Trends in Bioinformatics

ISSN 1994-7941

science alert

ANSInet
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Trends in Bioinformatics 8 (1): 1-13, 2015
ISSN 1994-7941 / DOI: 10.3923/tb.2015.1.13

Mutational Analysis on Human Granulocyte Macrophage-Colony Stimulating Factor Stability Using Computational Approaches

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ABSTRACT

Granulocyte Macrophage Colony Stimulating Factor (GM-CSF) is a 16.29 kDa cytokine that regulates the leukocyte production, migration and functions. The GM-CSF receptor ligand interaction stability plays vital role for prolonged differentiation of haematopoietic stem cell into granulocytes and monocytes. In the present investigation attempts were made to increase the number of stabilization centres in GM-CSF ligand using molecular simulation. This improves half-life stability of GM-CSF receptor ligand interaction complex. The numbers of stabilization centres were increased by amino-acid substitution which led to change in contact energy, hydrophobicity index and unfolding Gibbs free energy without altering receptor ligand interaction. Multiple sequence alignment of GM-CSF sequence using ClustalW with *Ovies aries*, *Homo sapiens*, *Mus musculus* and *Gallus gallus* species revealed the conserved domain regions and aminoacid dissimilarities in conserved and other regions. Based on the above, 21N, 25L, 42V, 55L, 56Q, 93E and 102T were mutated with its aminoacid substitution property. Different combinations of mutation were incorporated in the amino acid sequence and mutant proteins were modelled using structure of GM-CSF ligand (PDB ID: 1CSG) as a template by MODELLER. After mutation, the GLU21, LEU25, LEU55 and THR102 positions were identified as stability centre using SCide. Mutations at residues LEU55 and THR102 had 16.71% lesser energy value than the wild type GMCSF energy value which is 6831.73. The result suggested that, the stability of human GM-CSF has been increased (as the energy decreases) due to mutagenesis by computational tools.

Key words: GM-CSF, cytokine, stability, mutation, computational tools

INTRODUCTION

Cytokines are a large family of mainly soluble proteins and glycoproteins which function as the key regulators of the immune system. Cytokines were discovered on the initial observations that soluble biological factors could mediate inflammatory, cell stimulatory and anti-viral responses. Cytokines possess two important properties which play a crucial role in the development of treatment-associated adverse effects. Cytokines are pleiotropic in nature that, in fact, some cytokines able to stimulate cell types that mediate opposing biological effects (Vazquez-Lombardi *et al*., 2013). Further, cytokines have a short serum half-life and should be administered at high doses to achieve their therapeutic effects. Cytokines include interleukins (ILs), interferons (IFNs), growth factors, Colony Stimulating Factors (CSFs), the Tumor Necrosis Factors

(TNF) and chemokines (or chemotactic cytokines). Colony-stimulating factors (CSFs) are secreted glycoproteins that bind to receptor proteins on the surfaces of hemopoietic stem cells, thereby activating intracellular signaling pathways that can cause the cells to proliferate and differentiate into a specific kind of blood cell (usually white blood cells. For red blood cell formation, erythropoietin). They may be synthesized and administered exogenously. However, such molecules can at a latter stage be detected, since they differ slightly from the endogenous ones in, e.g., features of posttranslational modification. These are small signalling molecules (<30 kDa) mostly secreted by leukocytes and can also be produced by other cell types like endothelial cells, epithelial cells and fibroblasts etc. Cytokines functions as autocrine, paracrine or endocrine systems that stimulate or suppress the activity of target cell populations. Cytokines convey signals that are essential for generation, endurance and homeostasis of immune cells and also for the generation of immune responses upon external stimuli (Breitbach *et al*., 2011). Being natural immune modulators, many cytokines have been marked as suitable therapeutic agents for the treatment of a number of contagious, inflammatory, autoimmune and lethal diseases. Cytokine immunotherapy leads to the development of severe dose-limiting side effects. Even though it effectively enhances therapeutic efficacy, higher doses intensifies pleiotropic activities that acts as adverse effects in patients.

Most protein therapeutics suffers from sub-optimal stability and cytokines are no exception. Poor stability results in increased levels of protein unfolding, degradation and aggregation that leads to many issues affecting the constitution, mean shelf-life, immunogenicity and therapeutic efficacy (McCammon and Harvey, 1988). Cytokine stability can be improved by the mutation of free cysteines to serines, thus preventing undesired disulphide bond which leads to protein aggregation (Ng and Henikoff, 2006). Molecular engineering of cytokines with prolonged half-life enhances specificity or localized activity that is required to enhance the pharmacological properties of these proteins (Berrondo, 2010; Bishop *et al*., 2001). Mutagenesis can be employed to engineer cytokines with enhanced stability, half-life, specificity and activity (Rozwarski *et al*., 1996; Palma and Curmi, 1999). In early studies, scanning and deletion mutagenesis allowed the development of cytokines with modified activities (Arakawa *et al*., 1993). Protein engineering techniques approaches like rational design, directed evolution and computational modelling, allows for more efficient cytokine optimization (Marshall *et al*., 2003). Molecular engineering approach has been enforces on a number of cytokines which includes the commercially available form of recombinant human IL-2, Proleukin®. Cytokine stability can also be enhanced by the introduction of stabilizing mutations within "-helices. 4-helix bundle cytokines requires the mutation of residues with low "-helical propensity (e.g., proline, glycine) to residues with high "-helical propensity (e.g., alanine) (Luo *et al*., 2002). Previous studies suggests introduction of double and triple glycine-to-alanine substitutions resulted in the generation of a number of G-CSF variants that displayed enhanced resistance to chemical denaturation. The objective of this study were: (1) To identify of conserved domains, stabilizing centres, interacting residues and hydrophobic regions present in GM-CSF using computational methods, (2) To increase the number of protein stabilization centres using mutation and analyse the changes in protein stability using molecular force field energy calculation (Pikkemaat *et al*., 2002).

MATERIALS AND METHODS

Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF): Granulocyte-macrophage colony-stimulating factor (GM-CSF) is a pleiotropic cytokine that contains 144 amino acids and has

a molecular weight of 16294.7 Da. GM-CSF is produced by a number of cell types including monocytes, endothelial cells, fibroblasts, mitogen-stimulated B-cells, T-cells and LPS-stimulated macrophages. GM-CSF has been shown to amplify the response of the mature immune system to antigen. GM-CSF works in coordination with other cytokines, IL-5 and IL-3, increasing the granulocytic inflammatory response. It moderates the production and functional activation of haematopoietic cells and controls dendritic cell and T-cell function, thus linking innate and acquired immunity. A growing body of evidence now suggests that GM-CSF plays a key role in signalling emergency haematopoiesis (predominantly myelopoiesis) in response to infection that includes the production of granulocytes and macrophages in the bone marrow and their maintenance, endurance and functional activation at sites of injury or insult. GM-CSF has a recognized effect on granulocyte and macrophage maturation from hematopoietic precursors both *in vitro* and *in vivo*. In addition, GM-CSF also exerts effects on mature monocytes or macrophages and known to potentiate the release of various cytokines such as TNF-", IL-1$ and IL-12 after LPS stimulation without inducing these mediators by its own (Hercus *et al*., 2009). Because of its myeloproliferative and immuno-stimulatory properties, GM-CSF is widely used therapeutically in a number of clinical therapies such as myelosuppressive chemotherapy, bone marrow transplantation, neonatal sepsis, lung injury and wound healing. GM-CSF alters functions of mature monocytes and macrophages where it binds to high-affinity specific receptor found on most monocytes and has been proposed to enhance their activation potential. The effects of GM-CSF are mediated through a heteromeric receptor expressed on monocytes, macrophages and granulocytes (Hansen *et al*., 2008). The GM-CSF receptor, composed of GM-CSF specific " and a signal transducing $ chain that is common to receptors for GM-CSF (Kaushansky *et al*., 1989). Priming of neutrophils by GM-CSF increases antimicrobial functions, such as phagocytosis and oxidative burst. GM-CSF has multiple effects on mature DC that increased cross-presentation and increased uptake capacity. Atomic coordinates of Granulocyte-macrophage colony-stimulating factor (GM-CSF) protein was retrieved from protein data bank (PDB code: 1CSG, resolution 2.70 A°) (Walter *et al*., 1992).

Granulocyte-macrophage colony-stimulating factor (GM-CSF) protein molecule (PDB ID: 1CSG (recombinant) were downloaded from the Protein Data Bank (PDB). The three dimensional structure of the protein molecule was viewed using the PyMOL (Fig. 1) (Delano, 2002) (www.pymol.org) (Seeliger and de Groot, 2010). Figure 2 displays the various residues in the human GM-CSF. Table 1 details the position of different resides and their functions in structure of the protein.

Based on the results from Lyne *et al*. (1995) the highly conserved, stabilizing and interaction residues were identified in the wild type sequence and it was incorporated in PyMOL viewer, in which pink indicates the stabilizing residue, blue indicates the interacting residues, red indicates the highly conserved residues and white indicates both highly conserved and interacting residue (Fig. 3).

Multiple sequence alignment using CLUSTALW: One of the most widely performed bioinformatics analyses are multiple sequence alignments. The most widely used packages include Clustal W (Thompson *et al*., 1994) from which currently used clustal programs were derived. A novel position-specific scoring scheme and a weighting scheme for down weighting over-represented sequence groups are the attributes of Clustal W (Higgins *et al*., 1996). The W here indicated ‘weights’. Clustal could be run remotely from several sites using the Web or the programs might be downloaded and run locally on commonly used operating systems.

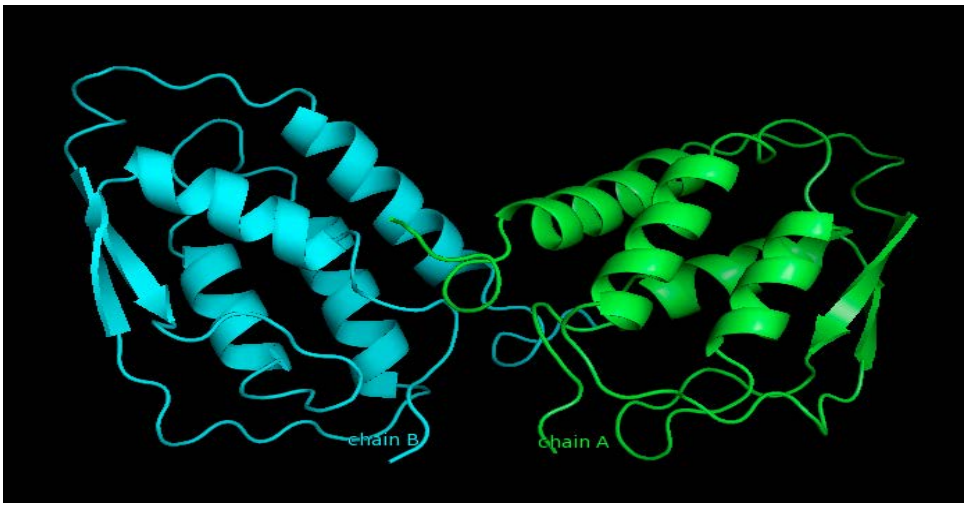

Fig. 1: Structure of recombinant Human GM-CSF (1CSG) in PyMOL viewer

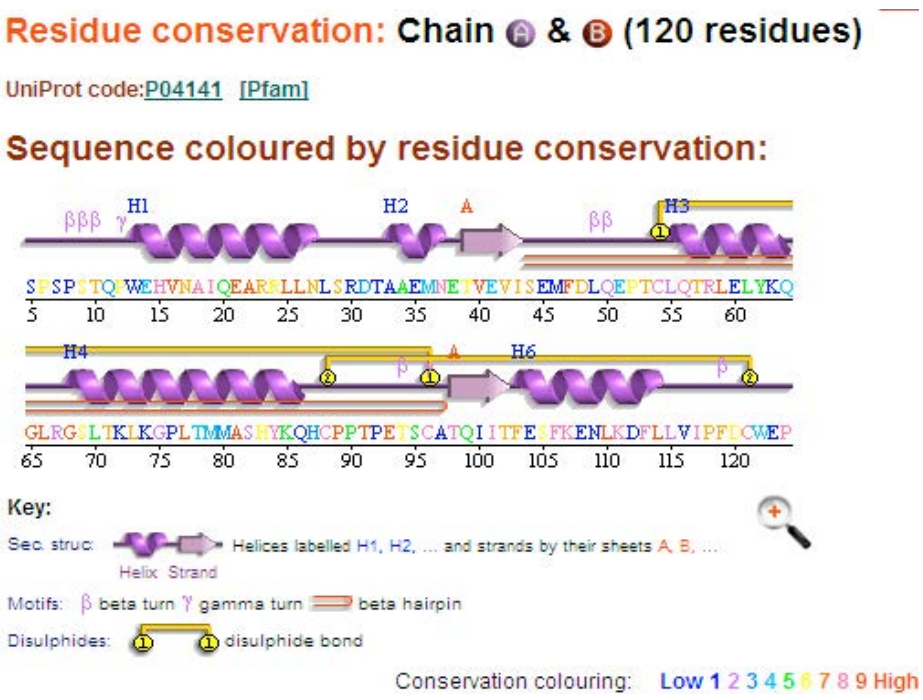

Fig. 2: Conformation of residues and structure elaboration of GM-CSF

The GM-CSF FASTA sequences of the various mammalian species: *Ovies aries, Mus musculus, Equus caballus* and *Homo sapiens* obtained from NCBI was given as input. The results of the multiple sequence alignment were obtained.

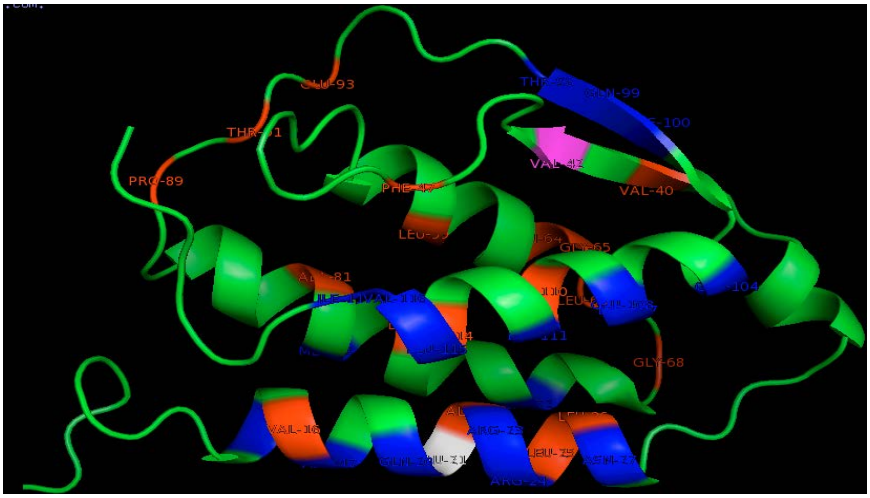

Fig. 3: Stabilizing, highly conserved and interacting residues in human GM-CSF

Table 1: List of amino acids at different position in human GMCSF and functions of the corresponding residues attributing to the protein structure

Colour	Corresponding residues		Function
Pink	V42		Stabilizing residues
Red	V16	L66	Highly conserved residues
	A22	G68	
	L25	L77	
	L26	A81	
	V40	P89	
	F47	T91	
	L59	E93	
	Q64	L110	
	Q65	L114	
Blue	N17	l100	Interacting residues
	Q20	E104	
	R23	K107	
	R24	E108	
	N27	K111	
	K72	L115	
	M79	V116	
	Q99	l117	
White	E21		Both interacting and highly conserved residues

Identifying possible amino acid mutations using CUPSAT: The multiple sequence alignment yields highly conserved sites and thus, to identify the sites where mutations are possible. Furthermore single point mutations will be introduced in the possible sites. Cologne University Protein Stability Analysis Tool (CUPSAT) (Parthiban *et al*., 2006) is a web tool used to analyze and predict protein stability changes upon point mutations (single amino acid mutations). The best possible amino acid substitutions at the particular sites were found. The favourable amino acids to be replaced were found by Bishop *et al*. (2001) which helped in comprehensive prediction using the software CUPSAT.

Modelling the mutant GM-CSF using MODELLER: The desired mutation was found from the CUPSAT results. Then the mutation was introduced at the corresponding position then, we modeled the protein sequence using MODELLER 9v4 program (Eswar *et al*., 2003) Modeled protein and then refined using loop refinement protocol of MODELLER. All the mutations were employed and different GM-CSF mutants were modelled.

Identifying the stabilization centres using SCide: Modelled protein structure further analysed to identify stabilization centre (Sali *et al*., 1995). The Supervisory Control Integrated Development Environment (SCide) is a tool to locate the stabilization centres from the known protein structures. Stabilizations centres are residues involved in co-operative long-range contacts which are formed between different regions of a polypeptide chain, or belong to different peptides or polypeptides in a complex protein. The SCide program selects stabilization centres which refer to the amino acids involved in contacts which are important in maintaining the stability of a protein (Dosztanyi *et al*., 2003). The stabilization centres for the mutated proteins were found using SCide.

Energy minimization: Modeled protein structure of mutated GM-CSF sequence was then subjected to biopolymer module of SYBYL 8.1 program. A higher temperature was applied to allow the system to rearrange from its present state and lower the temperature to bring the system into a stable state (Metropolis *et al*., 1953). The cycle is repeated several times so that multiple conformations may be obtained and later analysed using the Molecular Spread sheet. A reduced energy was obtained along with a model. Finally this structure was energy minimized using TRIPOS force field for 100 steps with staged minimization protocol in SYBYL. Simulation was done for all the different mutations and values were tabulated (Table 2). Accelrys studio was also used to perform energy minimization calculation. The modelled protein structure of mutated GM-CSF was reduced to stable state by optimization. The energy minimization was done using CHARMm (Brooks *et al*., 1983) force field. This procedure was carried out for all the different mutations and values were tabulated (Table 2).

RESULTS

Multiple sequence alignment: Multiple sequence alignment was performed across the four mammalian species, Homo sapiens, Mus musculus, Ovis aries and Gallus gallus using CLUSTAL W. The amino acid residues among the species were compared and the conserved regions were found.

Colons (:) positions indicating mutation with similar amino acid, within the four mammalian species. Following are the 26 positions with respective amino acid mutation observed in *Homo sapiens*: 3A, 11Q, 13W, 16V, 17N, 25L, 42V, 47F, 60Q, 55L, 56Q, 60E, 62Y, 64Q, 67R, 77L, 79M, 93E, 102T, 108E, 114L, 117I, 123E, 126Q (Fig. 4).

Mutational analysis by CUPSAT: CUPSAT mutational analysis results provided the amino acid residues with the favourable and stabilizing mutation with predicted)G (kcal moLG^{1}) was obtained. The possible mutations were found for the residues LEU55 and THR102 of the human GM-CSF. The output consists information about mutation site, its structural features and comprehensive information about changes in protein stability for 19 possible substitutions of a specific amino acid

Fig. 4: Multiple sequence alignment using ClustalW

mutation. Prediction of possible amino acid substitutions in the sequence of hGM-CSF at position 55 the possible substitutions for the amino acid LEU at the position 55, in which the substitution of LEU by VAL was found to be favourable (Fig. 5).

In hGM-CSF sequence Prediction results of possible amino acid substitutions for THR at position 102 shows SER was found to be stabilizing and favourable (Fig. 6). The CUPSAT analysis was performed on all the possible residues but these two amino acids provided stability to the molecule.

Stabilization centres using SCide: Identification of stabilization centres after mutation using the server Scide shows the stabilization centres after mutation using the server SCide, in which the residues GLU21, LEU25, LEU55 and THR102 were identified as stabilization centres (Fig. 7).

Energy minimization: The energy minimization was carried out using two different force fields: CHARMM and TRIPOS. Among that, the mutants (L55V and T102S) was comparatively found to be increasing than the wild type with a value of -286.431 in TRIPOS and -6945.95226 in CHARMm thereby making the mutation in the position amicable (Table 2).

DISCUSSION

Miyazawa and Jernigan (1994) studied four physicochemical factors which are responsible for the stability of a protein, like (1) change in specificity of amino acid local environment for initial and replaced amino acid which helped in analysing the GM-CSF wild type's specificity upon replacing an amino acid in the sequence, (2) Buriedness of the amino acid surface area those are inaccessible to water which helped in understanding of receptor interactions and formation of hydrogen bond

Mutation Site			
PDB	**Chain**	**Wild type AA**	**Residue ID**
1CSG	A	LEU	55

Structural Features		
SS element	**Solvent accessibility**	**Torsion angles (φ, ψ)**
Helix	0.55%	-66.5°, -48.0°

Amino Acid Mutations			
Amino acid	**Overall Stability**	**Torsion**	**Predicted ΔΔG (kcal/mol)**
GLY	Destabilising	Unfavourable	-7.82
ALA	Destabilising	Favourable	-4.1
VAL	**Destabilising**	**Favourable**	**-1.89**
ILE	Stabilising	Favourable	0.16
MET	Destabilising	Favourable	-2.64
PRO	Destabilising	Unfavourable	-7.42
TRP	Destabilising	Favourable	-5.58
SER	Destabilising	Unfavourable	-6.6
THR	Destabilising	Unfavourable	-6.16
PHE	Destabilising	Favourable	-1.11
GLN	Destabilising	Favourable	-7.59
LYS	Destabilising	Favourable	-14.17
TYR	Destabilising	No change	-4.47
ASN	Destabilising	Unfavourable	-4.27
CYS	Destabilising	Unfavourable	-2.31
GLU	Destabilising	Favourable	-7.01
ASP	Destabilising	Unfavourable	-3.98
ARG	Destabilising	Favourable	-9.45
HIS	Destabilising	Unfavourable	-6.94

Fig. 5: CUPAST analysis of position 55 in human GM-CSF

Mutation Site			
PDB	**Chain**	**Wild type AA**	**Residue ID**
1CSG	A	THR	102

Structural Features		
SS element	**Solvent accessibility**	**Torsion angles (φ, ψ)**
Sheet	33.17%	-74.4°, 157.0°

Amino Acid Mutations			
Amino acid	**Overall Stability**	**Torsion**	**Predicted ΔΔG (kcal/mol)**
GLY	Destabilising	Unfavourable	-0.62
ALA	Stabilising	Favourable	0.23
VAL	Stabilising	Unfavourable	1.67
LEU	Stabilising	Unfavourable	1.81
ILE	Stabilising	Unfavourable	2.17
MET	Destabilising	Unfavourable	-0.5
PRO	Stabilising	Favourable	3.18
TRP	Stabilising	Unfavourable	1.65
SER	**Stabilising**	**Favourable**	**0.27**
PHE	Stabilising	Unfavourable	1.18
GLN	Stabilising	Unfavourable	0.02
LYS	Stabilising	Unfavourable	1.96
TYR	Stabilising	Unfavourable	1.32
ASN	Stabilising	Unfavourable	0.09
CYS	Stabilising	Favourable	0.04
GLU	Stabilising	Unfavourable	2.45
ASP	Stabilising	Unfavourable	1.94
ARG	Stabilising	Unfavourable	1.31
HIS	Stabilising	Unfavourable	0.13

Fig. 6: CUPAST analysis at position 102 in human GM-CSF

in GM-CSF. (3) change in relative distance for original and replacement amino acid with respect to the protein molecule's centre of mass, in estimating the three dimensional mass of the protein entity and (4) Change in stability resulting from an amino acid substitution.

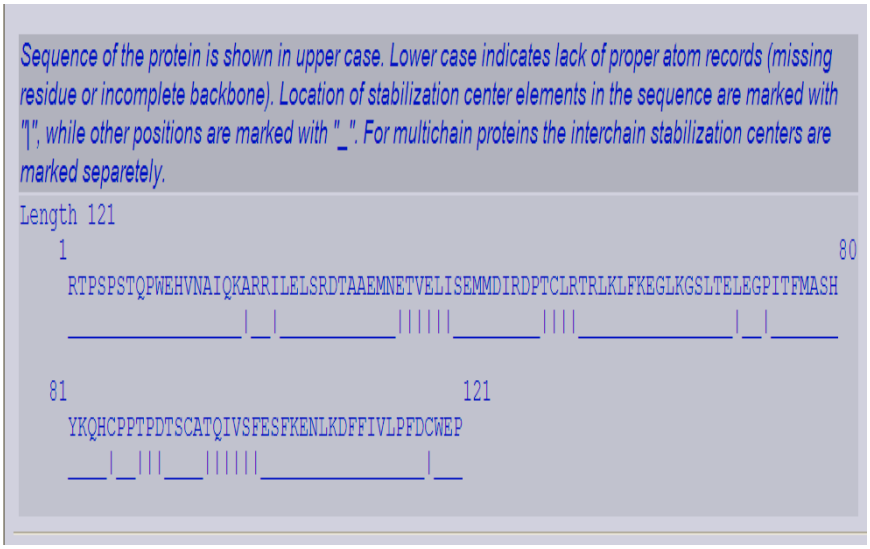

Fig. 7: Identification of stabilization centres after mutation using the server SCide

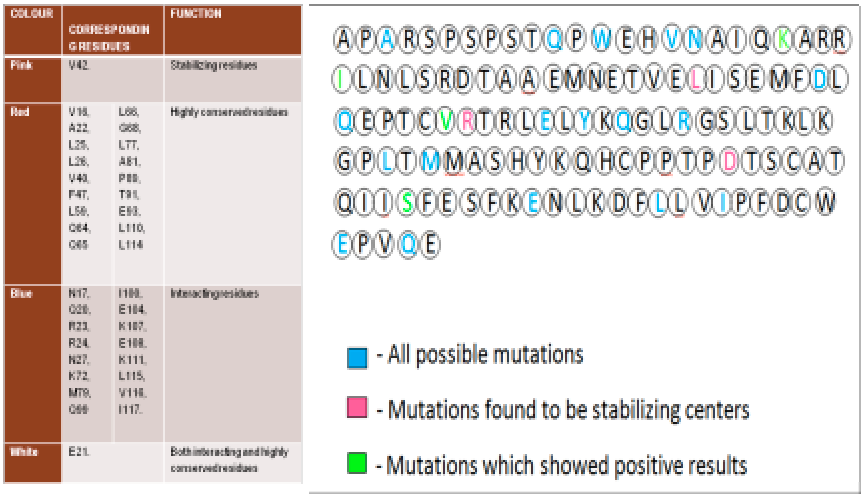

Fig. 8: List of amino acids in human GM-CSF with all the possible mutations that resulted in stabilizing and mutation of positive results

Miyazawa and Jernigan (1994) also provided us with the contact energy values obtained upon replacement of a particular amino acid. The Kolchanov and Shindyalov (1988) states that contact energy is responsible for the structural changes and stability of the molecule helped in concentrating on the contact energy change of the wild type as well as the mutated protein. The possible list of amino acids mutation and contact energy and possible amino acids replacements can be observed in Table 3.

Table 2: Energy minimization values for all possible mutations

Mutations	Minimized energy (Kcal/mol) Using tripos	Using CHARMm
Wildtype	-282.180	-6831.72812
V42L	-289.928	-6983.88120
L55V	-287.663	-6943.10531
Q56R	-289.234	-6895.09892
E93D	-287.738	-6902.64313
T102S	-287.653	-6887.03570
E21K	-285.465	-6723.47718
L25I	-284.234	-6892.63452
V42L and L55V	-287.716	-6964.39428
V42L and Q56R	-289.148	-6916.60819
V42L and E93D	-288.846	-6962.38638
V42L and T102S	-288.703	-6954.43597
L55V and Q56R	-288.308	-6889.48890
L55V and E93D	-286.444	-6900.32703
L55V and T102S	-286.431	-6945.95226
Q56R and E93D	-287.847	-6899.55081
Q56R and T102S	-288.391	6913.49923
E93D and T102S	-286.651	-6896.96613
V42L, L55V and Q56R	-289.535	-6885.54224
L55V, Q56R and E93D	-286.827	-6842.56348
Q56R, E93D and T102S	-286.793	-6903.92071
V42L, L55V and E93D	-287.748	-6931.73223
V42L, L55V and T102S	-287.716	-6964.39428
V42L, Q56R and T102S	-289.399	-6900.90094
V42L, Q56R and E93D	-289.148	-6916.21645
L55V, E93D and T102S	-285.331	-6903.48840
L55V, Q56R and T102S	-287.327	-6908.59759
V42L, L55V, Q56R, E93D and T102S	-286.985	-6859.60819

Table 3: List of amino acids mutation, the contact energy and possible amino acids replacements

Amino acid in wild type sequence and position	Possible list of substitute amino acid	Mutated amino acid	Contact energy
V42	I and L	L	0.70
L55	I and V	V	0.87
Q56	D, E, H, K, N, S, T, Y, W and R	R	0.20
E93	D	D	0.23
T102	D, E, H, K, N, R, S, Y, W and S	S	0.31

The evidences of the instability of the wild type GM-CSF cDNA has been provided in studies with Mycobacterium tuberculosis in (Elmore and Dougherty, 2001) it is suggested that mutation of the protein may lead to a decreased activity in macrophage maturation which triggers excess production of lytic enzyme leading to granuloma formation. This evidence is also supported by Cantrell *et al*. (1985) which discusses the stability enhancement and its regulatory mechanism in cell cycle and apoptosis.

The mutational studies on *Rattus rattus*, *Mus musculus* and *Equus caballus* in (Brown and Botstein, 1999) proved the faster degeneration of cDNA which helped in understanding the

evolution of the protein GM-CSF. The sequence of proteins in these organisms were analysed and the mutations were studied, in which, about 26 mutations were found to be done in the sequence of Human GM-CSF. The Chen *et al*. (2007), Diederichs *et al*. (1991) and Hercus *et al*. (1994), provided the physical evidence regarding binding sites of the receptor and ligand, interacting residues, highly conserved regions and stabilizing residues which was useful during introduction of favourable mutations (Fig. 8). The comparison shows the incorporated mutation leads to structural stability without disturbing the functional and conserved amino acids.

CONCLUSION

The work involves improving the stability of GM-CSF through site-directed mutations. The physical evidences were useful in determining the stabilizing residues and centres in which the residues GLU21, LEU25, LEU55 and THR102 were found to as stabilizing centres after mutation using the tool SCide. Based on CUPSAT results, LEU replaced by VAL at position 55 and THR replaced by SER at position 102 which were found to be favourable and stabilizing. The energy was found to be 16.7196% less for mutated sequence compared to the wild type using CHARMm and TRIPOS SYBYL.

ACKNOWLEGEMENT

The authors thank the Department of Biotechnology, Sri Venkateswara College of Engineering and the Management, Sri Venkateswara College of Engineering for their support.

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