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Ramachandran plot analysis: Conformational validation of NifA protein structure in nitrogen-fixing *Azorhizobium caulinodans*

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Abstract **-** With the increasing numbers of known protein structures and greater accuracy of ultra-high resolution protein structures in structural biology, high fidelity conformational information is used to explore the validation of three-dimensional protein structures using Ramachandran plots. In proteins structural determination method, amino acid residues (as nodes) and the close contact between the residues (as edges) have been used to explore basic network properties to study protein folding, its structural stability and for prediction of catalytic sites. In this study, *in silico* analysis and Ramachandran plot analysis of NifA protein using nitrogen-fixing *Azorhizobium caulinodans* was carried out on the basis of 3Drefine results. The percentage distribution of amino acids in different regions was recorded in I-TASSER and Raptor X models using Ramachandran plot analysis. In I-TASSER model, 79.9% of amino acid residues resided in most favoured red region and only 15.7% amino acid residues were found in the allowed (yellow region), out of total 523 amino acids analyzed in this model. On the other hand, 90.5% amino acid residues resided in most favoured (red) region in Raptor X model out of total 433 amino acids analyzed in this model. Only 6.7% amino acid residues were found in additional allowed (yellow) region, whereas only 1.2% residues were observed in generously allowed region. Thus, the number of amino acid residues belonging to "outlier, allowed, and favored" regions in Ramachandran plot analysis represents best quality metrics of experimental structure models before structure deposition.

Keywords **-** Ramachandran plot, NifA protein, Nitrogen fixation, I-TASSER model, Raptor X model, Amino acids, Structural stability

1. INTRODUCTION

Insight into the three-dimensional structures of macromolecules is crucial for our understanding of biological processes. Therefore, the validation and quality assessment of three-dimensional structures of proteins is an important issue in structural biology [1, 2]. Structure validation of proteins is thus an integral part in obtaining three-dimensional models of macromolecules in X-ray crystallography [3] and in cryoelectron microscopy [4]. It is also key in interpreting the quality of models from the Protein Data Bank (PDB) [5], as there is no formal structure quality requirement for acceptance to this repository. A key quality metric used in validation of the quality of atomic models of proteins is the Ramachandran plot [6]. Ramachandran plots describe the two-dimensional distribution of the torsion angles [phi (φ) and psi (ψ)] of the amino acids residues contained in a peptide, which constitutes protein backbone, and are one of the best quality metrics of experimental structure models [7]. By making a Ramachandran plot, protein structural scientists can determine which torsional angles are permitted and can obtain insight into the structure of peptides. Typically, validation software packages report the number of residues belonging to "outlier, allowed, and favored" regions [8]. The phrase ''no Ramachandran plot outliers'' is widely considered as the ''gold standard'' for a high-quality structure and is often found in the validation of most protein structures.

The input of nitrogen nutrient into agricultural soil largely depends on addition of chemical fertilizers, biological nitrogen fixation (BNF) and/or degradation of organic matter in soil [9-11]. In BNF process, nitrogenase enzyme in nitrogen-fixing bacteria reduces atmospheric nitrogen to plant utilizable ammoniacal form. Therefore, use of nitrogen-fixing bacteria as biofertilizer reduces the use of synthetic chemical fertilizers in agriculture farms and prevents public health hazards for healthy ecosystem functioning. Considering the importance of nitrogen fixation for different crops and environment safety [12], extensive analysis and appropriate stastistical modeling is required to understand the functioning of nitrogen fixation by diazotrophic bacteria under soil conditions.

Nitrogen-fixing bacterium *Azorhizobium caulinodans* forms nodules roots as well as on stem of *Sesbania* species, which is cultivated as a tropical legume shrub and green manure crop [13]. NifA protein has been reported to act as positive transcriptional activator of nitrogen fixation genes (involved in synthesis of nitrogenase enzyme) under microaerobic and nitrogen-limited conditions in different soil agri-ecosystems [14]. The use of phylogenetic tools, algorithms and *in silico* methods involved in modeling of

proteins in earlier studies has been considered as highly reliable and important techniques in biological sciences [15, 16]. Recently, *in silico* 3D structure of proteins based on homology modeling technique has emerged as one of the keys for understanding the biological processes at molecular level [17, 18]. Satyanarayana et al. [19] performed *in silico* and Ramachandran plot analysis of NifA protein using three other rhizobial strains and showed that 93.8% amino acid residues resided in the favoured (red) region, while 4.7% residues were found in allowed regions and only 1.5% residues were available in outlier or generously allowed region. Considering the importance of nitrogenase enzyme in agricultural field and involvement of NifA protein in its regulation, Ramachandaran plot analysis and *in silico* modeling of NifA protein was undertaken in *Azorhizobium caulinodans*.

2. MATERIALS AND METHODS

Efficient computational and bioinformatics tools afford novel opportunities for understanding biological information from genomic and proteomic databases [20]. In the present study, the quality of NifA protein structure was predicted by plotting of Ramachandran plots. We have shown that the simple counting of residue fractions that belong to favoured and outlier regions of the Ramachandran plot may provide basic information to validate protein backbone conformation.

2.1. Retrieval of NifA protein sequences in different nitrogen-fixing bacteria

In order to obtain complete information regarding the features of macromolecular structures, the protein data base (PDB) allows a wide spectrum of queries through data integration. Database similarity search tool, FASTA, was applied that work on heuristic method of database searching. BLAST was used for searching of GenBank and other sequence databases for sequence similarity and homology among different nitrogen-fixing bacteria [21, 22]. In the present study, amino acid sequences of NifA protein from 15 different nitrogen-fixing and nodule-forming bacterial strains were retrieved from NCBI GenBank and Uniprot KB Database.

Sequences of NifA protein varies in different rhizobial strains [14]. Amino acid sequence comparisons of NifA protein indicated that protein consists of three domains with variable conservation. The central domain of NifA protein contains about 240 amino acids, which play significant role in activation of other *nif* genes during functioning of nitrogenase [23]. The C-terminal domain contains helix-turn-helix motif that helps in binding to upstream activator sequences of other *nif* genes during nitrogen fixation by bacteria.

2.2. *In silico* modeling of NifA protein in *Azorhizobium caulinodans*

The amino acid sequences and structure of template were retrieved from PDB and UniProtKB database. The template and target sequences were aligned using CLUSTALW and pairwise sequence alignment script. *In silico* 3D models were built by creating backbone/threading based on template structure using I-TASSER and Raptor X.

I-TASSER server: I-TASSER based algorithms for prediction of protein structure and function are implemented through on-line platform I-TASSER server [24]. Use of I-TASSER based algorithms permits academic users to automatically create high-quality model predictions of 3D structure and biological function of protein molecules from their amino acid sequences [25, 26). I-TASSER Suite is downloadable package of independent PC programs, developed by Yang Zhang Lab for protein structure prediction and refinement, and structure-based protein function annotations (26). Additionally, I-TASSER structural and functional template library is weekly updated and freely accessible to the I-TASSER users.

Raptor X: Raptor X was developed by Xu group. It is protein structure prediction server, which predicts 3D structures for protein sequences without close homologs in the Protein Data Bank (PDB) (Fig. 1). Once input sequence has been provided, Raptor X predicts its secondary and tertiary structures, contacts, solvent accessibility, disordered regions and binding sites [27].

3Drefine web server: 3Drefine is interactive web server for consistent and computationally efficient protein structure refinement with capability to perform online statistical and visual analysis. 3D refine web servers takes into consideration protein structure refinement through text or file input submission, email notification, and is freely accessible without any registration. This server also provides comprehensive analysis of submissions through different energy and statistical feedback and interactive visualization of various refined models through JSmol applet that is equipped with various protein model analysis tools [28). The 3D refine web server has been made freely accessible, broadly tested and used by numerous users.

3. RESULTS

The amino acid sequences of NifA protein in different nitrogen-fixing bacteria were obtained in FASTA format. For instance, 519 amino acids are found in NifA protein of *Rhizobium leguminosarum* bv. *viciae*, whereas, 605 amino acids have been accounted in *Bradyrhizobium japonicum.* NifA protein of *Azospirillum brasilense* was reported to contain 626 amino acids.

Ramachandran plot analysis of different NifA protein models was carried out on the basis of 3Drefine results. Ramachandran plot of I-TASSER model indicated that 418 amino acid residues are distributed in most favoured region (red), whereas only 82 residues were observed in additional allowed (yellow) region (Fig. 2). Twelve residues were available in outlier or generously allowed region (yellow) and 11 residues were found in disallowed regions. Number of glycine residues was depicted as 42 and only 48 proline residues were shown in model. The percentage distribution

of amino acids in different regions of Ramachandran plots showed that 79.9% of amino acid residues resided in most favoured red region, out of total 523 amino acids analyzed in this model. Only 15.7% amino acid residues were found in the additional allowed (yellow region).

The amino acid sequences of the NifA protein in *Azorhizobium caulinodans* strain ORS 571 is provided below:

>***Azorhizobium caulinodans* ORS 571**

MPMTDAFQVRVPRVSSSTAGDIAASSITTRGALPRPGGMPVSMSRGTSPEVALIGVYEISKILTAPRRLEVTLANVVNVLSS
MLQMRHGMICILDSEGDPDMVATTGWTPEMAGQIRAHVPQKAIDQIVATQMPLVVQDVTADPLFAGHEDLFGPPEEATVSFI
GVPIKADHHVMGTLSIDRIWDGTARFRFDEDVRFLTMVANLVGQTVRLHKLVASDRDRLIAQTHRLEKALREEKSGAEPEVA
EAANGSAMGIVGDSPLVKRLIATAQVVARSNSTVLLRGESGTGKELFARAIHELSPRKGKPFVKVNCAALPESVLESELFGH
EKGAFTGALNMRQGRFELAHGGTLFLDEIGEITPAFQAKLLRVLQEGEFERVGGNRTLKVDVRLVCATNKNLEEAVSKGEFR
ADLYYRIHVVPLILPPLRERPGDIPKLAKNFLDRFNKENKLHMMLSAPAIDVLRRCYFPGNVRELENCIRRTATLAHDAVIT
PHDFACDSGQCLSAMLWKGSAPKPVMPHVPPAPTPLTPLSPAPLATAAPAAASPAPAADSLPVTCPGTEACPAVPPRQSEKE
QLLQAMERSGWVQAKAARLLNLTPRQVGYALRKYDIDIKRF

The one-letter abbreviation of various amino acids found in NifA protein are as follows: Alanine (A), Arginine (R), Asparagine (N), Aspartic acid (D), Cysteine (C), Glutamine (Q), Glutamic acid (E), Glycine (G), Histidine (H), Isoleucine (I), Leucine (L), Lysine (K), Methionine (M), Phenylalanine (F), Proline (P), Serine (S), Threonine (T), Tryptophan (W), Tyrosine (Y) and Valine (V)

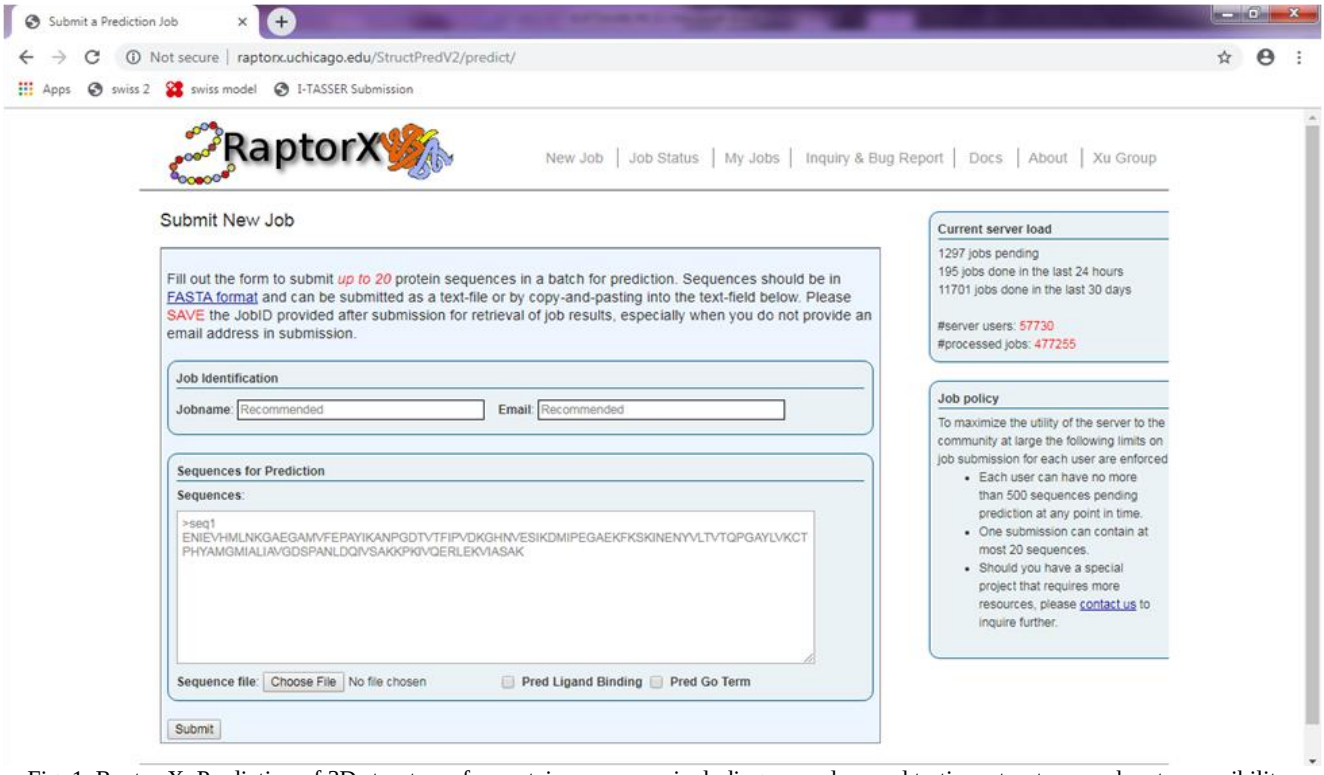

Fig. 1. Raptor X: Prediction of 3D structures for protein sequences including secondary and tertiary structures, solvent accessibility, disordered regions and binding sites

The Ramachandran plot analysis of NifA protein using Raptor X model showed that only 392 amino acid residues resided in most favoured (red) region, whereas only 29 amino acid residues were observed in additional allowed (yellow) region (Fig. 3). Five amino acid residues were present in the outlier or generously allowed (yellow) region and seven residues was located in the disallowed regions. The number of glycine residues was depicted as 38 and 29 proline residues were found in the model. The percentage distribution of amino acids in Raptor X model showed that 90.5% amino acids resided in most favoured (red) region, out of total 433 amino acids analyzed in this model. Only 6.7% amino acid residues were found in additional allowed (yellow) region, whereas only 1.2% residues were observed in generously allowed region.

SAVES results depicted that 33-69 amino acid residues were found in outlier region in five different models obtained using I-TASSER. In selected I-TASSER model, only 33 amino acid residues were found in the outlier

region. Similarly, number of amino acid residues varied in different models. Twelve amino acid residues were observed in outlier region of Raptor X model, whereas, only 14 residues were found in outlier region in Swiss model (data not shown).

4. DISCUSSION

Three-dimensional models of various macromolecules have been successfully described by a network of nodes, edges, ideal bond length and bonding angles in the last two decades. Ramachandran plot (also known as [φ, ψ] plot) provides a simple two-dimensional graphic representation of all possible protein structures in terms of torsion angles [6]. Although the Ramachandran plot was developed using theoretical methods, the importance of plot was realized with the beginning of mathematical calculations and *in silico* model building of the different protein structures [29]. The most important application of Ramachandran plot is the prediction of the quality of various protein structures determined using experimental methods such as X-ray crystallography, nuclear magnetic resonance (NMR) and cryoelectron microscopy (Cryo-EM). A good quality three-dimensional structure of macromolecule contains all the set of torsional angles in the allowed area where as, a bad quality (low resolution) protein structure is reflected as a number of torsional angles falling in the forbidden region [3]. Besides experimental methods, protein structure obtained using homology modeling or ab-initio methods are also routinely checked by plotting Ramachandran plot. From this perspective, the interacting amino acids in proteins may act like other self-organized networks, such as biological signaling pathways, metabolic networks, and scientific collaboration networks [30–32].

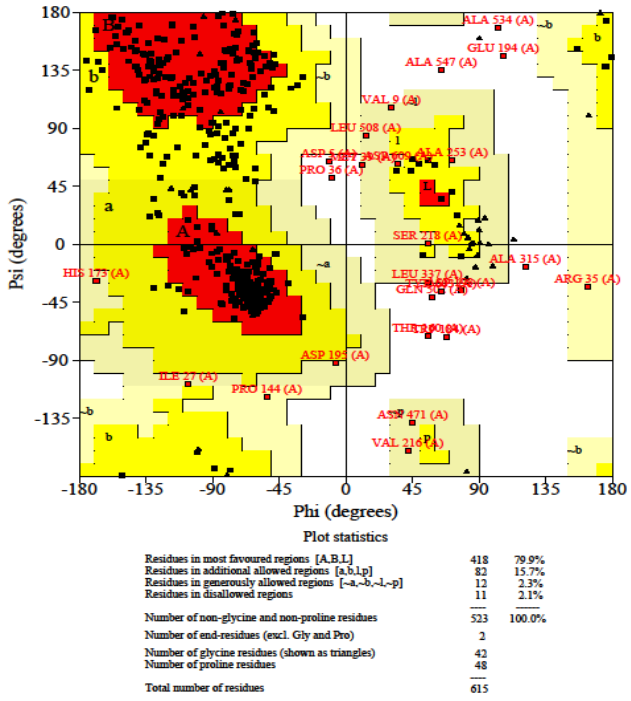

Fig. 2. Ramachandran plot of NifA protein using I-TASSER model

In this study, we have demonstrated the utility of the Ramachandran plot by counting of amino acid residue fractions that belong to favored and outlier regions to validate protein backbone conformation. Ramachandran plot analysis of NifA protein using I-TASSER model indicated that 418 amino acid residues (79.9%) are distributed in most favoured region (red), whereas only 82 residues (15.7%) were observed in additional allowed (yellow) region (Fig. 2), out of total 523 amino acids analyzed in this model. The percentage distribution of amino acids in Raptor X model showed that 392 amino acid residues (90.5%) resided in most favoured (red) region, out of total 433 amino acids analyzed in this model (Fig. 3). Only 29 amino acid residues (6.7%) were observed in additional allowed (yellow) region of NifA protein. In earlier studies, *in silico* analysis and homology modeling of NifA protein using three rhizobial strains was performed [19]. Ramachandran plot analysis showed that 93.80% residues resided in favoured (red) region, while 4.70% residues were found in allowed regions and rest 1.50% residues were available in outlier or generously allowed region. Similar observations regarding percentage distribution of amino acid residues in Ramachandran plots were reported for AmpC/ß–lactamase protein in *Pseudomonas aeruginosa* [33]. The distribution of more than 90% of residues in favoured region has been suggested as indicator of good quality model [34].

Analysis of Ramachandran plot during *in silico* structural analysis with 3D protein modeling of alkaline phosphatase enzyme in *Pseudomonas aeruginosa* indicated that 97.0% amino acid residues resided in favoured (red) region, while 4.70% residues were found in allowed (brown) regions and rest 2.60% residues were available in generously allowed regions (yellow) or outlier regions [35]. Such type of *in silico* homology modeling was also shown by several workers to predict a variety of 3D protein models of superoxide dismutase in extermophile *Exiguobacterium* [36], ACC deaminase enzyme in *Mesorhizobium* [37] and DNA ligase A protein of Gram-positive spirochete *Leptospira interrogans* [38].

The traditional X-ray crystallography or nuclear magnetic resonance methods used for identification of 3D structure of proteins are tedious and costly [18]. Therefore, *in silico* analysis of genes and proteins has been receiving greater attention to identify different pathogenic microorganisms, designing of effective chemicals and drugs, diagnosis of infectious diseases along with characterization of beneficial microbes for improving crop production [33, 38, 39]. In addition to structure validation of protein molecules using Ramachandran plot, engineering of the entire microbial or plant genomes is being performed using systems biology and synthetic biology approaches for obtaining desired novel functions and phenotypes in different agro-ecosystems [40–43]. Current advances at molecular and genomic level, development of new experimental methods, use of statistical and computational models, theoretical and *in silico* approaches along with multi-scale modeling and data integration along with validation and stability of

structural proteins will provide innovative technologies for future sustainable agricultural developments [16, 20, 44, 45]. Therefore, current scientific progress in the biological systems and their bioengineering holds enormous potential for improving crop production, human health and eco-friendly environment.

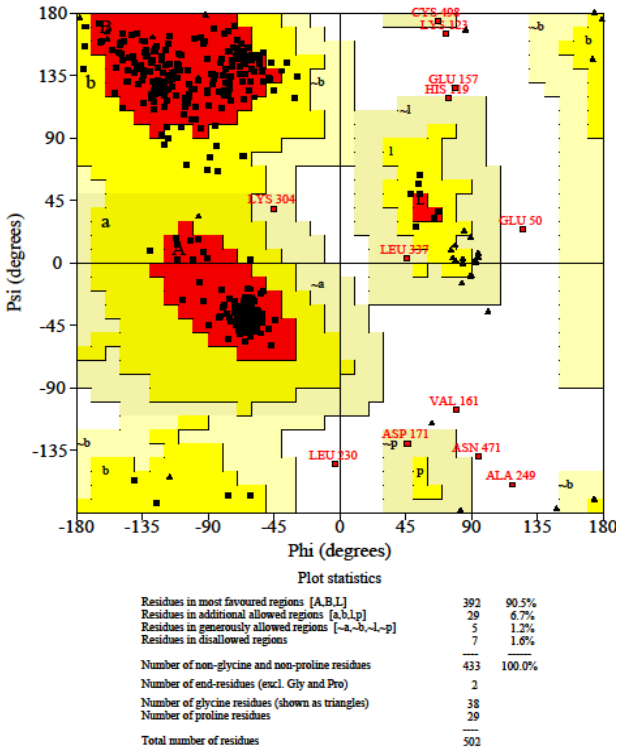

Fig. 3. Ramachandran plot of NifA protein using Raptor X model

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