Site directed mutagenesis of human Interleukin-2 gene to increase the stability of the gene product- A Bioinformatics Approach

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Abstract- Interleukin-2 (IL-2) is an immunoregulatory cytokine whose biological effects are mediated through interaction with specific receptors on the surface of target cells. Due to its presumed role in generating a normal immune response, IL-2 is being evaluated for the treatment of a variety of tumors, in addition to infectious diseases. Main drawback of human IL-2 is that the molecule is relatively unstable. Therefore, with the objective of increasing the stability of the molecule, site directed mutagenesis of human IL-2 gene was carried out. Early studies indicated that mutations at three Cysteine residues (58, 105, 125) which are in the active sites of human IL-2 resulted in the reduced stability as well as the biological activity of the molecule. Therefore, mutations were carried out at the positions of amino acid other than the receptor binding sites at 111Valine to Arginine, 117Lysine to Glutamine and 133 Threonine to Asparagine of the human sequence by comparing it with the bovine sequence which has higher stability than the human counterpart, using SWISS PDB tool. To understand the biological activity of the mutated IL-2, energy minimization studies were carried out using SWISS-PDB. Docking studies were performed to check the reliability of the results using HEX DOCK, ARGUS LAB and PATCH DOCK between the IL-2 receptor and its mutated Ligand. These docking results also confirmed that the reliability of these mutated IL-2 gene. Stability, half life and ADME characteristics of these mutants can be studied in a detailed manner in the in vivo studies.

Keywords: IL-2, in-silico, site directed mutagenesis, cytokine, bioinformatics

Introduction

Interleukin-2 (IL-2) is a type of cytokine immune system signalling molecule, which is instrumental in the body's natural response to microbial infection and in differentiating between the self and non-self antigens (foreign). IL-2 mediates its effects by binding to IL-2 receptors which are expressed by lymphocytes, the cells that are responsible for immunity. Interleukin-2 belongs to a family of cytokines, which includes IL-4, IL-7, IL-9, IL-15 and IL-21. IL-2 signals through a receptor complex consisting of IL-2 specific IL-2 receptor alpha (CD25), IL-2 receptor beta (CD122) and a common gamma chain (γc), which is shared by all members of this family of cytokines. Binding of IL-2 activates the Ras/MAPK, JAK/Stat and PI 3- kinase/Akt signalling modules. IL-2 is normally produced by the body during an immune response. When environmental substances (molecules or microbes) gain access to the body, these substances (termed antigens) are recognized as foreign by antigen receptors that are expressed on the surface of lymphocytes. Antigen binding to the T cell receptor (TCR) stimulates the secretion of IL-2, and the expression of IL-2 receptors IL-2R. The IL-2/IL-2R interaction then stimulates the growth, differentiation and survival of antigen-selected cytotoxic T cells via the activation of the expression of specific genes [12, 13]. As such, IL-2 is necessary for the development of T cell immunologic memory, one of the unique characteristics of the immune system, which depends upon the expansion of the number and function of antigen-selected T cell clones. IL-2 is also necessary during T cell development in the thymus for the maturation of a unique subset of T cells that are termed regulatory T cells (T-regs) [14, 15]. After existing from the thymus, T-Regs function to prevent other T cells from recognizing and reacting against "self antigens", which could result in "autoimmunity". T-Regs do so by preventing the responding cells from producing IL-2. Thus, IL-2 is required to discriminate between self and non-self, another one of the unique characteristics of the immune system. A recombinant form of IL-2 is Proleukin. It has been approved by the Food and Drug Administration (FDA) for the treatment of cancers (malignant melanoma, renal cell cancer), and is in clinical trials for the treatment of chronic viral infections, and as a booster (adjuvant) for vaccines. The role of IL-2 in HIV therapy remains to be fully determined [3, 6]. Many of the immunosuppressive drugs used in the treatment of autoimmune diseases such as corticosteroids, and organ transplant rejection (cyclosporine, tacrolimus) work by inhibiting the production of IL-2 by antigen-activated T cells. Others (Rapamycin) block IL-2R signalling, thereby preventing the clonal expansion and function of antigen – selected T cells. This shows the

importance of IL-2 and the need to increase its stability and thereby improving the activity of the compound. This has led the researchers to study the molecules genetics and induce mutations to increase its half life. Here in this study three mutations are to be induced based on a comparative study with the gene sequence of bovine IL-2. The main objective of this study is to induce site directed mutagenesis of human interleukin-2 gene to increase the stability of the gene product.

Background

In 1984, Lu SD et al [10] cloned the gene encoding human interleukin-2 (IL-2) from human spleen cells, peripheral blood lymphocytes, and the Jurkat cell line. Nucleotide sequence analysis of the gene revealed that the encoded IL-2 protein has three cysteines located at amino acid residues 58, 105, and 125 of the mature protein. Sitespecific mutagenesis procedures were used to modify the IL-2 gene by changing each of the cysteine codons individually to serine codons. Substitution of serine for cysteine residues at either position 58 or 105 of the IL-2 protein substantially reduced biological activity, indicating that the cysteines at these positions are necessary for maintenance of the biologically active conformation and may therefore be linked by a disulfide bridge. The modified IL-2 protein containing a substitution at position 125 retained full biological activity, suggesting that the cysteine at this position is not involved in a disulfide bond and that a free sulfhydryl group at that position is not necessary for receptor binding. In 1986, Anderson D et al [1] reported that Interleukin 2 (IL-2) cDNA clones have been isolated from both human and murine sources. This was accomplished by screening a cDNA library constructed from lectin-stimulated bovine lymph node cells, using a human IL-2 probe. Bovine IL-2 is composed of 155 amino acids and has a predicted molecular weight of 19,555. Alignment of the amino acid sequence with human IL-2 indicates that mature bovine IL-2 is composed of 135 amino acids and has a predicted molecular weight of 15,452. It has an amino acid homology of 65% with human IL-2 and 50% with murine IL-2. Bovine IL-2 is unique among IL-2 homologs in that it has a single N-linked glycosylation site. Biologically active bovine IL-2 was synthesized in an Escherichia coli expression system. In 1986, Barr P J et al [2] isolated a cDNA clone of the bovine interleukin 2 (IL-2) gene and demonstrated to be functional in the production of secreted bovine IL-2 protein when transfected into monkey cells. The bovine IL-2 clone is 791 base pairs in length and contains an open reading frame of 474 base pairs coding for a bovine IL-2 precursor polypeptide of 158 amino acids with an estimated molecular weight of 17,884. The putative hydrophobic leader or signal sequence of the precursor protein is 23 amino acid residues long, suggesting that, after removal by processing; the mature secreted bovine IL-2 protein contains 135 amino acids and has a molecular weight of 15,464. Comparisons of both the nucleotide sequence and the predicted amino acid sequence of bovine IL-2 with those of the human and mouse IL-2 show extensive regions of sequence conservation between the species, interspersed with other regions of less similarity. The 3' untranslated region of the bovine IL-2 gene shares as much, if not greater, sequence homology with the 3' untranslated regions of the human and mouse genes as do the transcribed coding regions of these genes, suggesting an involvement of this region in regulation. In particular, a tandemly repeated sequence, (TATT), found in the 3' untranslated tail of the bovine IL-2 clone is also found in the 3' untranslated region of the other known interleukin and interferon genes, as well as in similar regions of many other inducible genes of the lymphoid and immune response systems, suggesting a cell or tissue-specific regulatory function for these evolutionarily conserved sequences. In 1994, Berndt WG et al [4] used combinatorial cassette mutagenesis to investigate the functional role of a continuous five amino acid region of IL-2 suspected to interact with the intermediate-affinity IL-2 receptor. A limited random library of IL-2 mutants was constructed in which residues 17-21 (Leu-Leu-Leu-Asp-Leu) were simultaneously mutated. The proteins were produced in an *Escherichia coli* expression system and screened in a biological assay for their ability to mediate the proliferation of a murine IL-2-dependent cell line. From the over 2600 clones examined, only 42 exhibited significant activity, confirming the functional importance of this region. Selected clones were purified and further characterized by biological and receptor binding assays. Viewed in the context of the recently revised 2.5-A crystal structure for IL-2, these results suggest the following conclusions: both Asp20 and Leu21, as shown by their sensitivity to mutation, are the functionally more important residues in this region, but for different reasons. Asp20 is solvent accessible and likely plays a direct receptor contact role as previous studies have indicated. Leu21, in contrast, is completely buried in the hydrophobic core of the protein. Substitutions at this position, even a conservative Leu-->Val substitution, were found to perturb the precise hydrophobic packing arrangements that are critical for activity, resulting in a significant loss of function. In addition, one of the analogs identified in the screen was found to be 2-3 times more potent than the wild-type protein. In 2003, Margaret Speed Ricci et al [11] reported that the structure, stability, and interaction of the IL-2 molecule and its receptor are also critical factors

in the regulation of cellular trafficking. The IL-2 receptor is composed of α, β, and γ subunits, and mutational analysis of the Ligand has identified that residues are involved in receptor subunit binding. The α-subunit binding site is located in the short 310 helix of the Ligand, the β subunit is bound by the amino-terminal helix, and the γ-subunit binding site is in the carboxy-terminal helix. Cellular trafficking studies have shown that the α subunit of the receptor is recycled from endosomal compartments, whereas the βγ subunit complex is routed to the lysosomes for degradation. Mutations in the Ligand that disrupt binding to the βγ subunits or increase the affinity to the α subunit can result in greater Ligand recycling and enhanced mitogenic potency without changes to either the extracellular binding affinity or the internalization rate relative to wild-type IL-2 of these analogs has mutations L18M and L19S located in the amino-terminal helix near a site known to be essential for binding to the β subunit of the receptor .his mutant may have a greater endosomal affinity for the recycled α subunit of the receptor or a lower intracellular affinity for the βγ receptor complex, which undergoes degradation in the lysosomes.

Computational Methods

Swiss PDB

In SWISS PDB, initially the Ligand (wild type) in the pdb format was chosen from the file menu. Once, the tool displayed the 3D structure of the chosen ligand chosen. The corresponding receptor was chosen as a pdb format. Interaction between the ligand and the receptor was achieved by using the FIT- Magic fit option in the tool bar. Energy values corresponding to the ligand receptor interactions were displayed. Similar protocol was followed for mutant also. SWISS PDB viewer was used to view the interactions.

Patch Dock

The PDB structure of mutated ligand and receptor of IL-2 were loaded in the RECEPTOR MOLECULE and LIGAND MOLECULE tabs displayed in the tool bar. The results were obtained from the database through email which was viewed using PyMOL VIEWER. The geometric score, the desolvation energy, the interface area size and the actual rigid transformation were calculated for each solution.

Argus Lab

Binding sites residues of receptor and ligand were identified using the "**Q site finder tool".** Binding site residues were separated from the pdb structure with the help of control panel option from the **"Swiss PDB tool".** The amino acid from the ligand group was selected and pasted it in the receptor group for doing calculations. Docked poses with energy were displayed in the molecule tree view window. Most favourable result was the pose with high energy in the negative order.

Hex

In order to run a docking program in HEX Dock, the ligand and receptor sequence in pdb format was provided to the tool and docking was carried out. Docking between the ligand and receptor was progressed and results were obtained.

PROTPARM

ProtParam was used to compute the various physical and chemical parameters of our target given protein sequence. The computed parameters included the molecular weight, theoretical pI, amino acid composition, atomic composition, extinction coefficient, estimated half-life, instability index, aliphatic index and grand average of hydropathicity. This tool required "Swiss-prot" accession number. Once the accession number is entered, all the details about the IL-2 were obtained.

SWISS PROT

This tool was used to obtain the sequence information of a species. The amino acid sequence of Human IL-2 and Bovine IL-2 were obtained using this tool.

BLAST

In this study, protein blast was used to align the human IL-2 sequence with that of the bovine to determine the position were the point mutations can be induced.

Protein Data Bank

The Protein Data Bank (PDB) is a repository for the 3-D structural data of large biological molecules, such as proteins and nucleic acids [5]. The structure obtained can be viewed using VMD, MDL Chime, Swiss-PDB Viewer, StarBiochem and Sirius. Mutational and docking studies can be carried out using these structures.

Results and Discussion

BLAST and SWISS PDB Results

Amino acid sequences of Bovine IL-2 and Human IL-2 were compared using the BLAST tool and the results were shown in Figure 1. From the BLAST results three favourable amino acids with suitable positions as shown in the Table I were chosen. Single point and multi point mutations were carried out using SWISS PDB in the Ligand. The Ligand and the receptor were superimposed and energy minimization studies were carried out using the same tool. Wild type and mutant (multi point mutation at 111, 117 and 133) were superimposed with the receptor (Figure 2). Receptor was indicated by white colour and the

Ligand in violet colour, also Receptor was indicated in green colour and Ligand in blue colour. It was clearly seen that the mutations were induced at three points 111, 117 and 133 which were indicated by white boxes. Energy minimization results clearly showed acceptable energy values for the single point and multi point mutation (Table II). Energy simulation studies showed that mutant IL-2 was relatively more stable than the wild type IL-2 molecule and it was believed that biological activity of mutated IL-2 molecule does not differ from the wild type. In order to confirm whether the energy simulation results were reliable are not, docking was carried out using three bioinformatics tools namely PATCH DOCK, HEX DOCK and ARGUS LAB.

PATCH DOCK Results

Patch Dock results were viewed using PyMOL VIEWER. It was found from the PATCH DOCK result that first result had a very high value of area, atomic contact energy, score and transformation. First result of wild type and mutant Ligand docked with receptor was found to resemble each other. Therefore these results were used for viewing in PyMOL VIEWER. The PATCH DOCK result for mutant type and multi point mutations were clearly seen in Figure 3 and tabulated in Table III.

HEX DOCK Results

HEX DOCK tool gave the energy value for the docked protein. A number of solutions were obtained, out of which first solution was considered as the best fit. It had the highest energy value in the negative order. HEX DOCK results of wild type and mutant were given in the Table IV. The docking between the mutant Ligand (Multi point mutation Ligand) and the receptor was clearly studied (Figure 4).

ARGUS LAB Results

Argus Lab gave the binding energy for the docked molecule. It was not possible to dock large molecule using this tool. Therefore the amino acids present in the binding sites were docked with receptor molecule. The docking between the amino acid residue present in the Ligand binding site and the receptor was understood from this study (Figure 5). Table V shows the binding energy values of the docked structure for 4 different poses. Energy of the docked structure was found to be -5.98 kcal/mol. The binding energy values of mutant were found to be almost similar to the wild type. Therefore it was believed that biological activity of mutant must be similar to the wild type.

Conclusion

Interleukin-2 is a protein made by the human body. IL-2 makes infection-fighting cells multiply and mature. Patients who use IL-2 have large increases in their CD4 cell counts. IL-2 is therefore called as an immune modulator. . T-helper cells, a kind of white blood cell, produce IL-2 when they are stimulated by an infection. Interleukin-2 has been approved by the FDA for the treatment of some types of cancer. Human IL-2 was found to have a very low stability value when compared to other IL-2 counterparts like mice, rat, horse and bovine therefore mutational studies were carried out in the IL-2 sequence. Mutations carried out at receptor binding sites to increase the stability which resulted in the decrease of activity as well as its stability. Therefore, in this study mutations were carried out in the human IL-2 amino acid sequence at those positions other than the receptor binding sites by comparing it with the bovine sequence which has greater stability as well as a homology of 65 percent with the human. Mutations were induced at the positions 111Val to Arg, 117Lys to Gln and 133 Thr to Asn. Mutant Ligand was superimposed with the receptor and energy minimization studies were carried out using SWISS PDB tool. Energy minimization result showed the mutant to be relatively more stable than wild type. Energy results were then cross checked by performing docking studies between IL-2 molecule (wild and mutant type) and its receptor with the tools like Patch Dock, Hex dock and Argus lab. Patch Dock result showed that the atomic contact energy and score of mutant were more than the wild type. Relatively similar score and atomic contact energy between Ligand and the receptor showed the similarity in structural conformation of the bound complex before and after mutations. This showed the molecule to be stable over the wild type. Hex Dock and Argus lab results showed that the mutant molecule have more binding energy than the wild type. This indicated the mutant molecule were more stable when compared to wild type. Therefore from docking results it was inferred that mutant molecule was more stable than the wild type. The Half life, ADME characteristics and effectiveness of these mutations can be further studied if experiments are carried out on wet lab in the mere future.

References

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Tables and Figures

Figure 1: Blast Results
Figure 2: Wild type IL-2 Ligand superimposing with the IL-2 receptor and Mutant IL-2 Ligand (multi point mutation at 111, 117 and 133) superimposing with IL-2 receptor.
Figure 3: PATCH DOCK results for multiple point mutations induced in the IL-2 Ligand
Figure 4: Docking between mutant IL-2 Ligand and the receptor using HEX DOCK
Figure 5: Docked structure using ARGUS LAB
Table I: Mutation Positions and Energy values of amino acids
Table II: Total energy values of the wild type and mutated IL-2 Ligand and Multiple point mutation energy values
Table III: PATCH DOCK results used in PyMOL
Table IV: HEX DOCK energy values
Table V: Binding energy values

Figure 1

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 Score =  144 bits (363),  Expect = 6e-40, Method: Compositional matrix adjust.
 Identities = 101/155 (65%), Positives = 120/155 (77%), Gaps = 2/155 (1%)

Query  1    MYRMQLLSCIALSLALVTNSAPTSSSTKKTQLQLEHLLLDLQMILNGINNYKNPKLTRML  60
            MY++QLLSCIAL+LALV N APTSSST  T  +++ LLLDLQ++L  + N +N KL+RM
Sbjct  1    MYKIQLLSCIALTLALVANGAPTSSSTGNTMKEVKSLLLDLQLLLEKVKNPENLKLSRMH  60

Query  61   TFKFYMPK-KATELKHLQCLEEELKPLEEVLNLAQSKNFHLRP-RDLISNINVIVLELKG  118
            TF FY+PK  ATELKHL+CL EELK LEEVLNLA SKN + R  +D + NI  IVLEL+G
Sbjct  61   TFDFYVPKVNATELKHLKCLLEELKLLEEVLNLAPSKNLNPREIKDSMDNIKRIVLELQG  120

Query  119  SETTFMCEYADETATIVEFLNRWITFCQSIISTLT  153
            SET F CEY D T   VEFLN+WITFCQSI ST+T
Sbjct  121  SETRFTCEYDDATVNAVEFLNKWITFCQSIYSTMT  155
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Figure 2

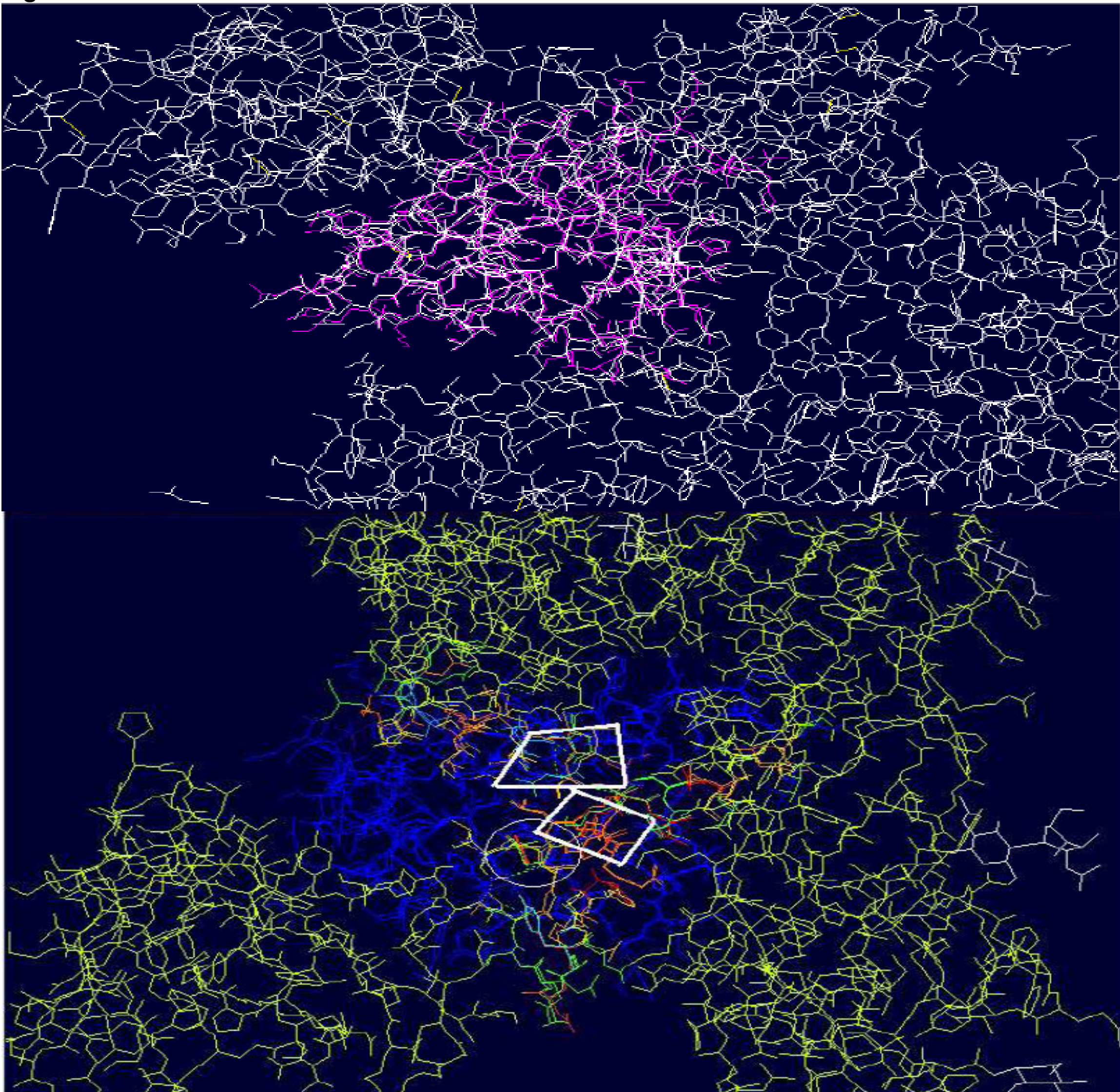

Figure 3

Solution No	Score	Area	ACE	Transformation	PDB file of the complex
1	17176	2058.60	454.82	0.39, 0.02, 2.36, -12.92, -11.45, -0.97	result.1.pdb
2	16576	2076.00	366.84	1.11, 0.32, 1.87, 23.51, -21.74, 22.60	result.2.pdb
3	16140	2388.80	196.43	2.51, 0.68, -1.78, 22.32, -34.85, 50.29	result.3.pdb
4	16050	2756.70	406.54	2.91, 0.24, -0.36, 23.13, 4.90, 60.04	result.4.pdb
5	15744	2238.80	482.53	-2.48, 0.58, -1.67, 47.81, 3.37, 65.51	result.5.pdb
6	15692	2568.60	289.75	-0.08, 1.13, -0.34, 18.79, -50.51, 41.72	result.6.pdb
7	15474	2059.50	258.58	-2.71, 0.11, 1.49, 2.12, -36.45, 77.89	result.7.pdb
8	15300	2622.80	464.12	1.17, 0.56, 1.38, 18.67, -15.13, 25.71	result.8.pdb
9	15224	2419.50	354.56	2.90, 0.68, -1.97, 22.47, -30.53, 60.31	result.9.pdb
10	15200	1845.50	445.27	-0.91, -0.66, 2.56, 30.90, -32.10, 26.14	result.10.pdb
11	15172	1893.80	422.72	-2.02, 0.25, -2.91, 31.16, -25.64, 82.47	result.11.pdb
12	15064	1946.10	291.69	0.88, -0.83, -0.74, -1.68, -19.15, 42.39	result.12.pdb
13	14976	2229.50	355.27	0.88, -0.83, -0.74, -1.68, -19.15, 42.39	result.13.pdb
14	14918	2173.10	403.30	-1.05, -0.13, 2.35, 46.79, -6.77, 72.60	result.14.pdb
15	14608	2433.10	353.65	0.25, -0.70, 0.82, 34.63, -45.73, 38.59	result.15.pdb
16	14532	1763.60	408.61	0.46, 0.46, 2.47, -22.95, -2.23, -2.15	result.16.pdb
17	14484	1773.50	354.74	-0.33, 0.40, -1.44, -35.21, -47.97, 43.62	result.17.pdb
18	14358	2082.90	459.00	2.62, 0.78, 1.19, -6.87, -21.36, 49.29	result.18.pdb
19	14348	2352.70	447.64	1.07, 0.39, 2.05, 22.46, -16.18, 22.70	result.19.pdb
20	14332	1786.10	396.88	1.44, 0.41, -0.64, 3.98, -23.92, 33.06	result.20.pdb

(RMSD: 4)

Figure 4

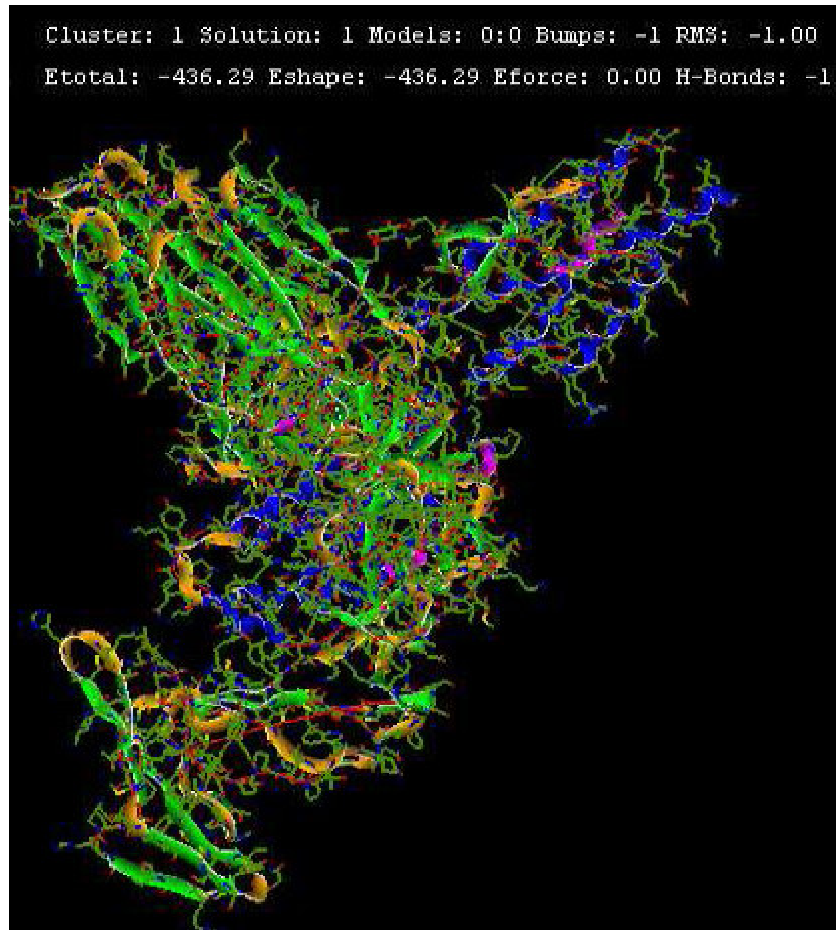

Figure 5

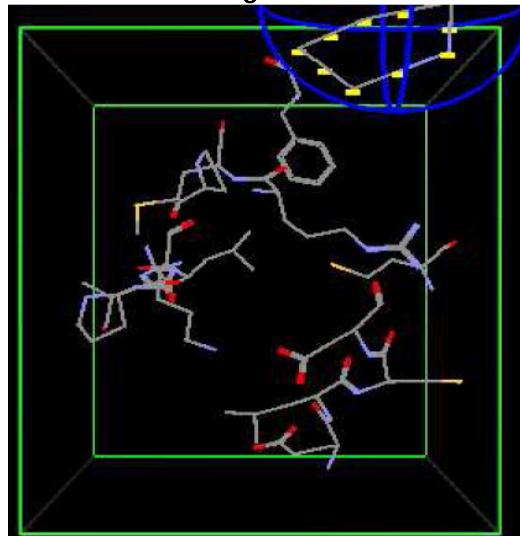

Table I

WILD TYPE AMINO ACID	**ENERGY (KJ/mol)**	**MUTANT AMINO ACID**	**ENERGY (KJ/mol)**
Valine(111)	-33.366	Arginine(111)	-300.251
Lysine(117)	9.442	Glutamine(117)	-161.825
Threonine(133)	-54.433	Asparagine(133)	-184.105

Table II

LIGAND TYPE	**TOTAL ENERGY**
WILD TYPE	-6560.032KJ/mol
ARGININE – 111 MUTANT	-6833.854KJ/mol
GLUTAMINE- 117 MUTANT	-6738.160KJ/mol
ASPARAGINE - 133 MUTANT	-6663.027KJ/mol

AMINOACID	**ENERGY (KJ/mol)**
Arginine(111)	-300.697
Glutamine(117)	-161.882
Asparagine(133)	-184.035
TOTAL(entire molecule)	**-7115.019**

Table IV

LIGAND TYPE	**ENERGY VALUES**
WILD TYPE	**-433.37**
MUTANT (111)	**-440.81**
MUTANT (117)	**-440.38**
MUTANT (133)	**-440.81**
MUTANT **(**Multi point mutation at 111, 117 and 133)	**-436.29**

Table III

TYPE OF LIGAND	SCORE	AREA	ACE	TRANSFORMSTION	PDB FILE OF THE COMPLEX
Wild type	23026	2979.6	453.28	-3.13 0.68 -0.58 18.61 3.43 59.48	result.1.pdb
Mutant (val 111-arg)	19276	3220.0	486.54	-1.98 0.53 -2.64 37.17 -15.04 74.77	result.1.pdb
Mutant (Lys117-Gln)	21208	3314.7	489.66	-2.05 0.44 -2.67 35.21 -20.05 76.95	result.1.pdb
Mutant (Thr133- Asn)	19276	3220.0	486.54	-1.98 0.53 -2.64 37.17 -15.04 74.77	result.1.pdb
Mutant (val111-arg, lys117-gln, Thr133-Asn)	19314	3216.20	477.35	-1.98 0.53 -2.64 37.17 -15.04 74.77	result.1.pdb

Table V

TYPE OF LIGAND	AMINO ACID RESIDUE OF LIGAND IN SITE 2 DOCKED WITH RECEPTOR SITE 2	POSE 1 Kcal/mol	POSE 2 Kcal/mol	POSE 3 Kcal/mol	POSE 4 Kcal/mol
WILD TYPE	**Cys A 58**	-5.45	-5.26	-5.24	-5.05
	His A 55	-5.75	-5.51	-5.33	-5.32
	Lys A 48	-5.86	-5.84	-5.83	-5.64
	Lys A 49	-5.84	-5.80	-5.79	-5.66
	Lys A 54	-5.87	-5.82	-5.74	-5.66
MUTANT (at 111,117,133)	**Arg 91**	-5.47	-5.37	-5.17	-5.16
	Asn 113	-5.87	-5.70	-5.60	-5.55
	Cys 58	-5.41	-5.17	-5.16	-5.03
	Gln 97	-6.20	-5.94	-5.82	-5.66
	His 55	-5.78	-5.66	-5.27	-5.22
	Lys 48	-5.79	-5.57	-5.53	-5.47
	Lys 49	-5.86	-5.82	-5.79	-5.72
	Lys 54	-5.89	-5.84	-5.83	-5.74
	Pro 47	-5.96	-5.70	-5.65	-5.64