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**Research Article**

# AN IN-SILICO STUDY OF EFFECT OF MUTATIONS ON AMP-BINDING (ACYL ACTIVATING) SITE OF ADENYLATE DOMAIN OF SURFACTIN SYNTHETASE

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**A R T I C L E I N F O**

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**A B S T R A C T**

Surfactin, cyclic lipopeptide is a powerful surfactant commonly used as an antibiotic and produced by gram positive endospore forming bacteria by Non-Ribosomal Protein Synthesis system. Surfactin was found to exhibit Characteristics like antibacterial, antiviral, antifungal and antimycoplasma. The optimum conformation of enzyme catalytic site in terms of geometry and potential energy with respect to amino acid change is helpful to optimize the catalysis. In present study surfactin synthetase enzyme's 3D structure and sequence of source bacteria, Bacillus subtilis 168 were retrieved from PDB and NCBI protein sequence database, respectively. It contains 3 domains - condensation, adenylation and thioesterase. Adenylate domain contains acyl activating conserved active site, which was considered for in-silico mutation by other amino acid. The residues in structural pattern were mutated by the software 'Swiss Pdb viewer ver. 4.12' (spdbv) present in the region between the residue number 611 to 624 [611Y-I-M-Y-T-S-G-T-T-G-K-P-K-G624] as well as its adjacent residue. The conformational changes at catalytic site were evaluated on the basis of changed potential energy and geometry. Gromas96 force field was used to calculate the molecular energy before and after geometry optimization. Increase or decrease in total energy (inclusive of bonding and non-bonding energy) with respect amino acid change to that of wild type, were the criteria to distinguish between favorable and non–favorable mutations. We observed that mutations at P609R, A610T, T611Q, I612L, M613F, Y614Q, T615N, S616R, G617E, T618R, T619R, G620N, K621R, P622Q, K623Q, G624T, N625Q, I626N, are found to be favorable mutations P609Y, A610R, T611L, I612P, M613Y, Y613K, T615I, S616G, G617P, T618P, T619G, G620P, K621G, P622Y, K623I, G624Y, N625R, I626P are non- favorable mutations. The reported mutations were subjected to (PAST) software for principle component analysis and correspondence analysis.



## INTRODUCTION

Surfactin was discovered by Arima *et al*. from the culture broth of *Bacillus subtilis* (K. Arima *et al* 1968). It was named thus due to its exceptional surfactant activity (Mor 2000). Surfactin is a very powerful surfactant commonly used as an antibiotic. It is a bacterial cyclic lipopeptide, largely prominent for its exceptional surfactant power (Bonmatin JM *et al*. 1999). Its amphiphilic properties help this substance to persist in both hydrophilic and hydrophobic environments. It is an antibiotic produced by the Gram-positive endospore-forming bacteria *Bacillus subtilis* (Pooja Singh *et al*. 2004). Surfactin have characteristics like antibacterial, antiviral, antifungal, anti-mycoplasma and hemolytic activities (Bonmatin JM *et al* 1999). A large number of bioactive oligopeptides are produced by bacteria and fungi through a unique nonribosomal mechanism. Large modular enzymes mention to as nonribosomal peptide synthetases catalyze the biosynthesis of these low molecular mass peptides. All these multimodular enzymatic assemblies carry out acyl chain initiation, elongation and chain termination, catalyzed by the protein molecules. Studies on the biosynthesis of surfactin began with the work of Kluge *et al*. (B. Kluge *et al*.1988) who proposed a nonribosomal mechanism catalyzed by multienzymatic thiotemplates constituting the surfactin synthetase. The surfactin synthetase complex com prise of four enzymatic subunits. Three of these are enzymes SrfA (E1A, 402 kDa), SrfB (E1B, 401 kDa), SrfC (E2, 144 kDa) and the fourth is SrfD (E3, 40 kDa), which plays an important role in the surfactin initiation reaction (S. Steller *et al*. 2004). Each module of the peptide synthetase consists of different domains and is responsible for the incorporation and modification of one specific amino acid into the peptide and module having co-linear sequence with the sequence of the peptide product. The surfactin synthetase

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complex is encoded by the inducible operon named srfA (25 kb), which is also responsible for sporulation and competence development (L.W. Hamoen *et al*. 2003).The module comprises three domains: (i) from the cognate amino acid and adenosine triphosphate (ATP), the adenylation domain catalyzes the formation of an aminoacyl adenosine and releases pyrophosphate, (ii) covalent bonding of the activated amino acid to 4'-phosphopantetheinyl prosthetic group present on the peptidyl carrier protein (PCP) via a thioester linkage, and (iii) condensation domain catalyzes the direct condensation of the thioesterified intermediate in a growing chain. Chain elongation chemistry in each module is different (K. Reuter *et al*. 1999). (fig.1)

The adenylation domain accountable for the specific recognition of amino acids and activation as adenylyl amino acids. The reaction catalyzed is aa + ATP -> aa-AMP + PPi. These domains are usually found as a part of multi-domain non-ribosomal peptide synthetases and are generally called "A-domains". A-domains are almost regularly support by "T-domains" (thiolation domains, PF00550) to which the amino acid adenylate is transferred as a thiol-ester to a bound pantetheine cofactor with the release of AMP (these are also entitled peptide carrier proteins, or PCPs. When the A-domain does not show the first module (analogous to the first amino acid in the product molecule) it is usually preceded by a "C-domain" (condensation domain, PF00668) which catalyzes the ligation of two amino acid thiol-esters from neighboring modules (RA Konz D *et al*. 1999). The surfactin synthetase enzyme (subunit3) contain adenylation domain and it is 550 amino acid residue long from 478-954. This domain have amp-binding, acyl activating conserved active site, this sites are the catalytic sites of this domain. This conserved site is formed of 12 amino acid from 612 to 623 [612I-M-Y-T-S-G-T-T-G-K-P-K623].

Mutation at catalytic site of the of enzyme lead to decrease in the rate of catalysis and hence decrease in the synthesis of surfactin. The mutation in the catalytic site AMP-binding (acyl activating enzyme consensus motif) conserved active site is responsible for the change in the rate of catalysis as well as geometry. At each position probable 20 possible mutations can happen by different amino acids. A amino acids mutated by amino acid with similar physico-chemical properties does not affect much to function and catalysis, but the amino acids physico-chemical different than the wild amino acid may bring change in structure and function of catalytic site of enzyme. Also the neighbouring amino acids to key residue are important in catalysis of enzyme.

These amino acids greatly influence the conformational properties of catalytic site. Stable conformation always has lowest potential energy. Therefore energy function measurement of enzyme with respect to specific residual mutation is key parameter to evaluate the favourable and unfavourable mutations. Database has very few sequences variants so this experiment was carried out to perform the *in-silico* mutations in protein structure with bound substrate, to generate mutant protein. These protein mutants were analyzed by energy function of 3 main categories - total energy, bonding energy and non-bonding energy. Molecular Energy were calculated after mutation and then after optimization of structure. The variation in protein structure due to residue mutation with respect to different types of energy was studied by multivariate statistical analysis.

The present study was carried out with aim to evaluate the various in-silico derived mutant of adenylate domain at key catalytic site through variation in potential energy function. The 20 mutations at each key positions of catalytic site may cause change in the rate of catalytic activity of the enzyme. In increase or decrease in total energy (inclusive of bonding and non-bonding energy) with respect to amino acid in wild type, are criteria to distinguish between favorable and unfavorable mutations.

## MATERIAL AND METHODOLOGY

### *Retrieval of protein structure*

The structure of surfactin synthetase enzyme as well as sequence were retrieved from the structure repository Protein Data Bank (PDB). Surfactin synthetase-subunit 3 (strain168) having (PDB-ID 2 VSQ) (fig.1). [11]

### *Structure analysis*

The structure has been subjected to mutational analysis .The sequential perspective to study the amino acid residual substitution, significant mutagenesis, and other functionality was derived from UniprotKB having accession no. 'Q08787'subjected to InterProScan for domain analysis of surfactin synthetase enzyme [12] .

### *Mutations and energy calculation*

As per the literature review, the AMP-binding (acyl activating enzyme consensus motif (catalytic site)) of surfactin synthetase was subsequently mutated at residual positions 612 to 623 [612I-M-Y-T-S-G-T-T-G-K-P-K623] using Swiss PDB Viewer to understand the effects, i.e. stability on the peptide during mutation [13]. The energy GROMOS 96 force field of the wild type structure, mutated structures was calculated using default parameters. The energy minimization of the wild type as well as the mutated structures was done using energy minimization tool of Swiss PDB viewer having steepest descent algorithm with a cutoff of 10 angstrom to check the proportionality of bond, angles, improper, torsions, electrostatic bonds and non-bonded.

### *Statistical Analysis*

Statistical analyses were carried out with the help of tool 'PAST'. Two multivariate methods, Correspondence Analysis and Principle Component Analysis (PCS) were used to evaluate the variation in energy of protein molecule at catalytic site with respect to mutations. Scatter plot of each point (each mutations) and eigenvalues loading of component were plotted to study the conformational variation and with respect to energy indicating the impact of mutation, which were compared with wild type structural pattern and its potential energy (A.Amadei *et al*. 1993).

### *Structural alignment*

Structural analysis is carried out with the help of tool 'RCSB chimera' (Pettersen EF *et al* 2004). Structural alignment is a valuable tool for the comparison of proteins with low sequence similarity, where evolutionary relationships between proteins cannot be easily detected by standard sequence alignment techniques. Structural alignments are mostly useful in answer data from structural genomics and proteomics efforts, and they can be used as comparison points to evaluate alignments produced by purely sequence

based bioinformatics methods. The outputs of a structural alignment are a superposition of the atomic coordinate sets and a minimal root mean square deviation (RMSD) between the structures. The RMSD of two aligned structures indicates their deviation from one another.

## RESULTS AND DISCUSSION

A study performed with an objective to find out favorable and non-favorable mutations at catalytic site and its effect on catalytic site. Study was carried out by mutating the protein structure at AMP binding site of catalytic pocket. The mutation was done at key residue of catalytic site and evaluated by the potential energy variation or protein which can be correlated with its confirmation change. Multivariate statistical analysis was carried out to classify the favorable and non-favorable mutations for either stability or catalytic activity.


**Fig 1** Fasta sequence of surfactinsynthetase subunit 168 (Accession no.Q08787)

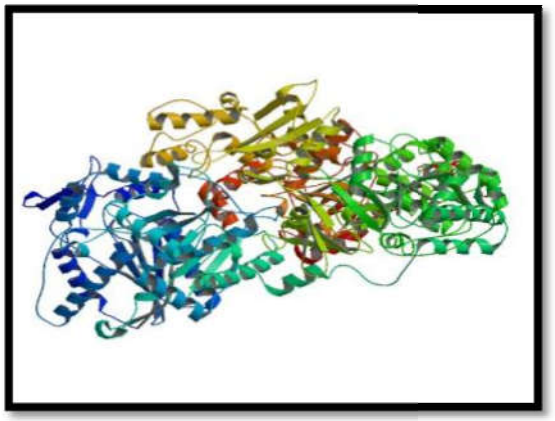

**Fig 2** 2D structural view of Surfactin synthetase enzyme (PDB ID: 2VSQ)

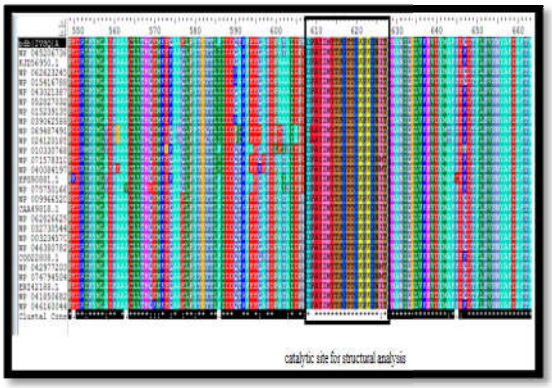


**Fig 3** Conserved catalytic site of different *Bacillus subtilis* strain 168

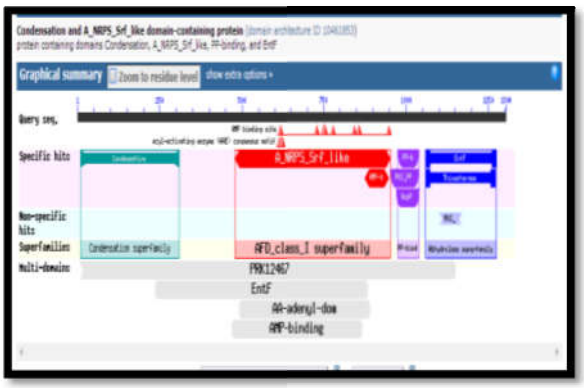


**Fig 4** NRPS _srf like domain containing domain


**Fig 5** Result of InterProScan showing domain region present in 1-1304 amino acid long sequence of surfactin synthetase subunit 3.

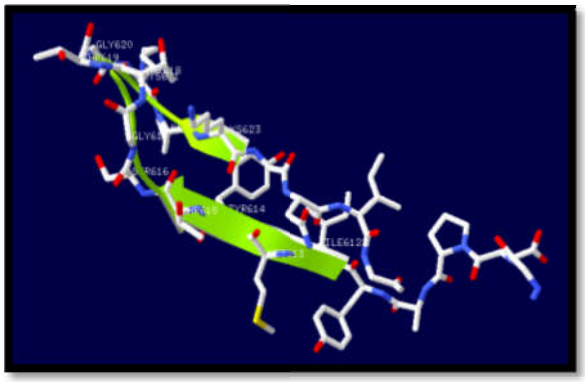

**Fig 6** Bacillus subtilis subunit 3 AMP-binding site pattern (acyl activating enzyme consensus motif) 612 {PAYIMYTSGTTGKPKGNT} 623

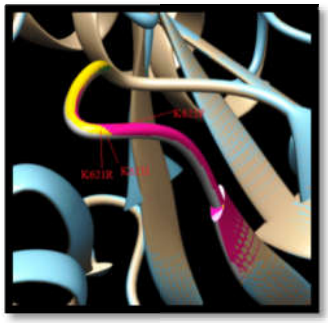

**Fig 7.1** Mutation at position Lys(K)621

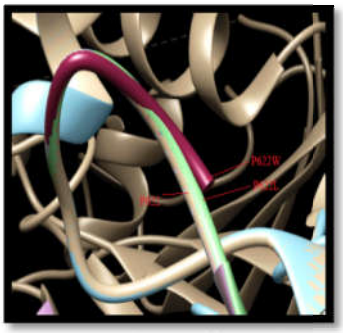

**Fig 7.2** Mutation at position Pro(P)622

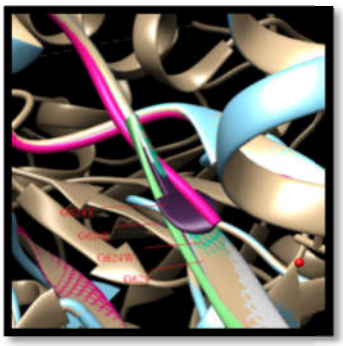

**Fig 7.3** Mutation at position Gly(G)624

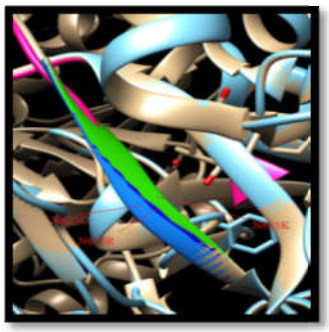

**Fig 7.4** Mutation at position Asn(N)625

**Fig 7** Structural Alignment of Mutated Structure

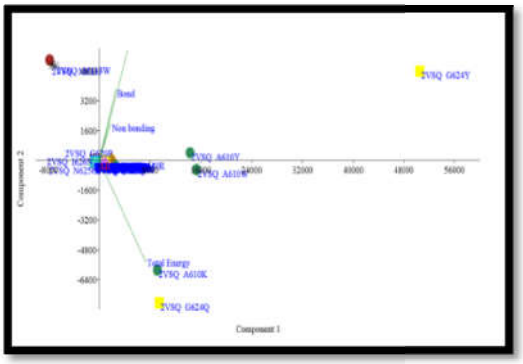

**Fig 8** Scatter plot (PCA), after energy minimization

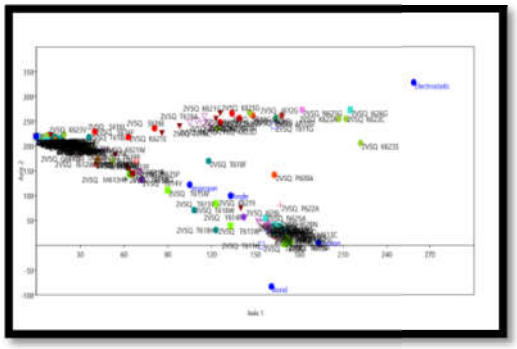

**Fig 9** scatter plot (CA) after energy minimization

### *Retrieval of surfactin synthetase enzyme structure and sequence*

Surfactin synthetase enzyme –subunit 3 structure was retrieved from the Protein Data Bank (PDB) of PDB ID: 2VSQ and Fasta sequence was retrievend from NCBI PROTEIN database of accession number 2VSQ _A of source organism *Bacillus subtilis* strain 168 It is involved in the biosynthesis of antibiotic surfactin. Fig 2 shows 2D structure and Fig 1 sequence of surfactin synthetase.

### *Conserved Catalytic Patterns of Surfactin Synthetase Enzyme*

Fig 3 is multiple sequence alignment of member sequences of Surfactin synthetase enzyme visualizing catalytic site. Pattern of catalytic site was generated from the multiple sequence alignment. According to the reference sequence as visualized in BioEdit, the pattern is spanning between the region amino acids 612 – 623, which is conserved catalytic site [IMYTSGTTGKPK] for AMP binding (acyl activating enzyme consensus motif).Pattern Sequence: 612 {IMYTSGTTGKPK} 623

### *Multiple Sequence Alignment*

Multiple Sequence Alignment of 28 surfactin synthetase sequences of different *Bacillus subtilis* strains was carried out through Clustal Omega software for detection of conserved sites (fig-3). Multiple Sequence Alignment of 28 sequences shows highly conserved at many regions throughout the alignment indicating similarity of surfactin synthetase sequences of different strains of *B. subtilis*. Separate clustering of surfactin synthetase sequences is observed indicating functional similarity. Variable regions throughout the alignment are highly conserved among the class of surfactin sequences. These regions are responsible for generation of variation in surfactin cyclic peptide leading to production of different types of surfactin. Regions of insertion/deletion can be observed in as presented by gap along the alignment of sequences. These insertion or deletions at specific regions are common to all member sequences of specific class of surfactin synthetase, which is at different position in other class.

### *Domain and Catalytic site Analysis*

Unit of surfactin synthetase sequence from *Bacillus subtilis* strain 168. Each fragment was analyzed for the presence of adenylation domain by the online tool "InterProScan", "prosite", "Pfam", "UniProt". The sequence of surfactin synthetase of *Bacillus subtilis* strain 168 domain for amino acid adenylation was separated and location is given in fig-4 and fig-5. Example of domain analysis by using the sequences of *Bacillus subtilis* strain 168 of accession no. Q08787. Domain analysis of surfactin synthase sequence of amino acid of 1-1304 by InterProScan has indicated the presence of 8 domains in query protein.

### *Mutation and Eenergy Ccalculation*

The residues at catalytic site as well as adjacent residue in structural pattern were mutated by the software 'Swiss Pdb viewer ver. 4.12' (spdbv) present in the region between the residue number 612 [P-A-Y-**I-M-Y-T-S-G-T-T-G-K-P-K**-G-N-T] 623. The conformational changes at catalytic site were evaluated on the basis of changed potential energy and geometry. Gromas96 force field was used to calculate the potential energy before and after geometry optimization.

**Table 1** list of favorable and non favorable mutation at catalytic site and adjacent site with their potential energy.

| RESIDUE NO. | MUTATION | TOTAL ENERGY | FAVORABLE /NOT FAVORABLE |
|---|---|---|---|
| 609 | P609R | -12472.962 | FAVORABLE |
| 609 | P609Y | -12123.44 | NON FAVORABLE |
| 610 | A610K | -1024.146 | FAVORABLE |
| 610 | A610W | 603.931 | NON FAVORABLE |
| 611 | T611Q | -12358.451 | FAVORABLE |
| 611 | T611L | -12010.841 | NON FAVORABLE |
| 612 | I612L | -21359.943 | FAVORABLE |
| 612 | I612P | -12217.121 | NON FAVORABLE |
| 613 | M613F | -20869.486 | FAVORABLE |
| 613 | M613Y | -11915.217 | NON FAVORABLE |
| 614 | Y614Q | -12324.743 | FAVORABLE |
| 614 | Y614K | -12044.14 | NON FAVORABLE |
| 615 | T615N | -12460.529 | FAVORABLE |
| 615 | T615I | -12156.801 | NON FAVORABLE |
| 616 | S616R | -12574.932 | FAVORABLE |
| 616 | S616G | -12195.523 | NON FAVORABLE |
| 617 | G617E | -12357.997 | FAVORABLE |
| 617 | G617P | -12190.036 | NON FAVORABLE |
| 618 | T618R | -12513.372 | FAVORABLE |
| 618 | T618P | -12127.358 | NON FAVORABLE |
| 619 | T619R | -12562.379 | FAVORABLE |
| 619 | T619G | -12223.406 | NON FAVORABLE |
| 620 | G620N | -12476.551 | FAVORABLE |
| 620 | G620P | -10340.024 | NON FAVORABLE |
| 621 | K621R | -12460.505 | FAVORABLE |
| 621 | K621G | -12206.972 | NON FAVORABLE |
| 622 | P622Q | -12494.275 | FAVORABLE |
| 622 | P622Y | -11798.328 | NON FAVORABLE |
| 623 | K623Q | -12441.77 | FAVORABLE |
| 623 | K623I | -12109.676 | NON FAVORABLE |
| 624 | G624T | -12038.104 | FAVORABLE |
| 624 | G624Y | 25839.027 | NON FAVORABLE |
| 625 | N625Q | -12141.948 | FAVORABLE |
| 625 | N625K | -11857.801 | NON FAVORABLE |
| 626 | I626N | -12452.05 | FAVORABLE |
| 626 | I626P | -12157.62 | NON FAVORABLE |

**Table 2** Potential Energy table of mutated residue (reference, before and after minimization)

| Energy Mini | Residue | Bond | Angle | Torsion | Improper | Non bonding | Electrostatic | Energy | Total Energy |
|---|---|---|---|---|---|---|---|---|---|
| Reference | 2VSQ A610 | 0.558 | 1.775 | 4.714 | 0.034 | -30.24 | -4.23 | -27.391 | -12274.793 |
| Before mini. | 2VSQ A610Y | 1.697 | 13.907 | 5.284 | 1.179 | 101657.48 | -14.06 | 101665.5 | 190903.266 |
| After mini. | 2VSQ A610Y | 3458.68 | 323.657 | 5.781 | 21.633 | 4016.57 | -59.93 | 7766.392 | -695.494 |
| Before mini. | 2VSQ A610W | 19.549 | 22.67 | 5.788 | 3.61 | 31767600 | -2.36 | 31767650 | 63522892 |
| After mini. | 2VSQ A610W | 56.898 | 195.57 | 24.434 | 1.259 | 762.62 | -7.85 | 1032.933 | -1024.146 |
| Before mini. | 2VSQ A610K | 1.527 | 2.633 | 5.603 | 0.114 | 394299.56 | -4.86 | 394304.6 | 776368.438 |
| After mini. | 2VSQ A610K | 56.898 | 195.57 | 24.434 | 1.259 | 762.62 | -7.85 | 1032.933 | -1024.146 |
| Reference | 2VSQ G620 | 0.281 | 1.544 | 5.909 | 0.021 | -16.8 | 38.97 | 29.926 | -12274.793 |
| Before mini. | 2VSQ G620P | 1.032 | 809.836 | 21.412 | 581.017 | 10665831 | -25.42 | 10667219 | 21320712 |
| After mini. | 2VSQ G620P | 231.072 | 751.535 | 10.302 | 325.63 | 386.03 | -23.35 | 1681.213 | -10340.024 |
| Reference | 2VSQ G624 | 1.243 | 2.192 | 3.326 | 0.001 | -23.1 | 28.01 | 11.673 | -12274.793 |
| Before mini. | 2VSQ G624Y | 2.697 | 14.278 | 3.657 | 1.161 | 65369348 | -33.51 | 65369336 | 130726320 |
| After mini. | 2VSQ G624Y | 17230.6 | 463.929 | 10.074 | 145.04 | 10211.46 | -28.35 | 28032.72 | 25839.027 |
| Before mini. | 2VSQ G624Q | 1.76 | 3.376 | 5.397 | 0.038 | 9824.53 | -157.89 | 9677.212 | 7208.104 |
| After mini. | 2VSQ G624Q | 51.022 | 151.627 | 6.786 | 10.787 | 10.787 | 265.69 | -172.48 | 313.432 |

Increase or decrease in total energy (inclusive of bonding and non-bonding energy) with respect amino acid change to that of wild type, were the criteria to distinguish between favorable and non–favorable mutations. Energy Minimization is a procedure that attempts to minimize the potential energy of the system to the lowest possible point. This can be a severe issue, as there is only one global minimum of the energy surface, but many local minima. If steps are not taken to avoid it, a Minimization algorithm might get stuck in a local minimum without ever finding the global minimum. In most cases, this is what will actually happen, finding a global minimum is often not necessary. Minimization algorithms examine the first (and in some cases second) derivatives to determine whether they are at a minimum. Some wrong contacts may cause large, unphysical variation in potential energy in subsequent dynamics simulations, or possibly other artifacts. A brief minimization can remove such potential problems. As a result, minimization is generally not done as an end unto itself, but to prepare for another type of calculation and must be done under the same conditions as the later calculation.

***Structural Alignment of Mutated Structure***

The products of a structural alignment are a juxtaposition of the atomic coordinate sets and a minimal root mean square deviation (RMSD) between the structures. The RMSD of two aligned structures indicates their divergence from one another. Difficulty arise in structural alignment because of the presence of multiple protein domains within one or more of the input structures, because changes in relative orientation of the domains between two structures to be aligned can artificially inflate the RMSD. Change in amino acid/ mutation at specific

position by physic-chemically unrelated amino acids may bring changes in conformation in protein either in terms increase or decrease in catalytic pocket size, change in secondary conformation resulting to loss or modification of catalytic activity. Sometimes energy may be minimum of mutated residue and protein after energy minimization/geometry optimization, due to conformational change. Therefore merely change in potential energy will not provide view of functional change, it's necessary to observe the geometry of protein. Structural alignment of mutants generated by various amino acids with wild amino acid protein was done by Chimera software and images of alignment focusing only specific mutant residue were taken (Fig-7)

***Principle component analysis***

Principal Component analysis gives the relationship of mutations with the respect to different energy components and its contribution in variation. Energy of the mutants before minimization is gathered around origin. Maximum mutations have shown the increased energies due to clash of side chains. After energy minimization the side chains of amino acids get geometry optimized and orient in such a way that it'll experience less steric hindrance and achieve lowest potential energy. Seven components of the total energy factor were analyzed through PCA. Few positions are observed to be very important with respect to stability and its role in catalysis. Positions, A610, G620, and G624 are important and sensitive for mutation by bulky and polar amino acids. Glycine and Alanine are small sized aliphatic amino acids, therefore the replacement of these residues by other bulky and polar amino acids always deform the 3D local structure, disturbing the catalytic pocket. The PCA (Fig. 8) scatter-plot shows that mutations, G624Y, G624Q are in opposite quadrant that of bond, non-bonding energy, indicating opposite correlation with respect to other mutations. Mutations A610Y, A610W, A610K are also away from the bond and non-bonding component indicating the high potential energy not in correlation with respect to other mutations. Mutations G620P is also on the side of bond and non-bonding energy but against the total energy component. Though this mutation has adjusted the local conformation but overall 3D structure of the protein is disturbed. Pro (P) is ring structured rigid amino acid responsible for disturbing the helix and beta strand conformation of the protein. These mutations are also presented in table-2 with its all energy components.

***Correspondence analysis***

In a similar way to principal component analysis, it gives a means of promoting a set of data in two-dimensional graphical form. All data should be nonnegative and on the same scale for CA to be applicable, keeping in mind that the method treats rows and columns equivalently. CA is a detailed technique; it can be applied to tables whether or not the statistic is appropriate. From correspondence analysis we find that residue I626G, K623C, N625G, T611G, P609A, K623S are clustered towards electrostatic energy. Residues G624Y, T618I, N625Q is clustered towards bonding, non bonding, and total energy located at axis 2. Residues N625C, M613C, N625A, and T615K is clustered towards torsion energy located at axis 1. So each mutation is clustered on the basis of different energy.

## CONCLUSION

All the positions were more or less affected by mutations, but mutations at P609, A610, Y614, T615, S616, G617, T618, G620, P622, K623 and G624 were observed to be more affected due to mutation by physico-chemically unrelated amino acids that have changed its conformation. Distortion in secondary conformation were observed in the mutant's structures mutated by the bulky aromatic residues at positions, P609, A610, G617, T619, P622 and G624. Polar charged and uncharged residues have greatly distorted wild type secondary conformation (β strand) at positions, P609, A610, S616, G617. Gly(G) a flexible amino acid has greatly influenced the secondary conformation at T619 and S616. The most key position sensitive for mutations by maximum amino acids are S616, G617, P622 and G624. Most deleterious mutations observed to be, G624Y, A610M, A610W, G620P, A610R.

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