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FAD MOLECULAR ADAPTABILITY AMONG SURROUNDING AMINO ACIDS AND ITS CATALYTIC ROLE IN GLUCOSE OXIDASEAND RELATED FLAVOPROTEINS

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ABSTRACT: Flavine adenine dinucleotide (FAD) based enzymes are used in medicinal industry. Like any enzyme, the flavoproteins show a pH - temperature dependent activity.The central point of the study is to find an answer to the question on the key role of the amino acid network surrounding the FAD moiety within the FAD-containing enzymes, glucose oxidase (GOx) included, on the catalytic activity of such enzyme(s) in different environmental conditions such as temperature, pressure and pH. Specifically, GOx is studied in the range ofpH4-8 and temperature domain from20C0 to 50Co.The FAD cofactor and surrounding amino acids were studied in different conditions and interrelations to each other. Mutation in amino acids structure is proposed for both the outer amino acids and those belonging to the binding pocket. In addition,a homology model and docking procedures were performed.

KEYWORDS: Flavine adenine dinucleotide , gluco oxidase, molecular adaptability, amino acid moiety, docking, molecular dynamics, quantitative structure activity relationship

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1.INTRODUCTION

Flavine adenine dinucleotide (FAD) based enzymes are used in medicinal industry. Like any enzyme, the flavoproteins show a pH - temperature dependent activity. The central point of the study is to find an answer to the question on the key role of the amino acid network surrounding the FAD moiety within the FAD-containing enzymes, glucose oxidase (GOx) included, on the catalytic activity of such enzyme(s) in different environmental conditions such as temperature, pressure and pH. Specifically, GOx is studied in the range of pH4-8 and temperature domain from20C^0 to 50C^o.The FAD cofactor and surrounding amino acids were studied in different conditions and interrelations to each other. Mutation in amino acids structure is proposed for both the outer amino acids and those belonging to the binding pocket. In addition, a homology model and docking procedures were performed. Flavine adenine dinucleotide (FAD) is a redox cofactor tightly bound to amino acid network of a given enzyme, more specifically a prosthetic group.FAD shows three redox states: hydroquinone, quinone and semiquinone [1], the conversion within these states being achieved by accepting or donating electrons. Fully oxidized form, the quinone FAD, accepts two electrons and two protons to give the hydroquinone, FADH2. If only one electron and one proton is transferred, it results asemiquinone, FADH. The redox function is realized on the isoalloxazineskeleton. FAD has an adenine dinucleotide or a mononucleotide (i.e., FMN) bridge, also involving phosphate groups [2]. Among flavoproteins, those with a covalently linked FAD represent only 5-10 %; these enzymes show a stronger redox power [3]. FAD provides structural support for the active sites and/or stabilization of intermediates during catalysis [4]. The number of flavoproteins encoded genes in the genome is species dependent and ranges 0.1-3.5% within humans, having 90 flavoproteins encoded genes [5]. FAD is the most complex and abundant form of flavine and it binds up to 75% of the total flavoproteins and 84% of human flavoproteins [6]. The glucose oxidase GOx enzyme is an oxidoreductase β-D-glucose:oxygen 1-oxidoreductase)that catalyzes the oxidation of α-D-glucose to D-glucono-σ-lactone, which is non-enzymatically hydrolyzed to gluconic acid. In the reaction, the FAD ring of GOx is reduced to FADH2, and the reduced GOx is re-oxidized by utilizing molecular oxygen to yield hydrogen peroxide (H2O2). GOx found many applications in biotechnology and modern diagnostics, typical assays including biosensors useful in determination of free glucose in body fluids [7,8]. Literature data suggest that FAD has different conformations **[9]**, in the different flavoprotein enzymes (with different reaction type catalysis and different substrate). Variation in enthalpy, entropy and Gibbs free energy of FAD and the constitutive amino acids (Aa) of the surrounding “capsule“, has been described in some papers [10**].** A significant decrease in GOx activity was reported when the enzyme is immobilized on a surface, e.g., the graphene or nanotube surface [11]. The published data also suggest that the catalytic activity depends on GOx orientation and exposure to solvent, type of immobilizing material (e.g., graphene),on pH and temperature, respectively. Recall that some optimized mutant GOx are commercially available. The optimized

GOx show the optimal enzymatic activity varying from pH7 at 25ºC to pH 5.1 at 35ºC. Existence of such an optimized enzyme suggests that GOx is susceptible to further optimization [12]. The ranges of pH and temperature, relevantin our study, are: (i) {26-27ºC, at pH 6.5}(the optimal activity domain for the wild enzyme); (ii){37-38ºC, at pH 7.45}(the human body domain, i.e., the euthermia domain) and(iii) {45-46ºC} (the temperature eventually reached by the bio-nano-device activity, also called the hyperthermia [13] domain). A comparison, in terms of stability, energy, activity and structure of GOx, within the three target condition domains,is needed. First, conformational studies are to be performed, to establish a correlation between FAD conformation and the enzyme optimal activity. Various protonation of the studied molecular structures would allow an insight to FAD-Aa system behavior; Molecular dynamics investigation, at various temperature, pH, pressure and dielectric constant, would complete these studies [14].

2. MATERIALS AND METHODS

In achieving an enzyme function optimization, computations were performed to establish if there exists a correlation between FAD geometry and the enzymatic activity of flavoproteins [15] .In this respect, a number of 15 flavoproteins (i.e., FAD-containing proteins) and the amino acids accompanying FAD were studied. The 15 flavoproteins herein computed are (by PDB ID): 1GPE, 4YNT, 4XWR, 2WDW, 1W1J, 2JBV, 2VFR, 3T37, 3TX1, 4H7U, 4KGD, 4MIF, 5EPG, 5HSA, and 3NVZ.The flavoproteins above listed originate in 12 microbial species, 1 comes from mammalian and two are human flavoproteins. All these protein files have been generated from crystallographic data (PDB files see the supplementary materials). For each PDB structure, the interaction between FAD and its surrounding amino acids, was computed as: total energy of interaction, external ligand interaction, protein-FAD interaction energy, FAD-Aa complex steric energy, water molecules - FAD interaction energy, FAD torsion energy, FAD steric energy, FAD-Aa complex at the enzymes binding pocket (hydrogen bonds, global (non-directional) energy, electrostatic energy and hydrogen bonds, were calculated; all energies are expressed in kcal/mol. Results are shown in Fig. 1. For a detailed view of FAD behavior in relation with the surrounding amino acids, particularly the outer amino acids network [16], which is theoretically more susceptible to environmental conditions changing, ”in silico” models of GOx were realized, using the PDB structure 1GAL. The crystallographic generated structure file, of 2.3 Å resolution, was energetically minimized, charges calculated, and atoms valences satisfied. To simulate the pH and temperature conditions, the structures were protonated in the pH range 4-8 while the temperature was varied from 20ºC to 50ºC.For the outer cell conditions, the pressure was set to 10 KPa, corresponding to interstitial pressure of most of the solid tissues [17] ;the dielectric constant of water was set according to the temperature (see the additional material) while the dielectric constant of GOx was set 3.The variables related to potential and kinetic energy (in kcal/mol) of the molecular system, at a time, selected pH and temperature domains, instantaneous pressure P (in KPa) of the system and instantaneous volume

(in cubic angstroms), were calculated. Thus for a pH domain from 4 to 8, 155 GOx 1 subunit PDB structures were generated. For structure equilibration, an NPH [18] (Nose-Poincaré-Hoover) and BER (Berendsen velocity/position scaling methodology) based algorithms were used.For the entire GOx system, the potential energy, kinetic energy, pressure and volume were represented for three temperature domains: 26-27 ^{o}C;37-38 ^{o}C; 45-46^{o}C and 4 pH states: 4,5,6 and 7, respectively. Similarly, FAD total energy, FAD heat of formation, FAD steric energy, hydrogen bonds energy, electrostatic interactions energy, total FAD-GOx-Aa interactions, torsional strain and steric energy of FAD alone, were computed. A homology model [19,20], having 1GAL as the sequence template, was developed; the identifier matrix was based on the following PDB and Uniprot amino acid sequence: 1GPE (587Aa), 1CF3A (581AA), 2JVB (529 Aa), 1JU2 (521 Aa) , U NQ16 (555 Aa), U 1BZ9 (552 Aa), U RXH5 (502Aa), U V371 (529 Aa), U 5751 (531 Aa). Ten protein models were developed (see the additional material) while the final model was obtained after energy minimization For the last model, the contact energy and electronic energy were calculated. A comparative docking study [21] on GOx monomer 1Gal and on homology model monomer was performed, with FAD and glucose as ligands; after the docking studies, the binding affinity was represented. A Molecular Dynamic study was performed on the three domains of interest (27^{o}C, pH 6.5); (37^{o}C, pH 7.4) and (46^{o}C, pH 7.4) respectively; RMSD of Debye-Waller factor (i.e., B factor) are computed. Entropic energy of FAD at optimal conditions, also at the body temperature and hyperthermia, contact areas, exposed areas, percent of exposed areas and close distances are calculated for the amino acids at the binding pocket, in order to model the optimal interactions at a chosen pH and temperature. Amino acids were mutated and free Gibbs energy was calculated to assessed the stability of the protein. In view of exploring the (inner and outer sets of) amino acids adaptability to temperature and pH conditions and subsequent GOx activity optimization, the contact areas and exposed areas of amino acids and percent of amino acid exposure occurring in FAD - Aa complex were calculated. Two states were particularly explored: {26.82~27 ^{o}C; pH 6.5 - the optimal activity state} and {46^{o}C; pH 7.5 -the hyper-thermal state}. For these two states, the parameters above mentioned were calculated. The conformational changes of amino acids at the FAD binding pocket would determine the changes in FAD structure/activity. Mutations [22] were made on 1GAL PDB in the purpose of optimizing amino acid contacts and exposure. The method used for replacing the amino acids is the replacing of one amino acid at hyperemic state with another one at the same hyper-thermal state but with contact or exposure areas approximately the same as for the optimal activity state. Those amino acids for which no corresponding mutation alternative was found, were further neglected (see Tables 1and 2 and the additional material). For the protein global stability [23,24], the Gibbs free energy variation, consecutively to amino acid mutation, between the wild type and mutated type, were calculated and represented in Figs. 11 and 12.In order to intrinsically study the Aa –FAD systems, Aa networks were constructed using Cytoscape and RIN application package. The resulted networks were analyzed.

Furthermore FAD within 15 molecules was analyzed. To find a relation between Aa network and FAD, in terms of the biological activity, a QSAR modeling was performed on the following Aa – FAD based networks computed parameters: clustering coefficient, number of connected components, network diameter, network centralization, shortest path, characteristic path length, average number of neighbors, number of nodes, network density and network heterogeneity respectively. FAD related data used in QSAR models (as independent variables) were: surface area, volume and hydration energy, respectively; three QSAR multiple linear regression(MLR) models were built up on the input data as a training set and validated by leave one out technique. When Z test was applied, no outliers have been detected.

3. RESULTS AND DISCUSSION

The interactions in FAD- amino acids complex are represented in Fig.1. One can see that GOx has the lowest steric energy for FAD - Aa (outer and inner) complexes; FAD hydrogen bonds energies are situated in the same energy domain as other flavoproteins (between-30 and -60 kcal/mol). The entire GOx complex shows an increased potential energy (see Fig. 2) with increasing the temperature. At pH 4 and 50^{o}C, GOx has the biggest energy, of-16723.979 kcal/mol; it decreases to -16690.881 kcal/mol, in going to pH 7 and 50^{o}C. Similarly, the kinetic energy of GOx is -8702.0293 at pH 4 and 50^{o}C, the highest obtained in the studied pH and temperature range. Kinetic energy values at pH 6 and 7 show approximately the same variation: from {-7882.8006kcal/mol at pH 6 and 20^{o}C} to {-8690.5771 kcal/mol at pH 6 and 50^{o}C} and from {-7881.0586 kcal/mol at pH 7 and 20^{o}C} to {-8687.5801 kcal/mol at pH 7 and50^{o}C). The volume of the system has the biggest value, of 180057.593 A^3, at pH 4 and50^{o}C; also its pressure shows the highest value at pH 6 and 50^{o}C, namely 1934583.90 kPa. Kinetic energy is pH and temperature dependent (see Fig.3). In order to increase the enzyme activity, the kinetic energy must be decreased. Our target energy ranges from -8043.7007 (at 26^{o}C) to -8.070.58915 (at 27^{o}C), averaged at-8057.1449 kcal kinetic energy; this mean value has to be modified to - 8568.0298kcal/mol (coming from -8554.583Kcal/mol at 27^{o}C and- 8581.477 at 46^{o}C, respectively). Thus, the kinetic energy must be decreased by 524.332 kcal/mol; it is achievable by a better covering of GOx with some added biopolymers, like PEI, PAMAM, etc. Pressure of GOx system varies in a pH and temperature dependent manner (see Fig. 4). In order to optimize GOx activity at pH interval 6-7 and temperature intervals 45-46^{o}Cthe pressure must be decreased with 13856.62 kPa. System volume needs a decrease with 14.297 A^3 at pH intervals 6-7 (Fig. 5); volume remains unchanged in respect to temperature conditions.

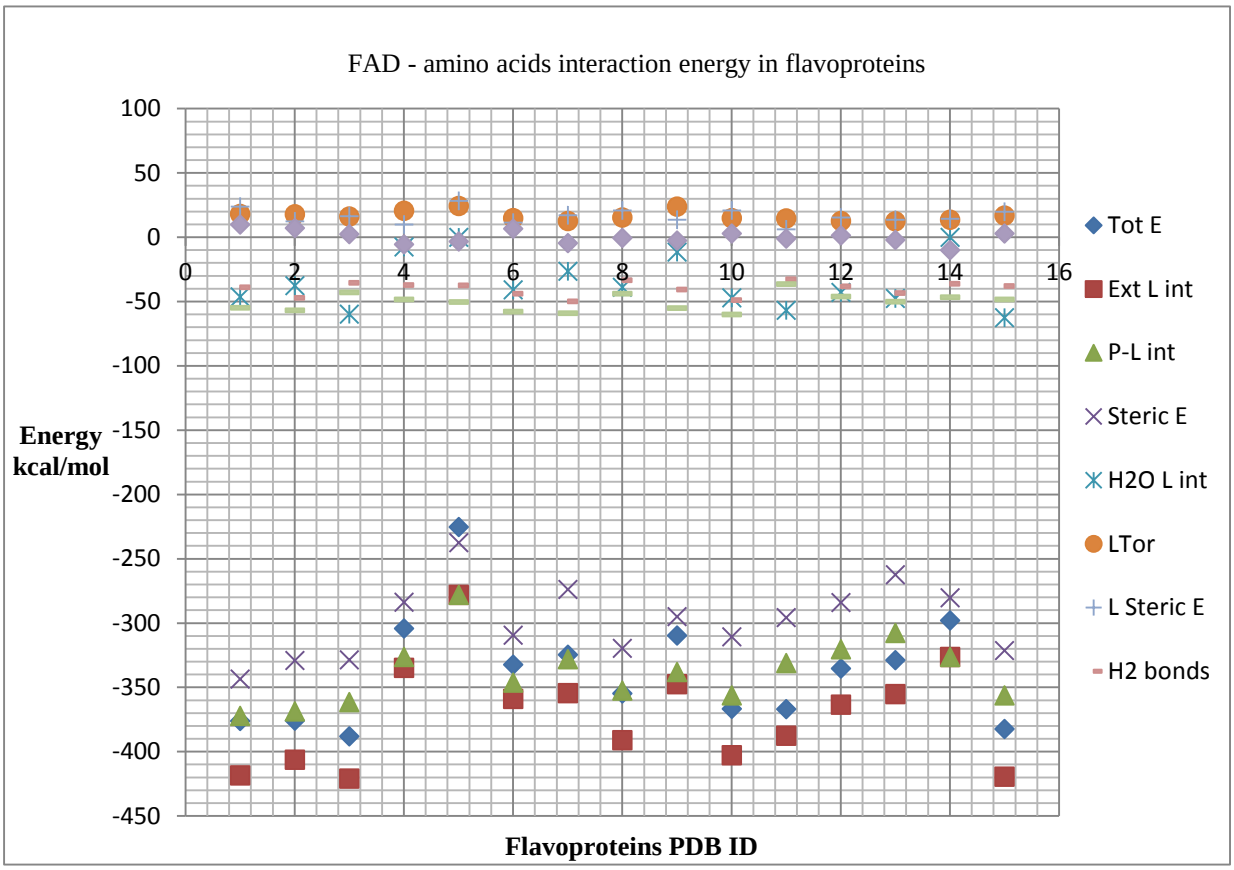


Figure 1: FAD - amino acids interaction energy for 15 flavoprotein structures. Legend (up to bottom): Tot. E = total energy of FAD - amino acid; Ext L int= External Aa - FAD interaction energy; P-L int=binding Pocket Aa- FAD interaction energy; Steric E = steric energy FAD -Aa (from the whole enzyme) complex; H2O L int= water - FAD interaction energy; L Tor = FAD torsion energy; L Steric E = FAD steric energy, H2 bonds = hydrogen bonds (FAD - Aa) energy, H2 bonds no dir=no directional/vectorial hydrogen bonds (FAD - Aa) energy; Elec. long = long range electrostatic FAD - Aa interaction energy. All energies are expressed in kcal/mol. The flavoprotein list is (from left to the right): 1GPE, 4YNT, 4XWR, 2WDW, 1W1J, 2JBV, 2VFR, 3T37, 3TX1, 4H7U, 4KGD, 4MIF, 5EPG, 5HSA, and 3NVZ (the first left term is 1GPE = GOx).

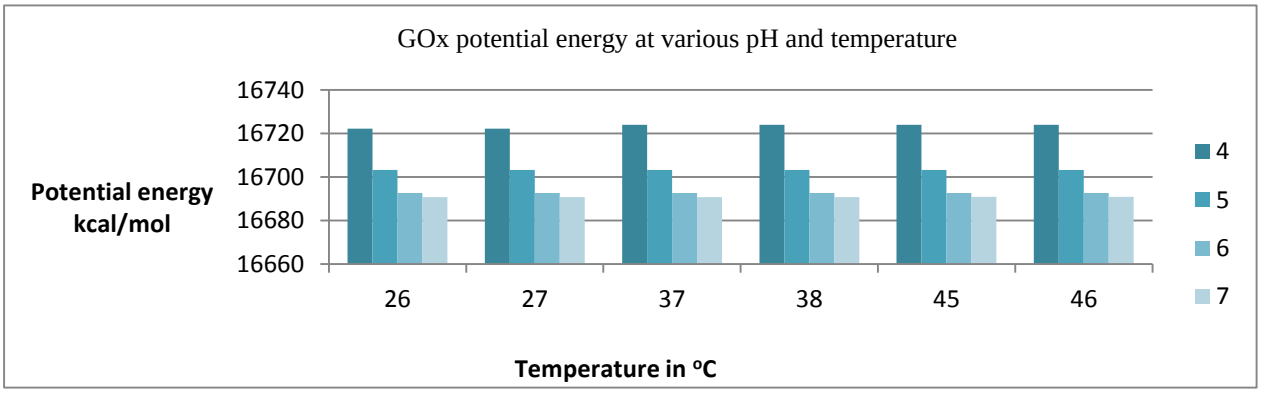


Figure 2: Variation of GOx potential energy, at different temperature and pH (4,5,6,7, represented in blue, red, green, and violet, respectively)

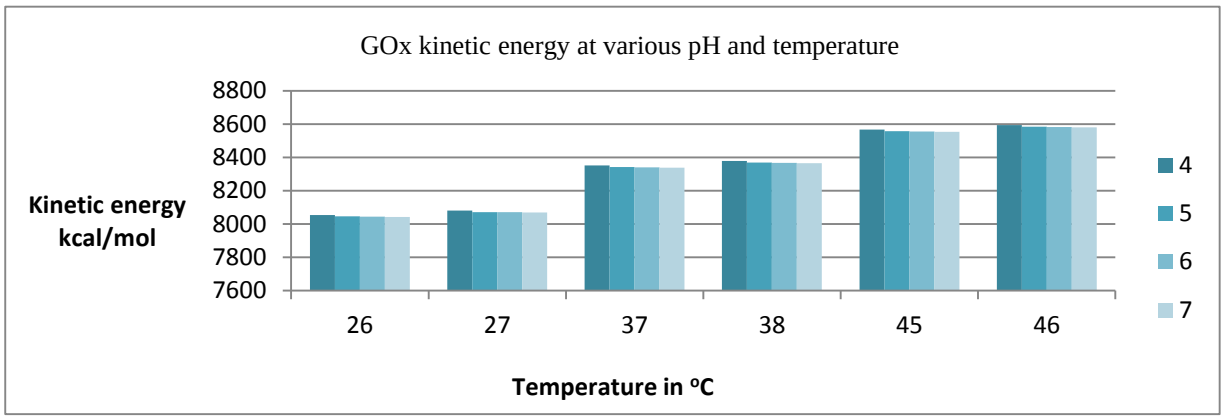


Figure 3: Variation of GOx kinetic energy, at different temperature and pH (4,5,6,7, represented in blue, red, green, violet respectively)

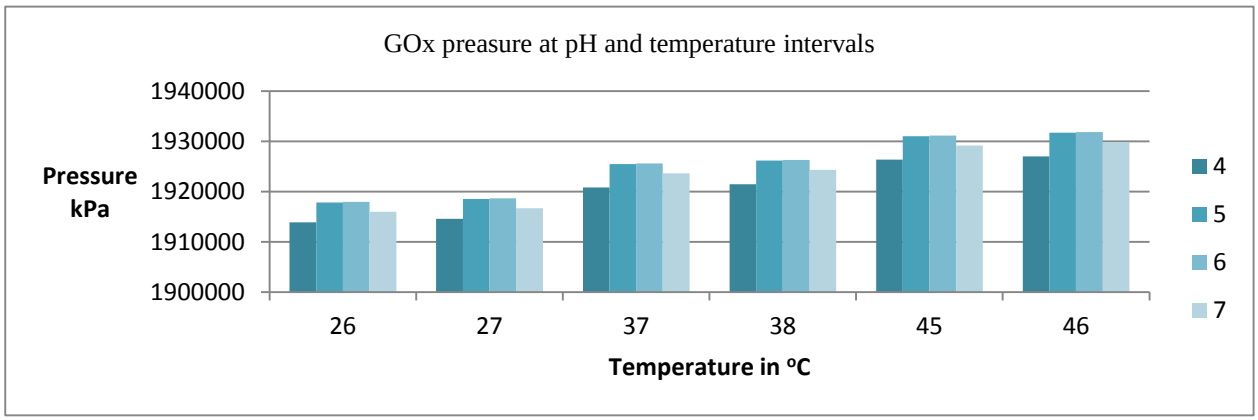


Figure 4: Variation of GOx pressure at different temperature and pH (4,5,6,7, represented in blue, red, green, violet respectively)

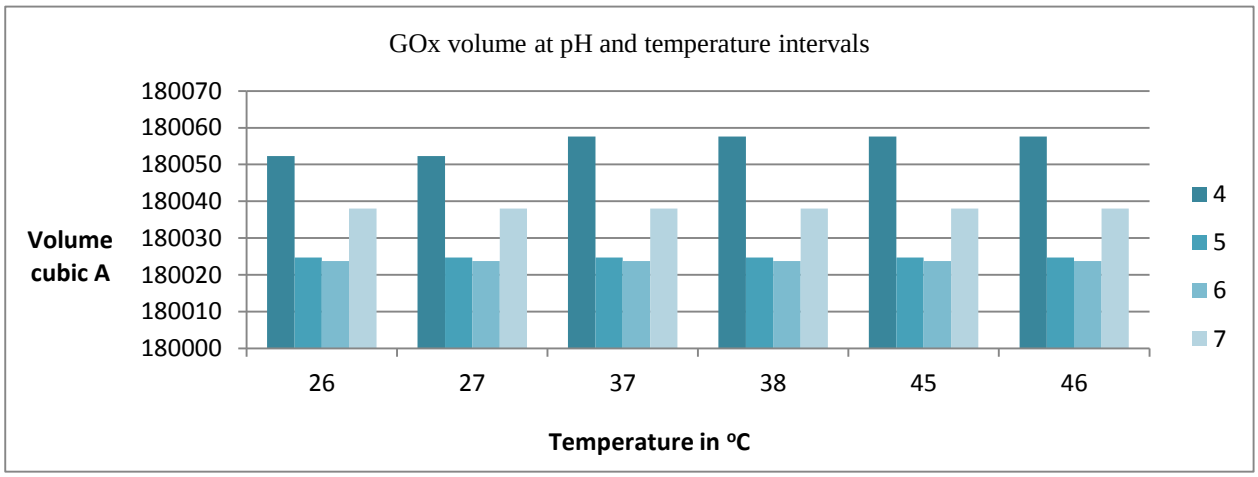


Figure 5: Variation of GOx volume at different temperature and pH (4,5,6,7, represented in blue, red, green, violet respectively)

FAD Total energy shows a temperature-pH dynamic with a general trend of increasing with temperature and pH. At pH 4 -5, the steric energy shows a significant peak. On pH intervals 6-7 at temperature intervals 25-28^{o}C and 44-47^{o}C, respectively, the system total energy shows a smooth plateau dynamic (Figs.6 and 7). Thus a decrease in FAD total energy, of 8.684 kcal/mol, will ensure

an optimal catalytic activity. Heat of formation shows two peaks where the energy could not be computed (Fig 8).

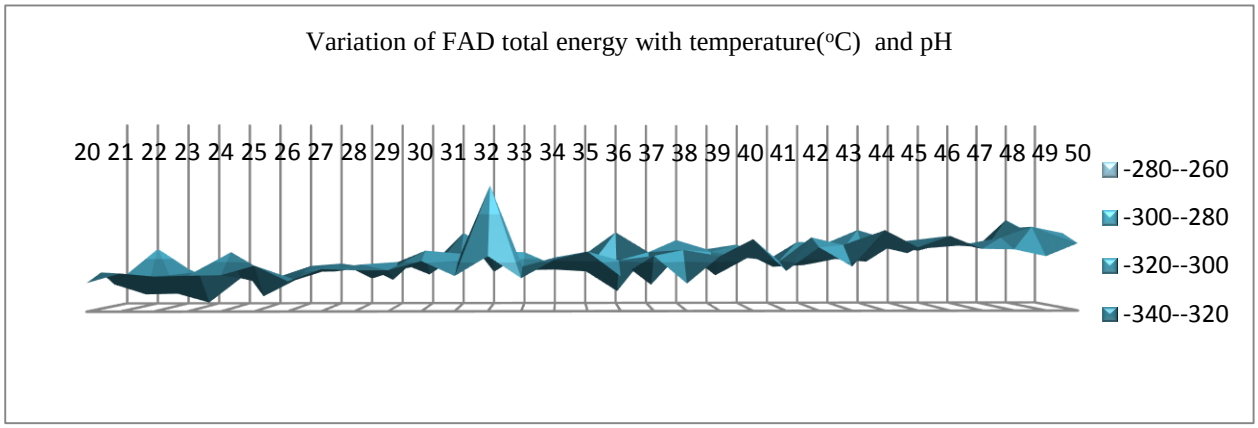


Figure 6: FAD total energy increasing with temperature expressed in °C

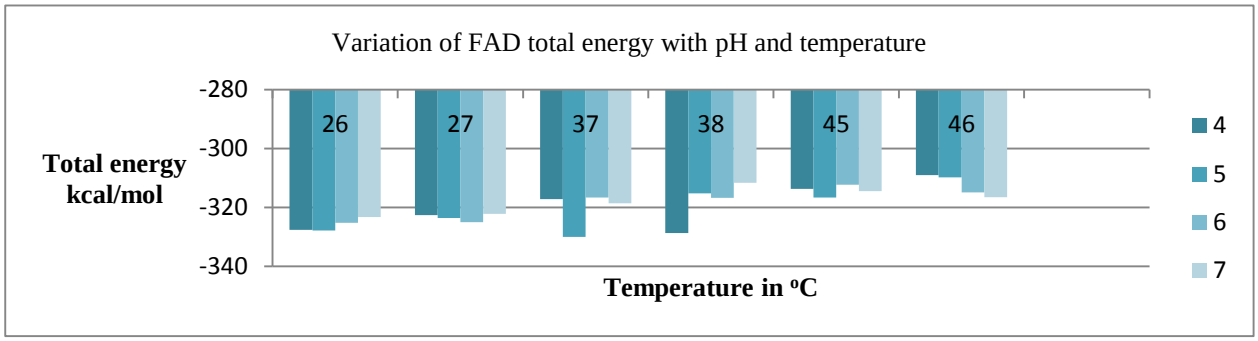


Figure 7: Variation of FAD total energy with pH and temperature.

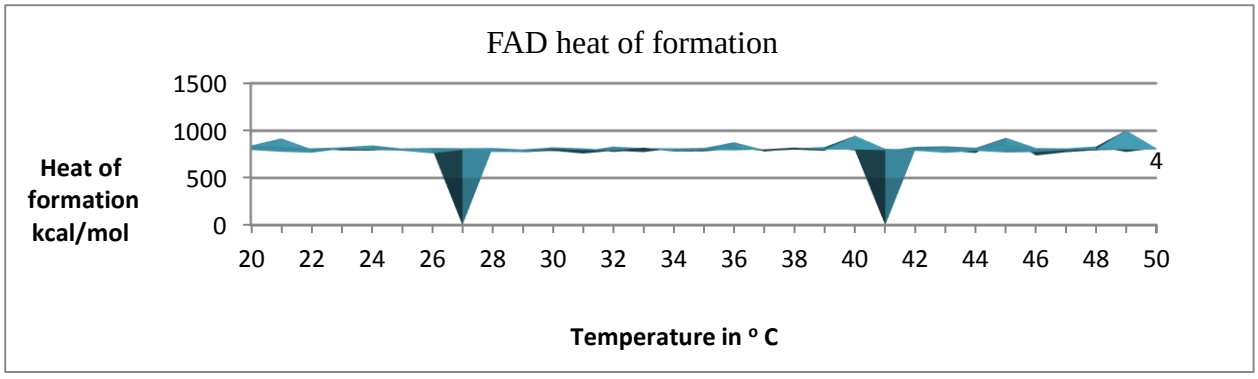


Figure 8: FAD heat of formation, on pH interval 4-7.

The homology model obtained by amino acids changes is shown in Fig.9, together with 1GAL(represented without FAD and other cofactors). The resulted model has 3 bonds and 4 rotamersas outliers. Molecular dynamic simulations showed that, at the hyper-thermic state, GOx's B factor reaches values situated between the values at optimal activity and normothermic state (see Fig. 10). Binding affinity(or Gibbs free energy[23] was computed for:(i) FAD in 1GAL; (ii)FAD in water 1 GAL and (iii) FAD homology "Aa moiety" model, respectively. FAD has the lowest binding affinity in the homology model (see Fig. 11). FAD conformation entropy [25] increases, starting from a value of -0.2125 kcal/mol (at optimal activity) to 1.116 kcal/mol, at hyperthermia.

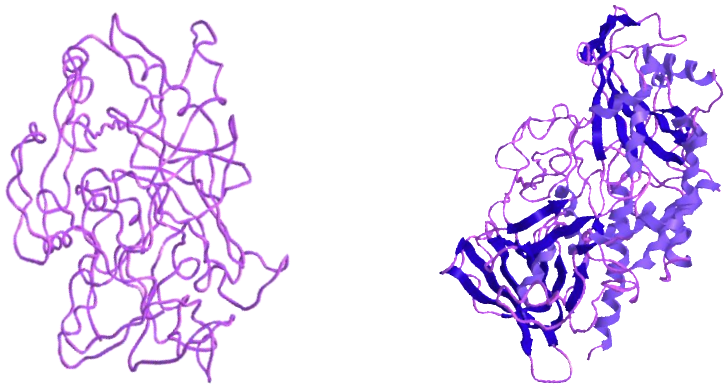


Figure 9: Homology model of 1GPE: the generated model (left) and 1GPE (right)

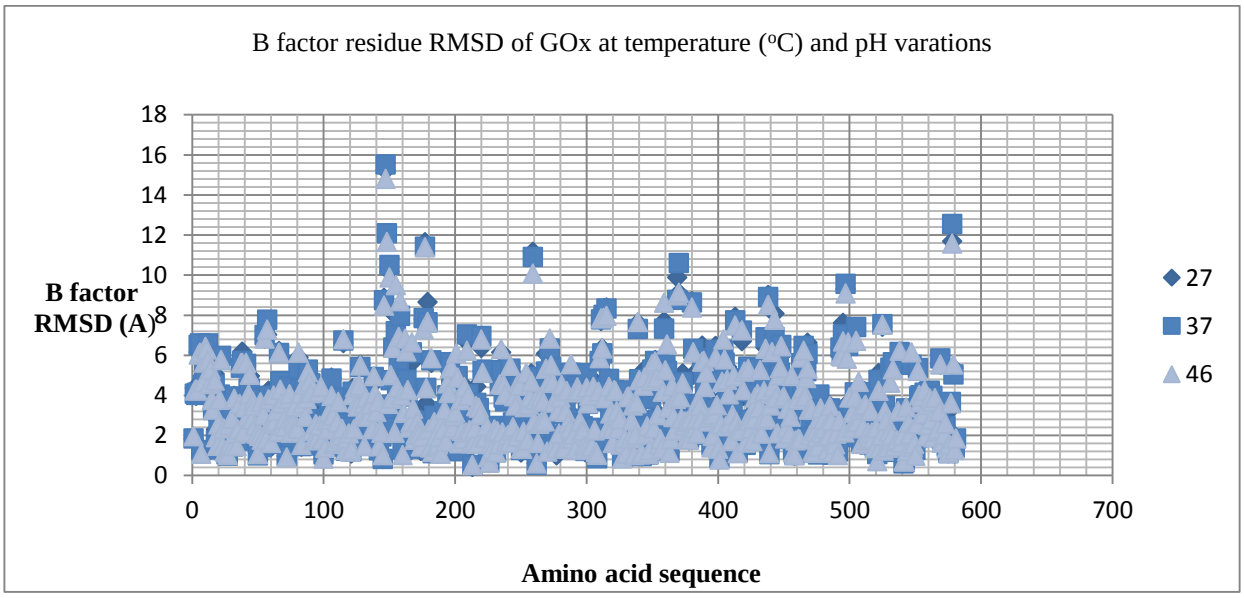


Figure 10: B factor residue of GOx at variable pH and temperature conditions (blue = optimal conditions; red = body temperature; green = hyperthermia[26].

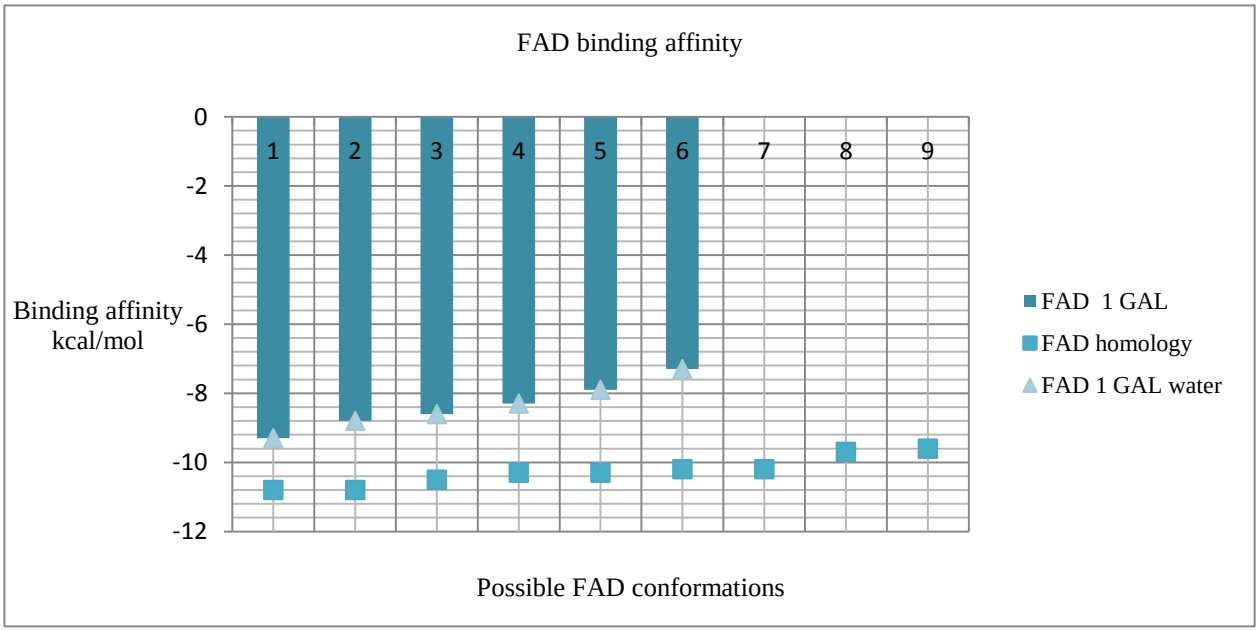


Figure 11. FAD binding affinity in the studied models.

Contact areas, exposed areas, percent of exposed areas, closest distance of the amino acid to FAD [27] **,**were calculated, as shown in Figs. 12 to 15 (and the additional material). In some cases, mutated amino acids have more favorable contact energy than the wild type. E.g. in the case of His 514, its mutation cf. contact areas (in blue) and exposed areas (in light blue) resulted in energetically more favorable conformations. In some cases, mutated amino acids have more favorable contact energy than the wild type. E.g. in the case of His 514, its mutation cf. contact areas (in blue) and exposed areas (in light blue) resulted in energetically more favorable conformations (figure 15).

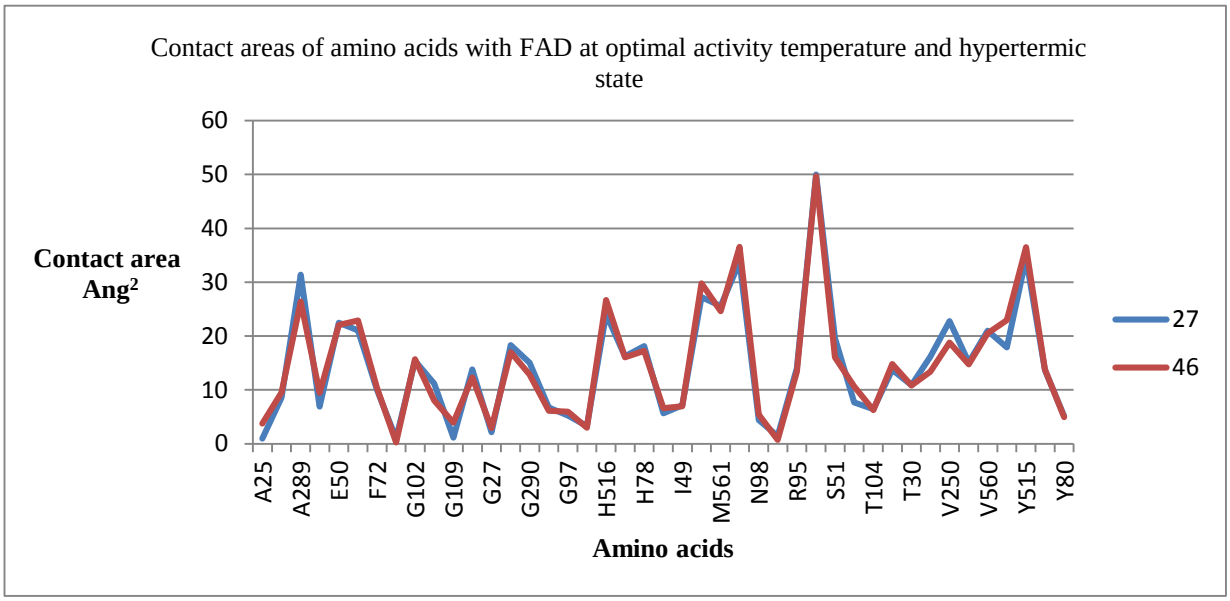


Figure 12: Contact areas of FAD and amino acids at the binding site at optimal activity and hyper-thermic state

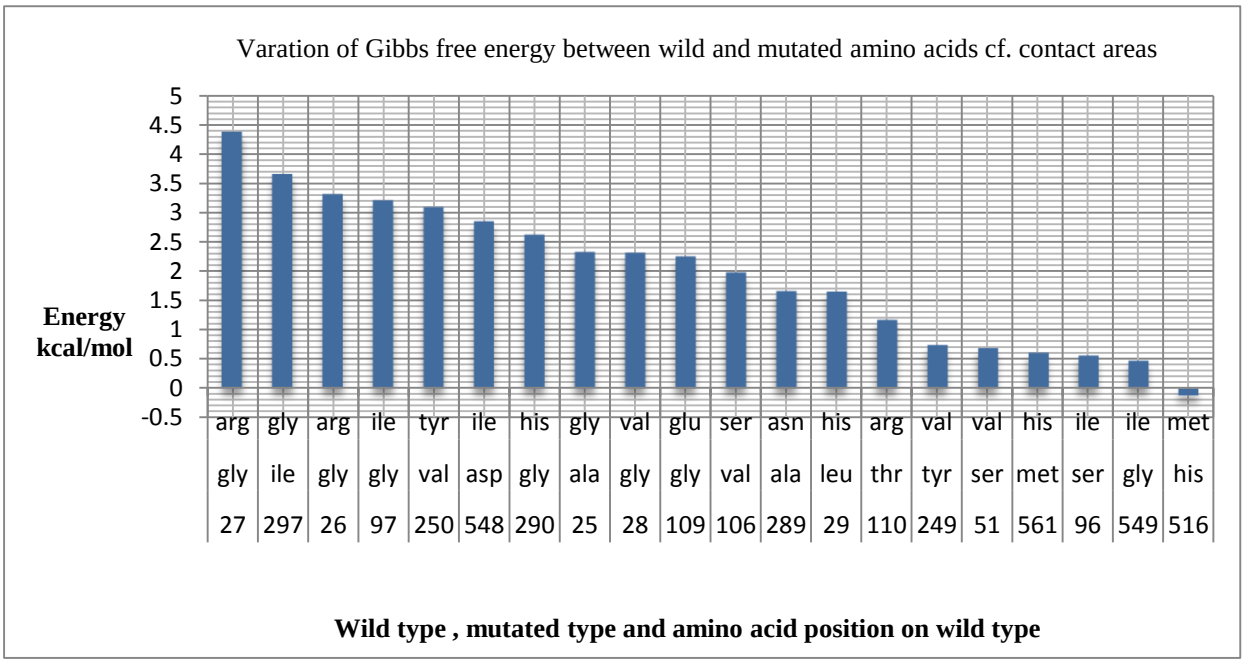


Figure 13: Variation of Gibbs free energy subsequently to amino acid mutation. First line under the horizontal axis represents mutated amino acids, second line gives the type of wild amino acids with

their position in the third line – e.g.,Gly 27 was mutated with Arg, resulting a variation in Gibbs energy, after mutation, of 4.383 kcal/mol.

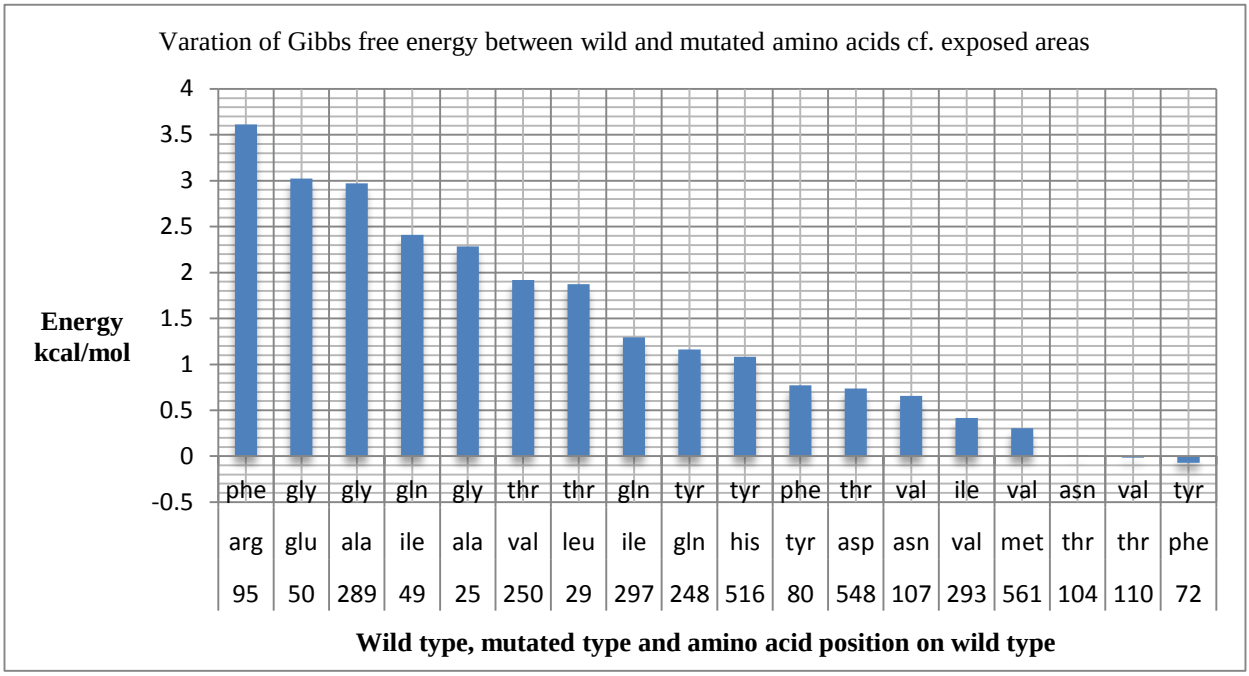


Figure 14: Variation of Gibbs free energy consecutively to amino acid mutation. First line below the horizontal axis represents mutated amino acids, second line are the wild type amino acids with their position in the third line – e.g.,Arg 27 was mutated with Phe, resulting a variation in Gibbs energy, after mutation, of 3.612 kcal/mol.

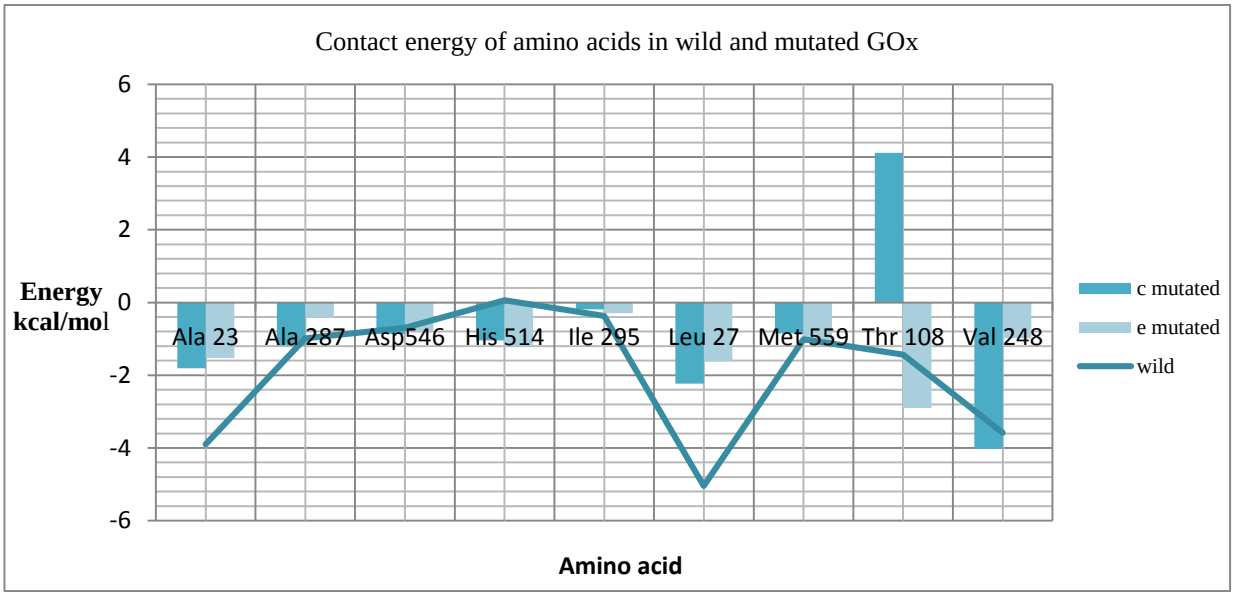


Figure 15: Contact energy of wild type and mutated amino acids in GOx. Wild type energy is represented as a dark blue line.

Networks computed using Cytoscape showed a similar pattern with the cofactor FAD highly connected with the nodes composing the enzyme network (see supplemental materials for the other FAD enzyme networks). To envisage FAD relation with the surrounding Aa networks, two types of

networks were generated: i(MNu) the union of all components of network; ii(MNd), the difference between GOx network (1GPE} and all other networks (see supplimental materials).

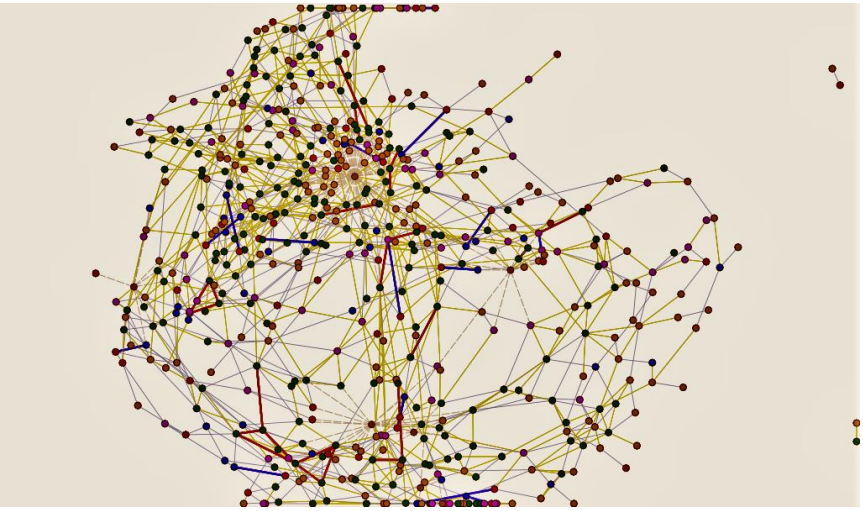
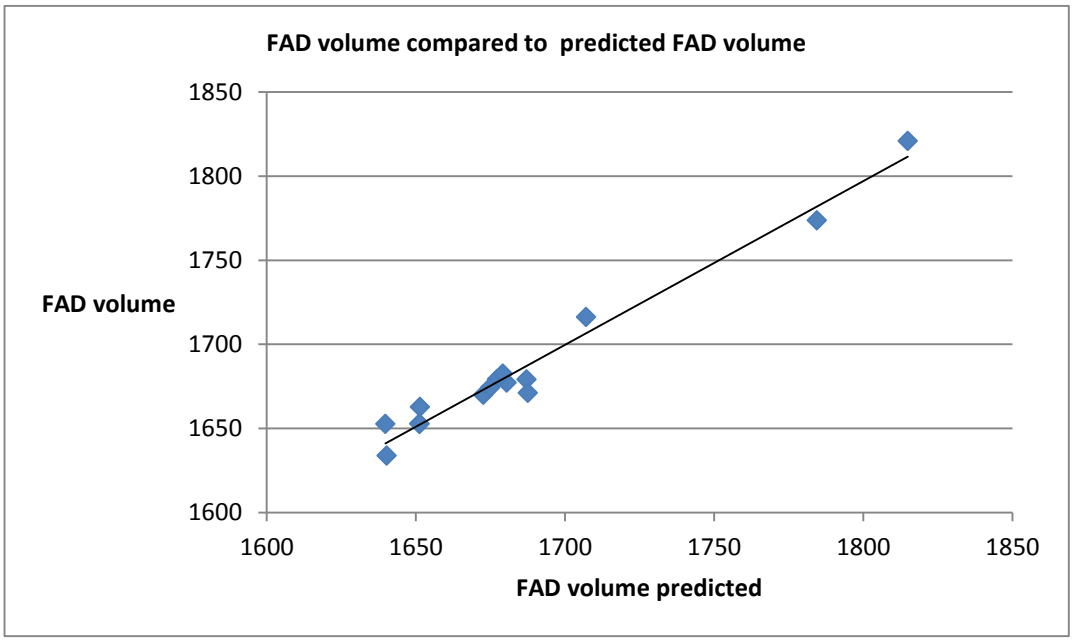


Figure 16. Glucose oxidase (1GPE) \ network showing FAD as the enzyme center highly connected to the surrounding aminoacids Aa.

QSAR equations are as follows: (model 1) y=45.5608+0.972987*x, with Pearson correlation R=0.986; (model 2) y=85.9525+0.912035*x, of R= 0.955(model 3)y=-0.924656+0.9724373*xof R=0.986. The predicted data for FAD parameters (in "union" and "difference" networks, respectively) are as follows: (a) predicted volumes:-22114A3 and 1448.04A3; (b) predicted surface areas: -12145.4A2 and 716.076A2;(c) predicted hydration FAD energy: 203.369kcal/mol and 43.4186kcal/mol respectively.



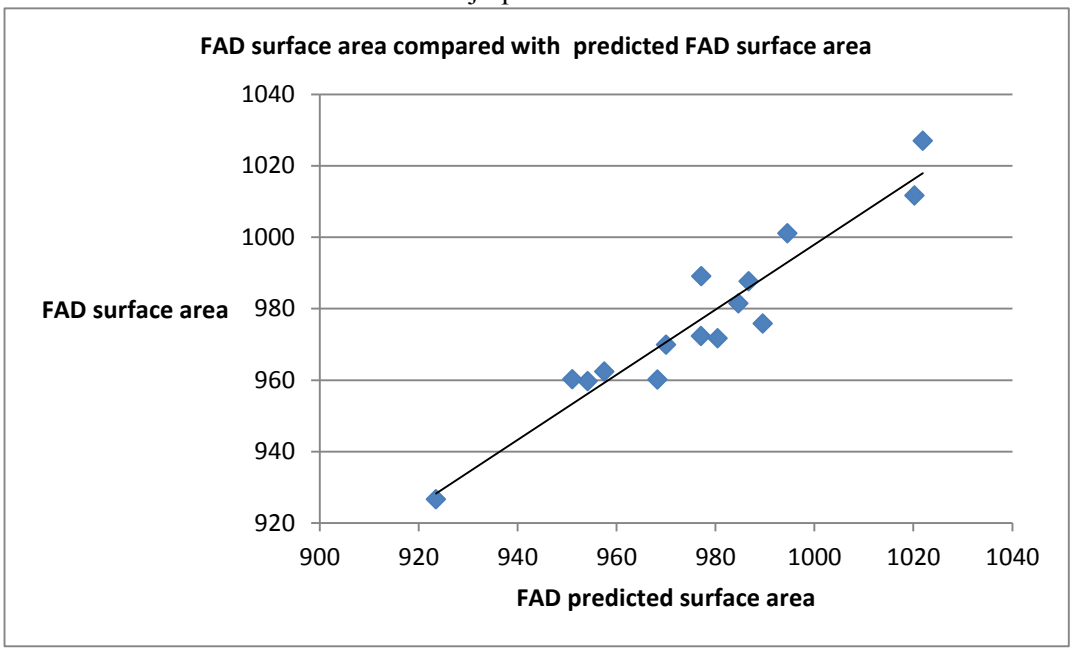


Figure 17. FAD (known vs predicted) volume and surface values, respectively, by MLR models.

A low protein - FAD interaction energy means that the GOx cofactor (i.e., FAD) is poorly ”meshed” by amino acids. To have a more clear view, the FAD – amino acids relation was represented in Fig.1 (see also the supplementary material,for all 15 enzymes).Flavoproteins with similar substrate affinity are represented cf. hydrophobic and electrostatic interactions: 1GPE(GOx = glucose oxidase); 2WDW(hexose oxidase) and 4YNT (glucose dehydrogenase). As discussed above, in the case of GOx, the FAD “surrounding” by amino acids is discreet in comparison with the other herein considered structures. In the homology model, FAD takes the lowest, more favorable energy, because of model flexibility(in the model, disulfide bonds between chains were avoided). GOx potential energy, as represented on pH and temperature intervals, suggests a pH- related variation, independent of temperature(see Fig.2).It means that a decrease in potential energy of about 0.08305 kcal/mol (cf. the target potential energy =-16691.6689 kcal/mol; the mean potential energy to be modified = -16691.7519kcal/mol) will preserve the GOx activity at pH 7 and 50^{o}C. FAD total energy, mainly the steric energy, increases with temperature(see the additional material). The two picks of heat of formation may represent, by chance, transition states [28] of FAD. However this computational result may simply represent a failure of the used force field. B factor (or the temperature factor) suggests that GOx have, at euthermia state, the highest flexibility. At hyperthermia state, a certain atom immobility is expected, according to a decrease in B values, probably producing a decrease in enzyme activity. In other words, due to protein “immobility”, the contacts between FAD and amino acid network tend to increase, resulting an increase in the total and steric energy of the FAD - Aa system; the energy of amino acids does not correspond to their optimal energy, thus FAD will have an unfavorable conformation for catalyzing the oxidation of beta –D-glucose. Mutation within the binding pocket amino acids can modify the FAD behavior to external changes. FAD, after the inner

amino acid mutation, reacts more at the inner changes, without ,,seeing'' the entire network.Figs.16 to 18 illustrate how FAD is contacted by amino acids. The upper diagram shows FAD surrounded by the outer amino acids, in the presence of water molecules; the external surface is represented by a dotted line across FAD.

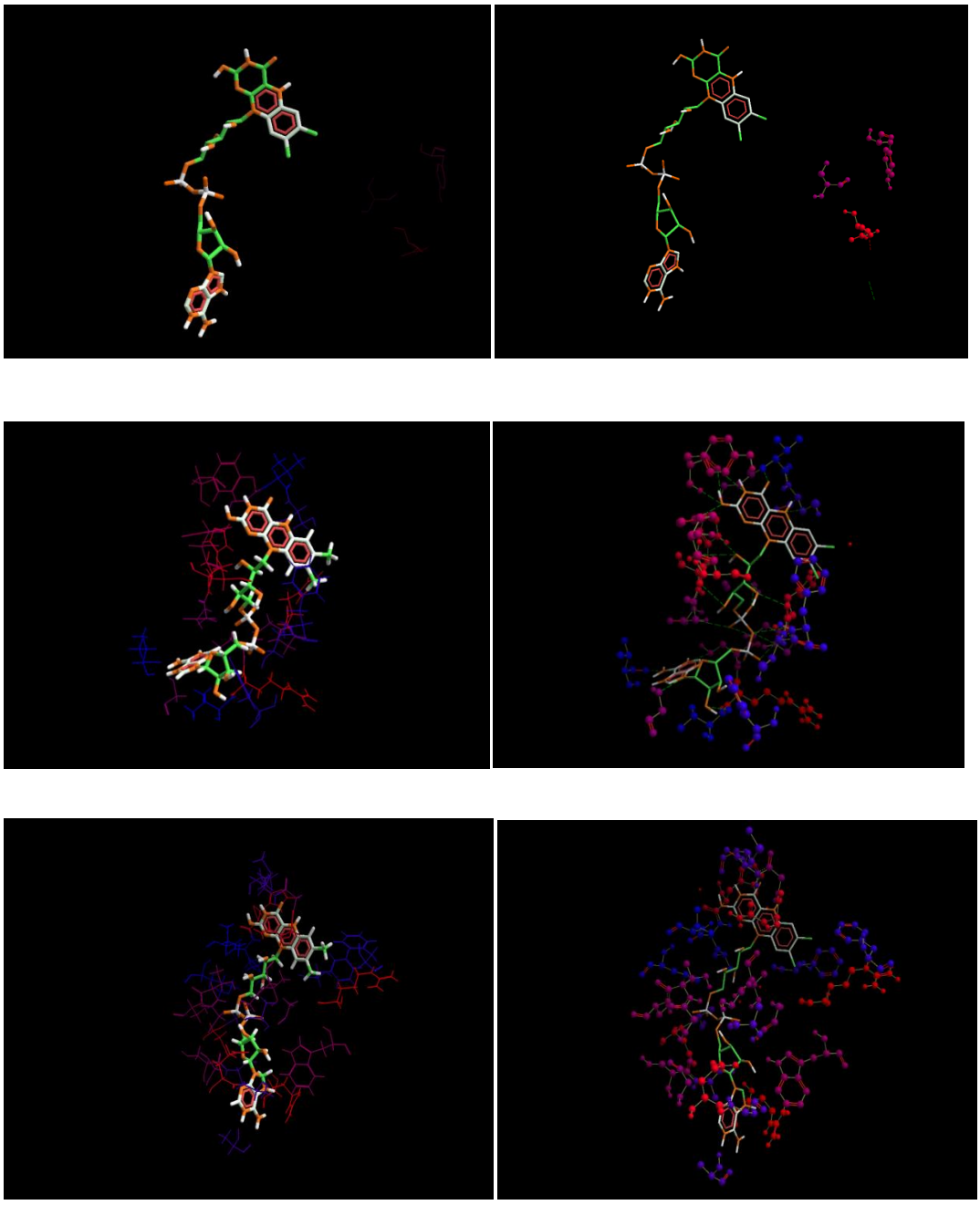
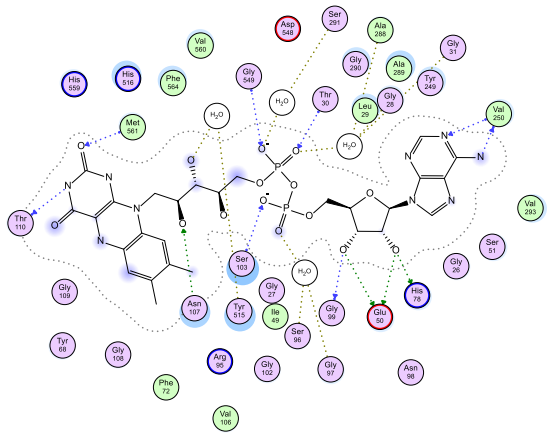
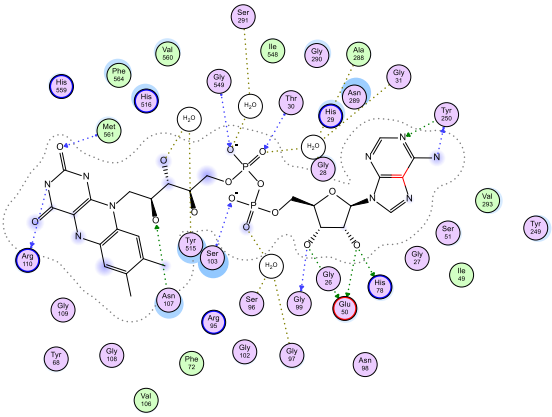


Figure 18: From top to bottom, FAD - amino acid,, meshing'': 1GPE, 2WDW, 4YNT,by hydrophobicity (left column) and by electrostatic interactions (right column). FAD is represented with the flavine group orientated approximately in the same way.

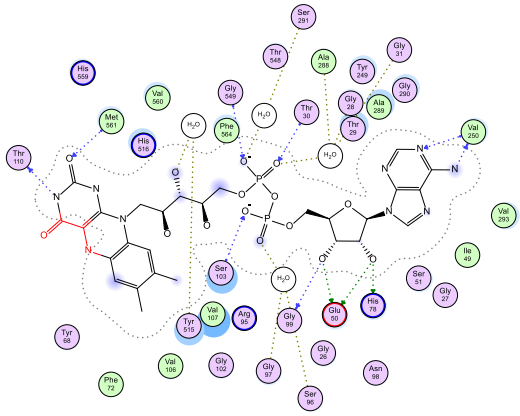


FAD - amino acid interactions in GOx wild type



FAD - amino acid interaction in GOx

Cf. contact areas mutated



FAD- amino acid interaction in GOx

Cf. exposed areas mutated

Figure 19: FAD amino acid interaction in GOx (wild and mutated, respectively).

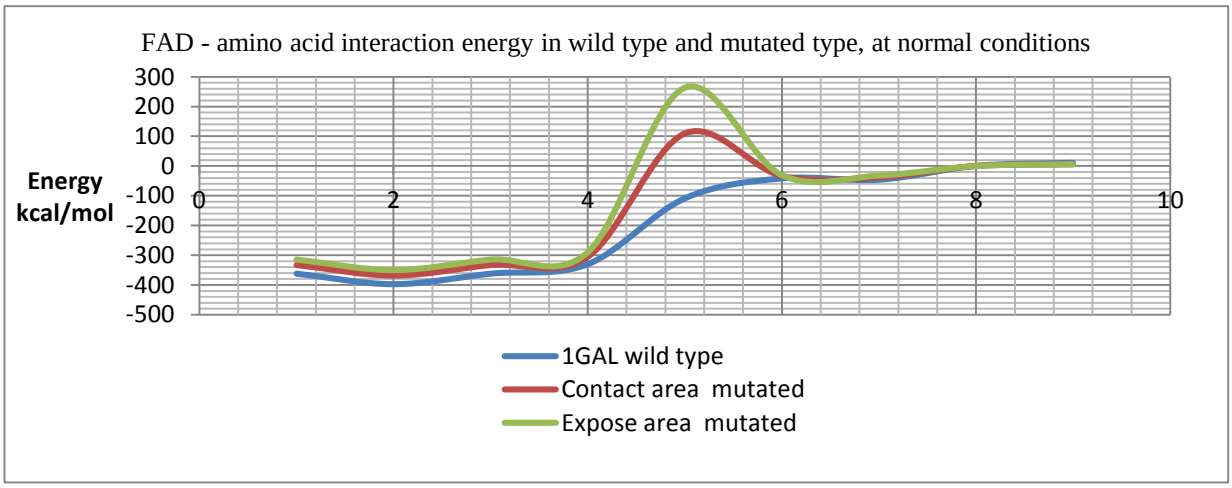


Figure 18: FAD amino acid interactions in wild and mutated type. The three structures are represented at standard normal conditions. Mutated protein cf. contact area is more close to the wild type energetic favorable conformation

Calculations of FAD volume , surface area and hydration energy predictions for the two merge networks (union and difference, respectively) suggested the following conclusions. In the case of merged united network, FAD suffers a drastic ,, compression'' with "negative" values for both volume and surface area and big positive values for hydration energy. Note that normal hydration energy values (in case of non ionic molecules) are negative. Such values shows how easy (if negative value) or how difficult (if positive value) a molecule hydrates, in a bad and good, respectively, exposure to a solvent. The ratio of surface area to volume (of FAD) shows relatively constant values (see supplemental materials):the upper limit promotes an oxidation while the lower limit in favor of a reduction reaction.

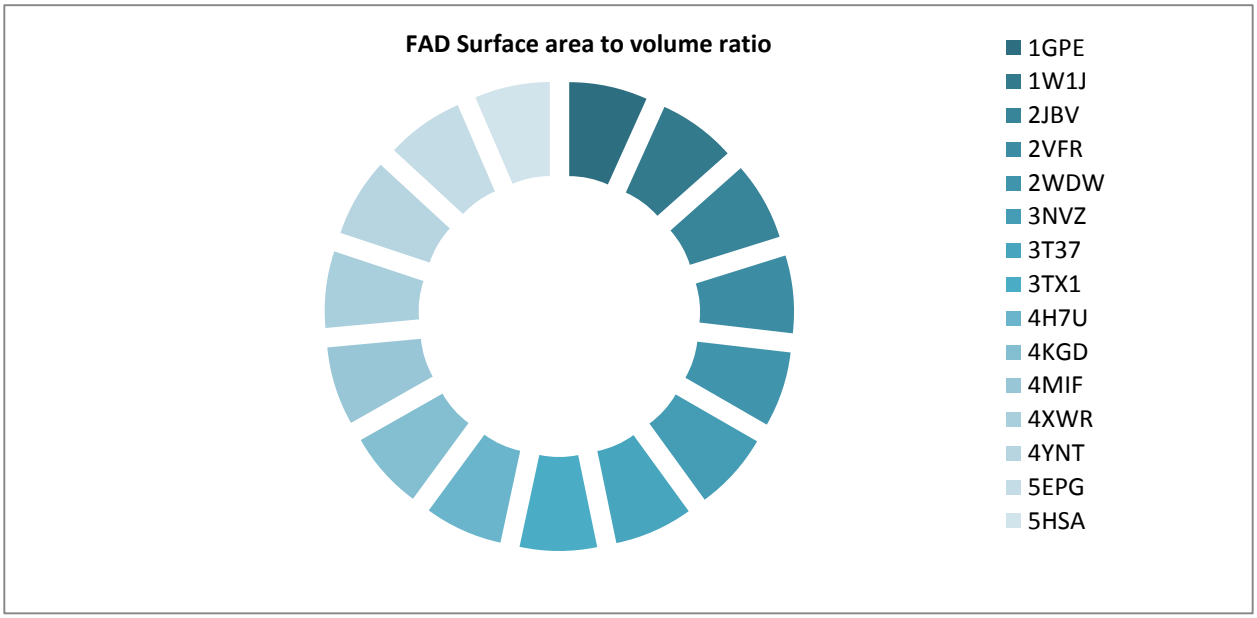


Figure 20. FAD surface area to volume ratio in different FAD based enzymes (in increasing ratio (and color), from 5HSA to 1GPE).

4. CONCLUSION

FAD plays a significant role in modulating flavoproteins (i.e., enzymes having FAD as a cofactor) activity, determining the type of substrate and type of reaction. FAD can take various conformations, in other words, it has an ,,adaptable" structure to amino acid ,,meshing", thus providing its own ,,response'', by appropriate conformational and energetic diverse geometries. As expected, the outer conditions can produce changes in FAD geometry by interacting with the amino acid inner (at the binding site) and outer(all other amino acids) networks. Optimization of amino acid network can mask or make more "visible"(for FAD) a part of the inner or outer network. Augmenting these two amino acid networks allows changes in FAD adaptability and consecutively in enzyme behavior, in terms of stability, substrate specificity and performance. There exist a close relationship between the protein function (in this case GOx) and its structure, energetically mediated. GOx is a packed macromolecule that may undergo a variety of conformational changes during its function performance, with energetic answers, accordingly. Therefore, we can say that interactions,

particularly the binding energies, will determine the stability, structure, but also the type of transformed substrate. FAD showed conformational changes, (inside amino acid network) at temperatures up to 46oC meaning that FAD can realize its catalytic function on hyper-thermia temperature intervals. Amino acids, as shown by MD studies, are susceptible to conformational changes with no loss in their stability. Thus GOx, as a whole, can perform its function on a hyper-thermia domain and its function can be predicted.

CONFLICT OF INTEREST

The authors have no conflict of interest.

ACKNOWLEDGEMENT

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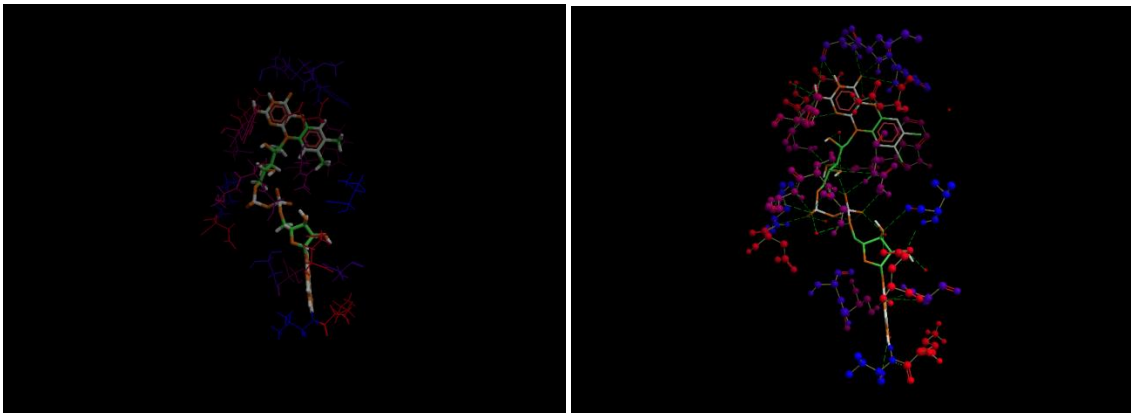
Supplementary Files

FAD proteins and FAD conformations

5HSA



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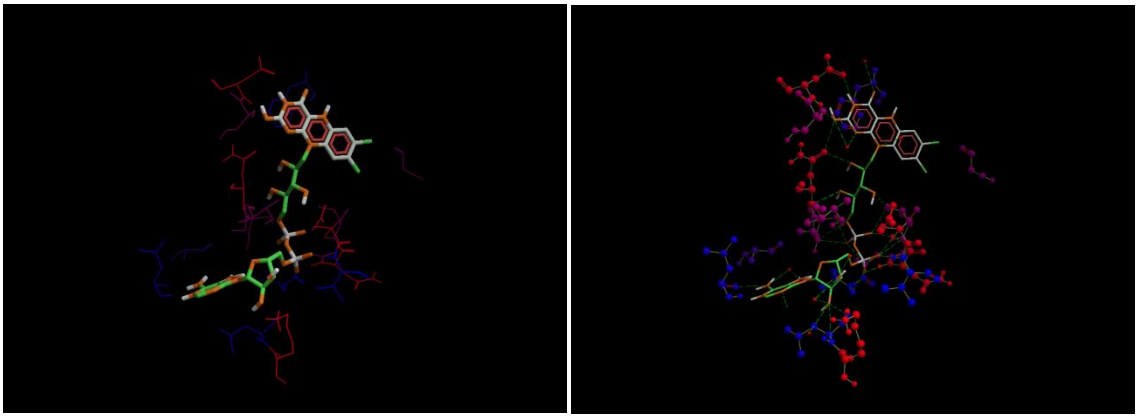

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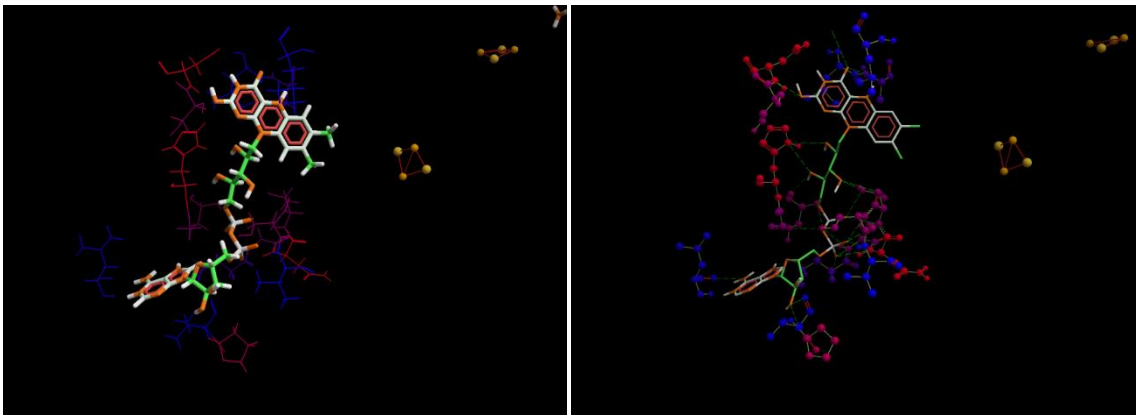

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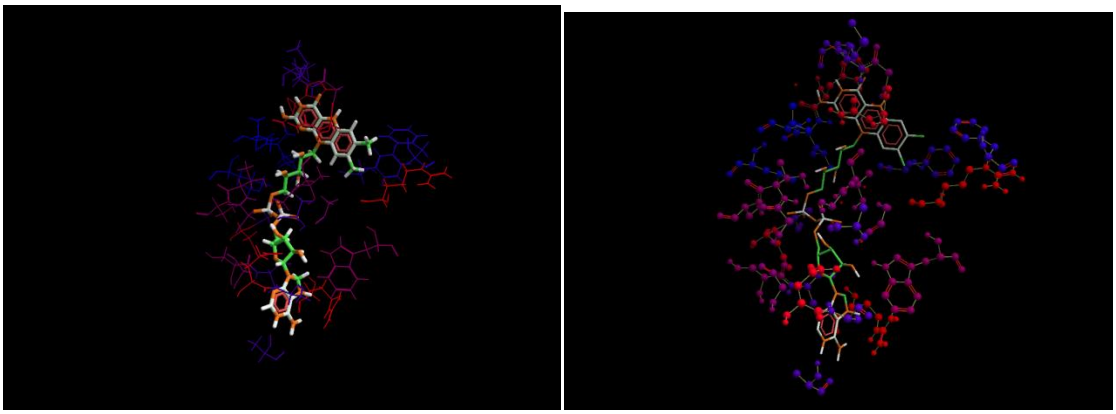

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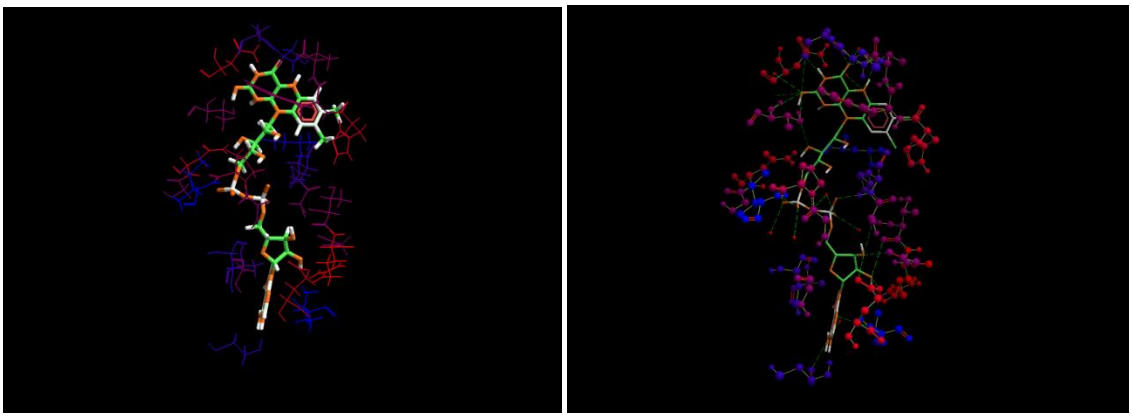

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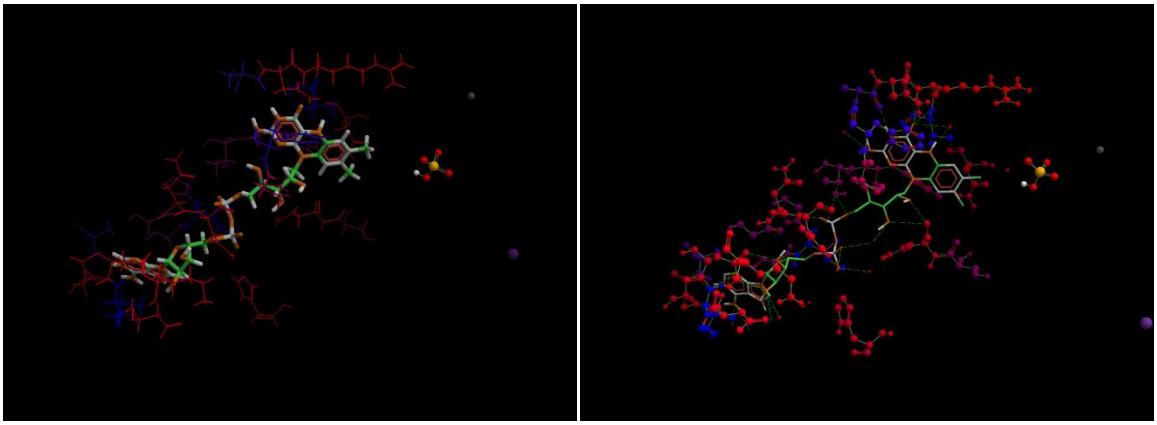

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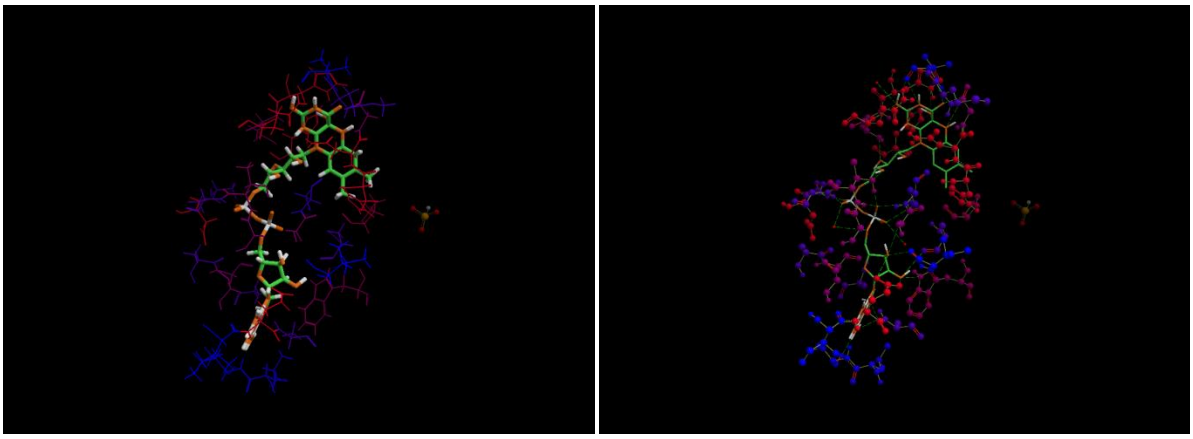

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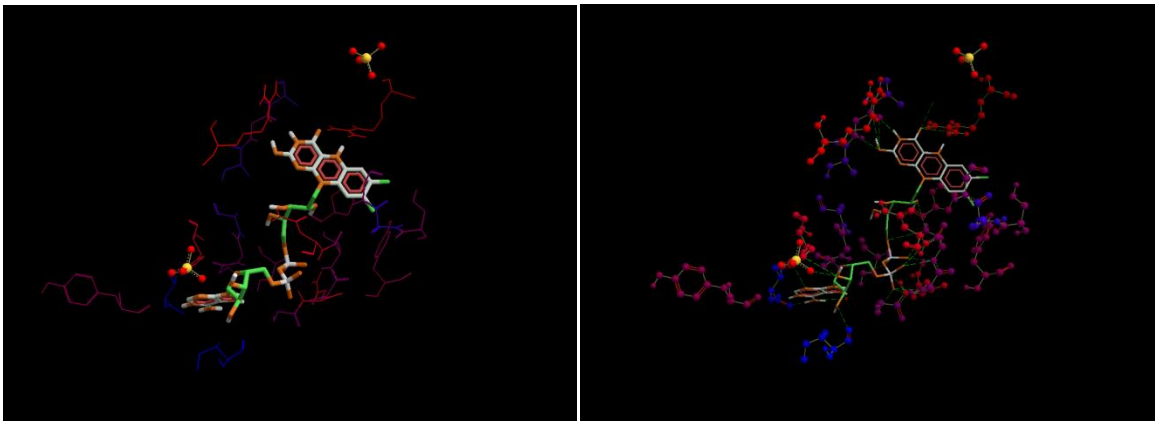

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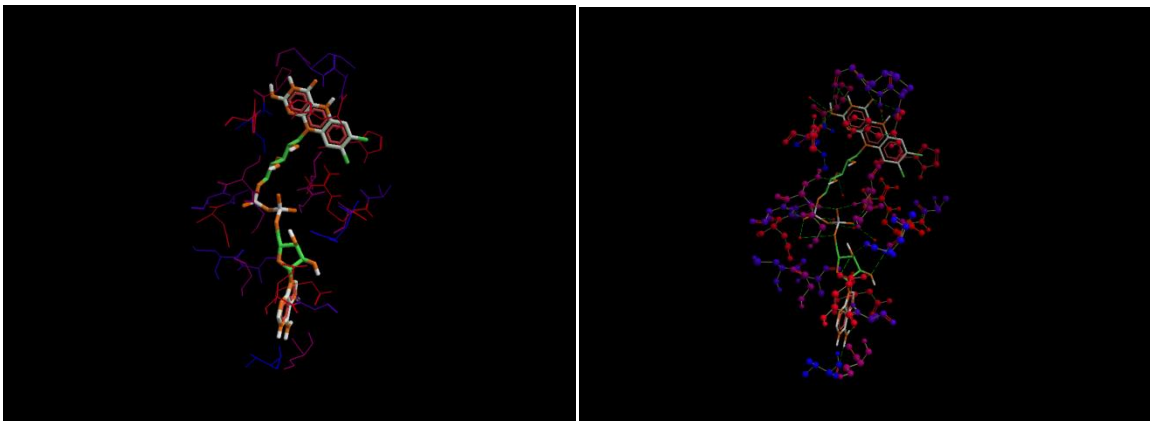

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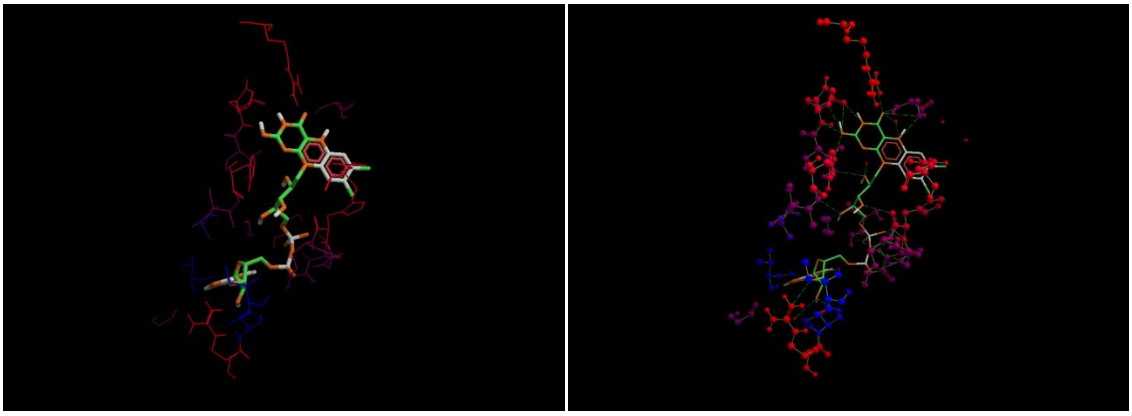

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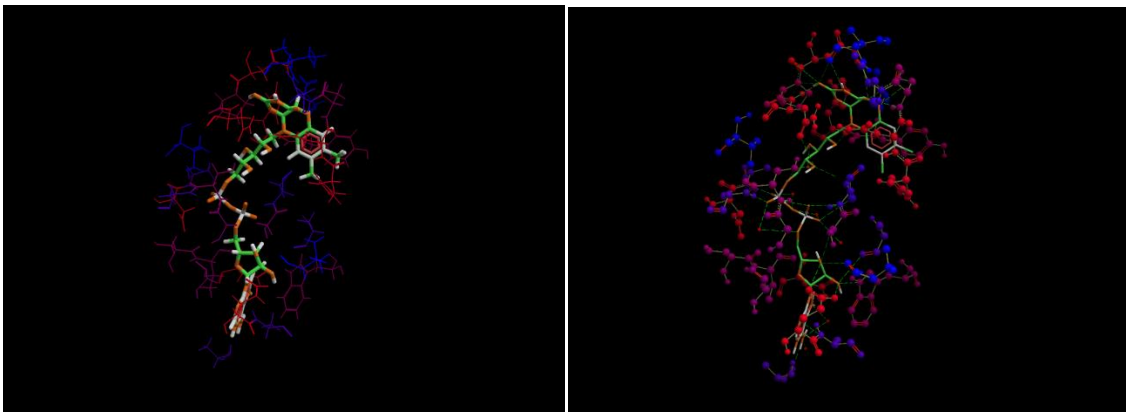

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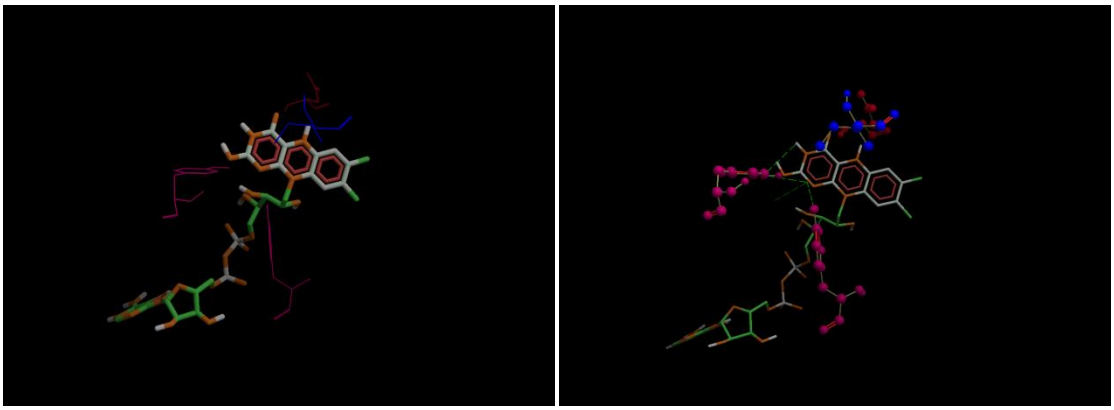
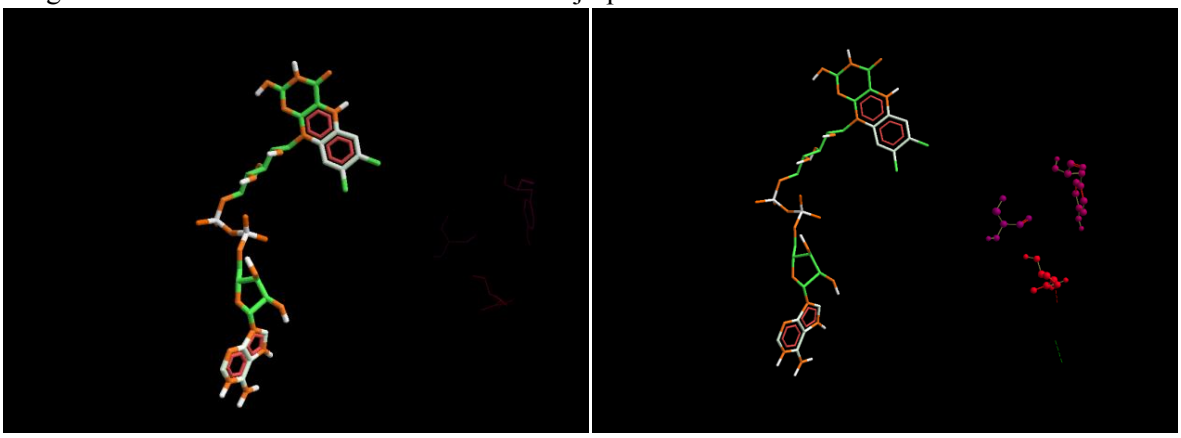


1gal



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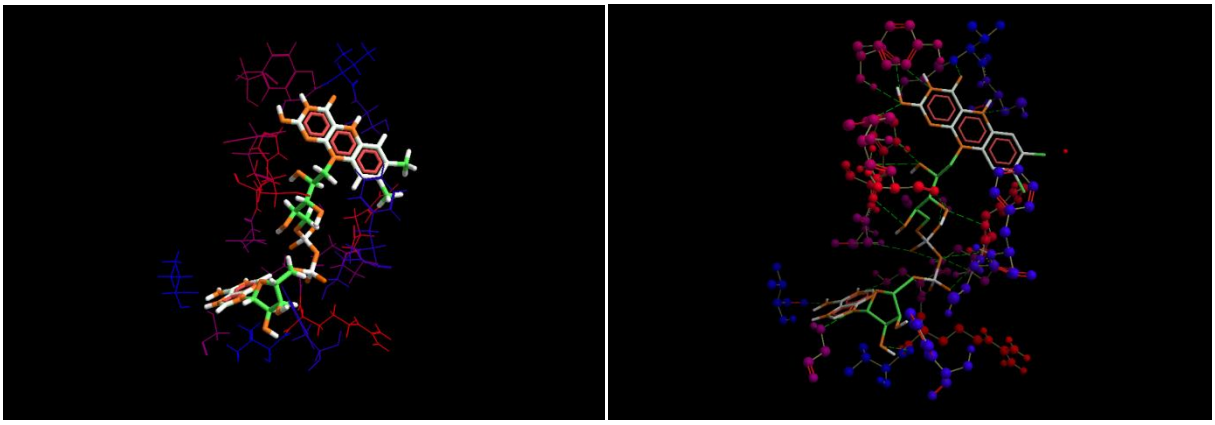

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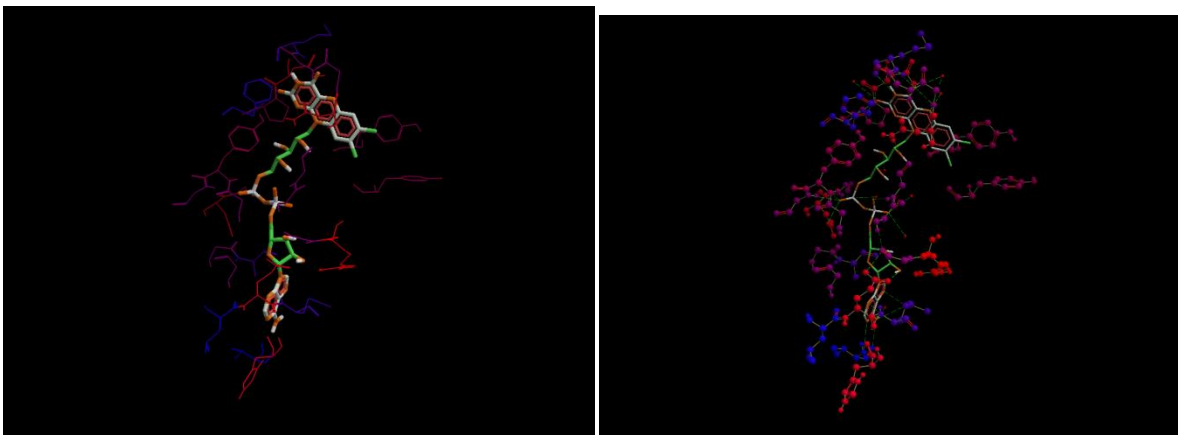
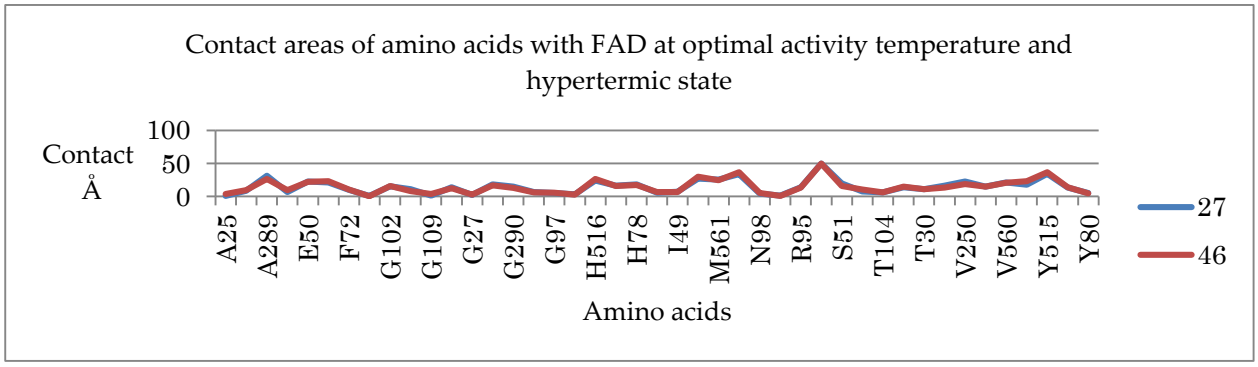
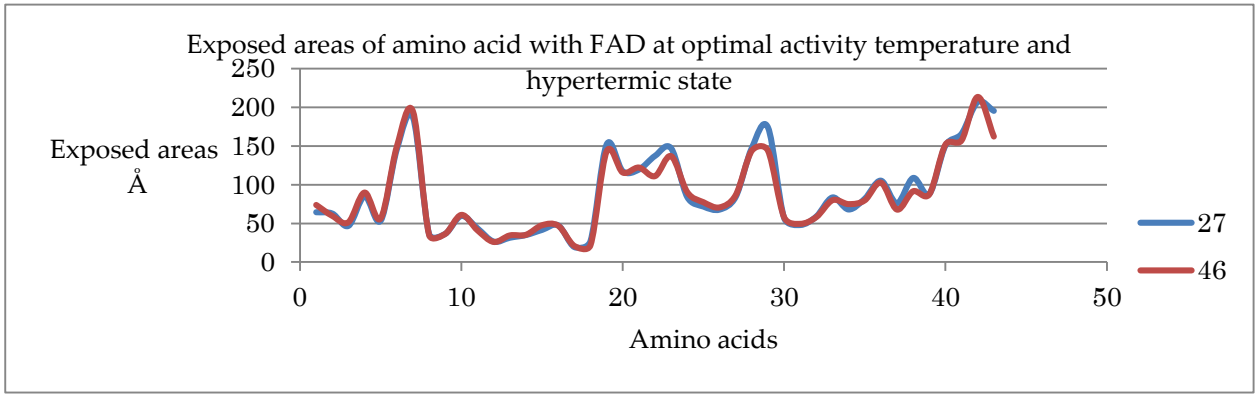
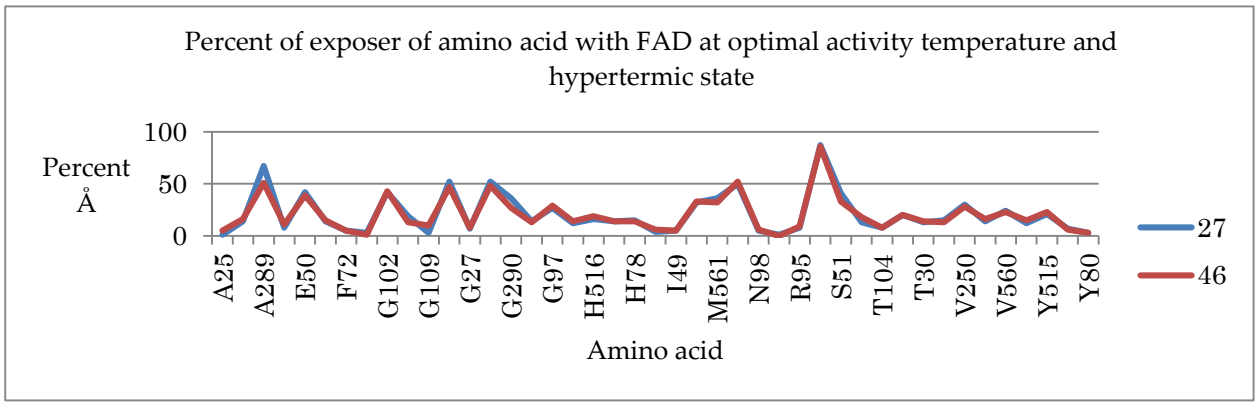
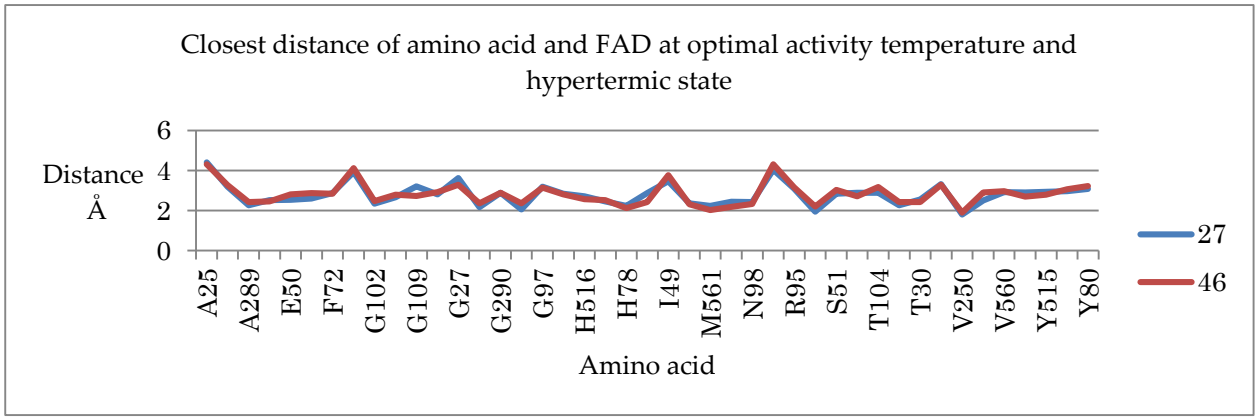


Amino acids exposure graphs









Amino acids mutation energy

	WAa	Sc pol	Scch	Hy /MW	MAa	Sc pol	Scch	Hy MW
C A²	Ala25	nonpolar	neutral	1.8/89	Gly 101	nonpolar	neutral	-0.4/75
	Ala 289	nonpolar	neutral	1.8/89	Asn 107	polar	neutral	-3.5/132
	Asp 548	Acid polar	negativ	-3.5/133	Ile 297	nonpolar	neutral	4.5/131
	Gly 109	nonpolar	neutral	-0.4/75	Glu 248	Acid polar	negativ	-3.5/133
	Gly26	nonpolar	neutral	-0.4/75	Arg 95	Basic polar	positive	-4.5/174
	Gly27	nonpolar	neutral	-0.4/75	*Arg95*			
	Gly28	nonpolar	neutral	-0.4/75	Val250	nonpolar	neutral	4.2/117

	Gly290	nonpolar	neutral	-0.4/75	His599	Basic polar	P10/n90	-3.2/155
	Gly 549	nonpolar	neutral	-0.4/75	Ile297	nonpolar	neutral	4.5/131
	Gly 97	nonpolar	neutral	-0.4/75	Ile297	nonpolar	neutral	4.5/131
	His 516	Basic polar	P10/n90	-3.2/155	Met 561	nonpolar	neutral	1.9/149
	His 78	Basic polar	P10/n90	-3.2/155	Val250	nonpolar	neutral	4.2/117
	Ile 297	nonpolar	neutral	4.5/131	Gly 97	nonpolar	neutral	-0.4/75
	Leu 29	nonpolar	neutral	3.8/131	His516	Basic polar	P10/n90	-3.2/155
	Met561	nonpolar	neutral	1.9/149	His516	Basic polar	P10/n90	-3.2/155
	Asn107	polar	neutral	-3.5/132				
	Ser51	polar	neutral	-0.8/105	Val560	nonpolar	neutral	4.2/117
	Ser 96	polar	neutral	-0.8/105	Ile 49	nonpolar	neutral	4.5/131
	Thr110	polar	neutral	0.7/119	Arg 95	Basic polar	positive	-4.5/174
	Val106	nonpolar	neutral	4.2/117	Ser 51	polar	neutral	-0.8/105
	Val250	nonpolar	neutral	4.2/117	Tyr249	polar	neutral	-1.3/181
	Tyr249	polar	neutral	-1.3/181	Val250	nonpolar	neutral	4.2/117
	Tyr515	polar	neutral	-1.3/181				
Ex A2	Ala 25	nonpolar	neutral	1.8/89	Gly108	nonpolar	neutral	-0.4/75
	Ala289	nonpolar	neutral	1.8/89	Gly549	nonpolar	neutral	-0.4/75
	Asp548	Acid polar	negativ	-3.5/133	Thr104			
	Glu 50	Acid polar	negativ	-3.5/133	Gly108	nonpolar	neutral	-0.4/75
	Phe 72	nonpolar	neutral	2.8/165	Tyr515	polar	neutral	-1.3/181
	His516	Basic polar	p10/n90	-3.2/155	Tyr249	polar	neutral	-1.3/181
	Ile297	nonpolar	neutral	4.5/131	Gln248	polar	neutral	-3.5/146
	Ile49	nonpolar	neutral	4.5/131	Gln248	polar	neutral	-3.5/146
	Leu29	nonpolar	neutral	3.8/131	Thr104	polar	neutral	0.7/119
	Met561	nonpolar	neutral	1.9/149	Val250	nonpolar	neutral	4.2/117
	Asn107	polar	neutral	-3.5/132	Val250	nonpolar	neutral	4.2/117

	Gln248	polar	neutral	-3.5/146	Tyr249	polar	neutral	-1.3/181
	Arg95	Basic polar	positive	-4.5/174	Phe72	nonpolar	neutral	2.8/165
	Thr104	polar	neutral	0.7/119	Asn98	polar	neutral	-3.5/132
	Thr110	polar	neutral	0.7/119	Val250	nonpolar	neutral	4.2/117
	Val106	nonpolar	neutral	4.2/117				
	Val250	nonpolar	neutral	4.2/117	Thr104	polar	neutral	0.7/119
	Val293	nonpolar	neutral	4.2/117	Ile297	nonpolar	neutral	4.5/131
	Tyr515	polar	neutral	-1.3/181				
	Tyr68	polar	neutral	-1.3/181				
	Tyr80	polar	neutral	-1.3/181	Phe72	nonpolar	neutral	2.8/165

Table 1: CA^2 - Contact area, Ex A^2-Exposed area, WAa- wild type Aa, Sc pol - side chain polarity, Scch- side chain charge at ph 7.4, Hy/MW- Hydropaty index / molecular weight, MAa-mutated amino acid, p10/n90-positive 10% negative 90%, Blanked spaces in table represent situations where no substitute was found for Aa.

CA^2					
Protei ID	Chain	Residue	Wild type	Mutant type	ddG
1gal	a	25	ala	gly	2.331kcal/mol
1gal	a	289	ala	asn	1.664 kcal/mol
1gal	a	548	asp	ile	2.856kcal/mol
1gal	a	109	gly	glu	2.258kcal/mol
1gal	a	26	gly	arg	3.316kcal/mol
1gal	a	27	gly	arg	4.383kcal/mol
1gal	a	28	gly	val	2.320kcal/mol
1gal	a	290	gly	his	2.629kcal/mol
1gal	a	549	gly	ile	0.476kcal/mol
1gal	a	97	gly	ile	3.214kcal/mol
1gal	a	516	his	met	-0.125kcal/mol
1gal	a	297	ile	gly	3.664kcal/mol
1gal	a	29	leu	his	1.657kcal/mol
1gal	a	561	met	his	0.6118kcal/mol
1gal	a	51	ser	val	0.6869kcal/mol
1gal	a	96	ser	ile	0.5624kcal/mol
1gal	a	110	thr	arg	1.167kcal/mol
1gal	a	106	val	ser	1.986kcal/mol

1gal	a	250	val	tyr	3.1000kcal/mol
1gal	a	249	tyr	val	0.7447kcal/mol
ExA²					
1gal	a	25	ala	gly	2.2830kcal/mol
1gal	a	289	ala	gly	2.9700kcal/mol
1gal	a	548	asp	thr	0.7364kcal/mol
1gal	a	50	glu	gly	3.0230kcal/mol
1gal	a	72	phe	tyr	-0.0715kcal/mol
1gal	a	516	his	tyr	1.081kcal/mol
1gal	a	297	ile	gln	1.296kcal/mol
1gal	a	49	ile	gln	2.411kcal/mol
1gal	a	29	leu	thr	1.873kcal/mol
1gal	a	561	met	val	0.3041kcal/mol
1gal	a	107	asn	val	0.6582kcal/mol
1gal	a	248	gln	tyr	1.1610kcal/mol
1gal	a	95	arg	phe	3.6120kcal/mol
1gal	a	104	thr	asn	0.007929kal/mol
1gal	a	110	thr	val	-0.01441kcal/mol
1gal	a	250	val	thr	1.9200kcal/mol
1gal	a	293	val	ile	0.4170kcal/mol
1gal	a	80	tyr	phe	0.7724kcal/mol

Table 2: CA² -Contact area, ExA²- Exposed area,Protein ID-molecule PDB ID , Chain- chain a of GOx molecule, Residue-Aa residue number, Wild type- wild type Aa, Mutant type- mutant Aa, ddG- difference between Gibbs energy of wild type and mutated type.