Trends in Bioinformatics

ISSN 1994-7941
Mutational Analysis on Human Granulocyte Macrophage-Colony Stimulating Factor Stability Using Computational Approaches

S. Naga Vignesh, Shrinath Narayanan and M. Sivanandham
Department of Biotechnology, Sri Venkateswara College of Engineering, Affiliated to Anna University, Pennalur, Sriperumbudur Taluk, Tamilnadu, 602 117, India

Corresponding Author: S. Naga Vignesh, Department of Biotechnology, Sri Venkateswara, College of Engineering, Affiliated to Anna University, Pennalur, Sriperumbudur Taluk, Tamilnadu, 602 117, India

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 Ovis 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
Trends Bioinform., 8 (1): 1-13, 2015

(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 (McCammom 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-$\alpha$, IL-1$\beta$ 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 $\alpha$ and a signal transducing $\beta$ 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 Å) (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 residues 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

**Residue conservation:** Chain \( A \) & \( B \) (120 residues)

*UniProt code: P04141 [Pram]*

**Sequence coloured by residue conservation:**

```
SPITOQVCNMAQCPAGLLNLHPRAMLQVEVISPMPLQELCALIVLIEQ
```

**Key:**
- Sec. struc.
- Helix
- Stand
- Motifs: \( \beta \) beta turn, \( \gamma \) gamma turn, \( \beta \) beta hairpin
- Disulphides

Conservation colouring: 1 2 3 4 5 6 7 8 9 High

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.
Table 1: List of amino acids at different position in human GM-CSF 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, I100              | Interacting residues         |
|        | Q20, E104              |                              |
|        | R23, K107              |                              |
|        | R24, E108              |                              |
|        | N27, K111              |                              |
|        | K72, L115              |                              |
|        | M79, V116              |                              |
|        | Q99, I117              | Both interacting and highly conserved residues |
| 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 moL G) 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
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.
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 Using tripos | Minimized energy (Kcal/mol) |
|------------------------|-----------------------------|
| 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 -288.148 | -6961.60819                 |
| 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 | -6900.90994                 |
| 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.

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

REFERENCES
Arakawa, T., S.J. Prestrelski, L.O. Narhi, T.C. Boone and W.C. Kenney, 1993. Cysteine 17 of
recombinant human granulocyte-colony stimulating factor is partially solvent-exposed.
J. Protein Chem., 12: 525-531.
Berrondo, M., 2010. Predicting the structure and function of protein mutants. Ph.D. Thesis, Johns
Hopkins University, Maryland.
Bishop, B., D.C. Koay, A.C. Sartorelli and L. Regan, 2001. Reengineering granulocyte
colony-stimulating factor for enhanced stability. J. Biol. Chem., 276: 33465-33470.
Breitbach, C.J., J. Burke, D. Jonker, J. Stephenson and A.R. Haas et al., 2011. Intravenous
delivery of a multi-mechanistic cancer-targeted oncolytic poxvirus in humans. Nature,
477: 99-102.
Brooks, B.R., R.E. Bruccoleri, B.D. Olafson, D.J. States, S. Swaminathan and M. Karplus, 1983.
CHARMM: A program for macromolecular energy, minimization and dynamics calculations.
J. Comp. Chem., 4: 187-217.
Brown, P.O. and D. Botstein, 1999. Exploring the new world of the genome with DNA microarrays.
Nat. Genet., 21: 33-37.
Cantrell, M.A., D. Anderson, D.P. Cerretti, V. Price and K. Mckereghan et al., 1985. Cloning,
sequence and expression of a human granulocyte/macrophage colony-stimulating factor.
Proc. Natl. Acad. Sci. USA., 82: 6250-6254.
Chen, S., P.G. Schultz and A. Brock, 2007. An improved system for the generation and analysis
of mutant proteins containing unnatural amino acids in Saccharomyces cerevisiae. J. Mol. Boil.,
371: 112-122.
DeLano, W.L., 2002. The PyMOL User’s Manual. DeLano Scientific, San Carlos, CA., USA.,
Pages: 452.
Diederichs, K., T. Boone and P.A. Karplus, 1991. Novel fold and putative receptor binding site of granulocyte-macrophage colony-stimulating factor. Science, 254: 1779-1782.
Dosztanyi, Z., C. Magyar, G. Tusnady and I. Simon, 2003. SCide: Identification of stabilization centers in proteins. Bioinformatics, 19: 899-900.
Elmore, D.E. and D.A. Dougherty, 2001. Molecular dynamics simulations of wild-type and mutant forms of the *Mycobacterium tuberculosis* MscL channel. Biophys. J., 81: 1345-1359.
Eswar, N., B. John, N. Mirkovic, A. Fiser and V.A. Ilyin *et al*., 2003. Tools for comparative protein structure modeling and analysis. Nucleic Acids Res., 31: 3375-3380.
Hansen, G., T.R. Hercus, B.J. McClure, F.C. Stomski and M. Dottore *et al*., 2008. The structure of the GM-CSF receptor complex reveals a distinct mode of cytokine receptor activation. Cell, 134: 496-507.
Hercus, T.R., B. Cambareri, M. Dottore, J. Woodcock and C.J. Bagley *et al*., 1994. Identification of residues in the first and fourth helices of human granulocyte-macrophage colony-stimulating factor involved in biologic activity and in binding to the "- and $-chains of its receptor. Blood, 83: 3500-3508.
Hercus, T.R., D. Thomas, M.A. Guthridge, P.G. Ekert, J. King-Scott, M.W. Parker and A.F. Lopez, 2009. The granulocyte-macrophage colony-stimulating factor receptor: Linking its structure to cell signaling and its role in disease. Blood, 114: 1289-1298.
Higgins, D.G., J.D. Thompson and TJ. Gibson, 1996. Using CLUSTAL for multiple sequence alignments. Methods Enzymol., 266: 383-402.
Kaushansky, K., S.G. Shoemaker, S. Alfaro and C. Brown, 1989. Hematopoietic activity of granulocyte/macrophage colony-stimulating factor is dependent upon two distinct regions of the molecule: Functional analysis based upon the activities of interspecies hybrid growth factors. Proc. Natl. Acad. Sci. USA., 86: 1213-1217.
Kolchanov, N.A. and I.N. Shindyalov, 1988. Single amino acid substitutions producing instability of globular proteins: Calculation of their frequencies in the entire mutational spectra of the "- and $-subunits of human hemoglobin. J. Mol. Evol., 27: 154-162.
Luo, P., R.J. Hayes, C. Chan, D.M. Stark and M.Y. Hwang *et al*., 2002. Development of a cytokine analog with enhanced stability using computational ultrahigh throughput screening. Protein Sci., 11: 1218-1226.
Lyne, P.D., P. Bamborough, D. Duncan and W.G. Richards, 1995. Molecular modeling of the GM-CSF and IL-3 receptor complexes. Protein Sci., 4: 2223-2233.
Marshall, S.A., G.A. Lazar, A.J. Chirino and J.R. Desjarlais, 2003. Rational design and engineering of therapeutic proteins. Drug Discov. Today, 8: 212-221.
McCammion, J.A. and S.C. Harvey, 1988. Dynamics of Proteins and Nucleic Acids. Cambridge University Press, Cambridge, UK., ISBN-13: 9780521356527, Pages: 248.
Metropolis, N., A.W. Rosenbluth, M.N. Rosenbluth, A.H. Teller and E. Teller, 1953. Equation of state calculations by fast computing machines. J. Chem. Phys., 21: 1087-1092.
Miyazawa, S. and R.L. Jernigan, 1994. Protein stability for single substitution mutants and the extent of local compactness in the denatured state. Protein Eng., 7: 1209-1220.
Ng, P.C. and S. Henikoff, 2006. Predicting the effects of amino acid substitutions on protein function. Annu. Rev. Genom. Hum. Genet., 7: 61-80.
Palma, R. and P.M.G. Curmi, 1999. Computational studies on mutant protein stability: The correlation between surface thermal expansion and protein stability. Protein Sci., 8: 913-920.
Trends Bioinform., 8 (1): 1-13, 2015

Parthiban, V., M.M. Gromiha and D. Schomburg, 2006. CUPSAT: Prediction of protein stability upon point mutations. Nucleic Acids Res., 34: W239-W242.

Pikkemaat, M.G., A.B.M. Linssen, H.J.C. Berendsen and D.B. Janssen, 2002. Molecular dynamics simulations as a tool for improving protein stability. Protein Eng., 15: 185-192.

Rozwarski, D.A., K. Diederichs, R. Hecht, T. Boone and P.A. Karplus, 1996. Refined crystal structure and mutagenesis of human granulocyte-macrophage colony-stimulating factor. Proteins, 26: 304-313.

Sali, A., L. Potterton, F. Yuan, H.V. Vlijmen and M. Karplus, 1995. Evaluation of comparative protein modeling by Modeller. Proteins: Struct. Funct. Bioinform., 23: 318-326.

Seeliger, D. and B.L. de Groot, 2010. Ligand docking and binding site analysis with PyMOL and Autodock/Vina. J. Comput. Aided Mol. Des., 24: 417-422.

Thompson, J.D., D.G. Higgins and T.J. Gibson, 1994. CLUSTAL W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res., 22: 4673-4680.

Vazquez-Lombardi, R., B. Roome and D. Christ, 2013. Molecular engineering of therapeutic cytokines. Antibodies, 2: 426-451.

Walter, M.R., W.J. Cook, S.E. Ealick, T.L. Nagabhushan, P.P. Trotta and C.E. Bugg, 1992. Three-dimensional structure of recombinant human granulocyte-macrophage colony-stimulating factor. J. Mol. Biol., 224: 1075-1085.