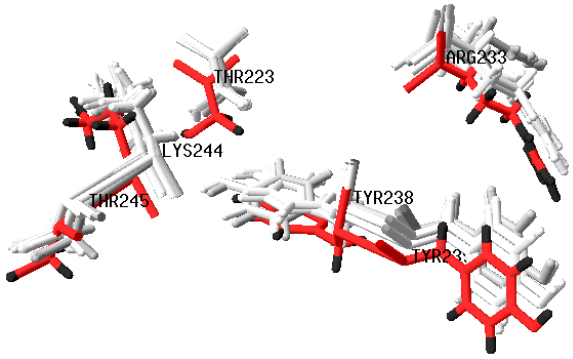


The Figure 20 corresponds to the PDB average taken to the stable zone in a simulation time of 100 ns, it shows six amino acids directly responsible for creating CNN will hydrogen bonds, between DNA-Protein. Each amino acid has a specific position and its interaction depends largely on how is interacting its amino acid neighbour. i.e. ARG233 can only create a hydrogen bond with C424 (Figure 16) because the other Van der Waals forces and *steric impediments* are interrupting everything.

Particular situation was analyzed in tyrosine's 238 and 239 where its electrostatic repulsion, dihedral angle in respect of the DNA and big electrostatic surface, caused an increment in the atomic fluctuation (Figure 22) when compared with an AmtR protein mutated model.

The tyrosine's amino acids are responsible greatly for preserving the specificity of amino acids with some nucleotides, because the strategic position in α-helix 3 (holding the monomer bind to DNA) attracts or repels influencing to all hydrogen bonding (Figure 21).

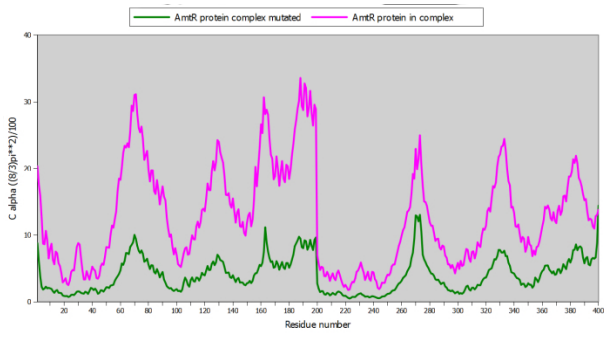
Figure 21. Schematic trajectory in 50 ns of all amino acids responsible for the hydrogen bonds in complex



The Figure 21 shows the trajectory taken from five average PDB files during 50 ns in the stable zone. Each view represents 10 ns, where the red color is for the firsts 10 ns and so on up to the gray color for the last. Note that the trajectory shows all amino acids scrolling up in the opposite direction to the DNA, where a small rotation of TYR238 in dihedral angle Chi2 in β Carbon causes loss of interaction in ARG233 around to 26 ns (see Figures 11 and 16) also THR223 and the rest shows high bond instability (see the *Results* section).

The previous situation could be normal, if we consider that the AmtR protein signal transduction is a member of the tetracycline repressors family of transcriptional regulators, where this class of proteins binds to the DNA when Nitrogen is present in surplus. This preliminary study was made without the influence of external signals as adenosine monophosphate (AMP) and GlnK protein. In other words, without induction process.

Figure 22. Comparative plot of Atomic Fluctuations in alpha carbons for AmtR protein complex (pink) and mutated (green)



The Figure 22 compares two protein complexes with the same characteristics except that the protein mutated has changed the amino acids TYR38 and 39 in the first

monomer and TYR238 and 239 in the second, both by serine amino acid.

It can be observed between the amino acids number ~20 up to 40 and ~220 up to 250, that several amino acids has changed the position and now are near to the binding zone and also some amino acids seem to have stability, all this because there is not the TYR repulsion.

V. CONCLUSION AND OUTLOOK

As shown in *Results* and subsequently in *Discussion*, this analysis is a starting point to understand how the AmtR signal transduction protein bind to specific DNA sequence in a molecular dynamic. This analysis revealed six amino acids located in a correct position in the binding zone, that they permit to interact strongly with the DNA, however, although they have hydrogen bonds with some nucleotides in the zone CTAT and corresponding base pairs GATA, e.g. C432-THR23 and A434-TYR38, this analysis can't conclude that only the interaction is in these zones, because each monomer formed hydrogen bonds with others nucleotides outside of CTAT and GATA DNA sequences.

Also it could be discovered the spectacular role of amino acid tyrosine in the protein binding. When the phenol adopts a horizontal position with respect to the DNA, the high electrostatic surface causes repulsion and triggers protein destabilization.

On the other hand, when phenol adopts a vertical position, the hydrogen in the alcohol group is attracted by the oxygen in the DNA phosphate group causing stabilization. This discovery in the molecular dynamics could be the first key for understanding how the AmtR signal transduction protein lost affinity with the DNA under nitrogen limitation. However, several simulations will be necessary, in particular with mutated models or directly with a model from experimental procedures (X-Ray), with the purpose of determining what affects the dihedral angles variation in tyrosine amino acids.

This analysis concludes and provides key information about the complex AmtR-DNA, but the only possibility to understand and to demonstrate the *binding ON* or *binding OFF* is simulating the induction mechanism using the homotrimer complex Adenosine monophosphate (AMP) with the GlnK protein signal (Nolden et al., 2001).

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