



Investigations on the role of π – π interactions and π – π networks in eNOS and nNOS proteins



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ABSTRACT

π – π Interactions play an important role in the stability of protein structures. In the present study, we have analyzed the influence of π – π interactions in eNOS and nNOS proteins. The contribution of these π – π interacting residues in sequential separation, secondary structure involvement, solvent accessibility and stabilization centers has been evaluated. π – π interactions stabilize the core regions within eNOS and nNOS proteins. π – π interacting residues are evolutionary conserved. There is a significant number of π – π interactions in spite of the lesser natural occurrences of π -residues in eNOS and nNOS proteins. In addition to π – π interactions, π residues also form π – π networks in both eNOS and nNOS proteins which might play an important role in the structural stability of these protein structures.

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1. Introduction

Noncovalent interactions play vital roles in many aspects of chemistry, biology and consequently, various types of experimental and theoretical approaches have been used to study these forces [1]. Interactions between aromatic molecules represent one of most important intermolecular forces in chemical and biological system. Interactions involving π systems are pivotal to biological events such as protein–ligand recognition. The side-chains of phenylalanine (Phe), tyrosine (Tyr) and tryptophan (Trp) residues in proteins are frequently found to be involved in pair wise interactions. These occur both within repeating elements of secondary structure and in tertiary and quaternary interactions. It has been suggested that they are important in protein folding and stability. These π – π interactions have an important influence in the base-stacking interactions which determine, the sequence dependent structure and properties of DNA [2,3], the tertiary structure of proteins [4] and the intercalation of different drugs into DNA [5]. The aggregation of aromatic rings has been quite well known in bio macromolecules, the two modes π – π and C–H \cdots π engages themselves in competitive manner [6]. The isolated two benzene or π -rings is the simplest prototype of π – π interactions and has been used to understand the fundamentals of these interactions as they are observed in more complex systems [3,7].

Nitric oxide (NO) is a ubiquitous molecule that is involved in the regulation of almost all aspects of cellular function, including metabolism, cell growth, proliferation and cell death. NO is synthesized from L-arginine by a family of Nitric oxide synthase (NOS) which includes neuronal Nitric Oxide Synthase (nNOS), endothelial Nitric Oxide Synthase (eNOS) and inducible Nitric Oxide Synthase (iNOS). The nNOS and eNOS are constitutive enzymes that are regulated by the Ca21-dependent binding of CaM to the CaM-binding sequence. Their activity is physiologically controlled by local changes in the Ca21 concentration. In contrast, iNOS binds CaM in essentially a Ca21-independent, irreversible manner, and its activity is transcriptionally regulated by cytokines rather than by changes in the Ca21 concentration [8,9]. The nNOS isoform is first identified in neurons, has also been found to be constitutively expressed in cardiac myocytes [10] and vascular smooth muscle cells [11]. The eNOS isoform is expressed both in endothelial cells and in cardiac myocytes [12].

Daniel Escudero demonstrated that the T-shaped aromatic interaction between pyridine and benzene can be modulated by using σ -interactions via the nitrogen atom of the pyridine ring [13]. Recently, the contribution of π – π interactions in the mechanism of removal of paracetamol from water by vegetable wastes is reported [14]. There are reports of noncovalent interactions for their role in the enhancement of stability of thermophilic protein [15,16], folding of polypeptide [17] and the stability of membrane protein [18,19]. The stability and specificity of protein DNA complexes [20,21], RNA binding proteins [22], TNF and interleukins [23], adhesion molecules [24], bacterial exotoxins [25], metallo-proteins [26] and iNOS [27] are reported on the basis of cation– π interactions. In our previous analysis we have analyzed the role

Abbreviations: eNOS, endothelial Nitric Oxide Synthase; nNOS, neuronal Nitric Oxide Synthase; ASA, Accessible surface area; NCI, Non-canonical interactions; PDB, Protein Data Bank; SC, stabilization center.

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of cation- π interactions in eNOS and nNOS proteins [28]. In continuation of that work, we report here our results on the studies on π - π interactions in eNOS and nNOS proteins.

Even though many studies are done on the molecular aspects [29–32], there are no reports on the systematic analysis of π - π interactions in eNOS and nNOS proteins. Thus, considering the above facts, we thought it would be useful to investigate the role of π - π interactions in eNOS and nNOS proteins by bioinformatics approaches. In the present study, we have investigated the structural stability patterns of π - π interactions in eNOS and nNOS proteins in relation to other environmental preferences like secondary structure, solvent accessibility, sequential distance, stabilization centers and conservation patterns.

2. Material and methods

2.1. PDB structures employed for the analysis of π - π interactions

All available crystal structures of eNOS and nNOS from Protein Data Bank [33] were considered for our study. There were 94 eNOS and 113 nNOS structures in PDB. Considering the fact that analysis will be scientifically relevant when the redundancy in the dataset is removed, the following criteria were employed to filter the protein structures

- (i) The structures were solved with <2.5 Å resolution.
- (ii) The sequence identity among the majority of proteins in the dataset was less than 40%.

These selection criteria resulted in a non-redundant dataset of four eNOS and six nNOS proteins. The PDB ID's of eNOS proteins included in data set are 1D0C [34], 1M9J [32], 1P6M [35], 1RS8 [36] and the nNOS proteins are 1F20 [37], 1K2R [38], 1OM4 [38], 1P6I [39], 1QW6 [40] and 1TLL [41]. The details of data set are given in Table 1.

2.2. π - π Interactions

The stand alone program NCI (Non-canonical interactions) server [42] was used to identify π - π interactions in a protein. NCI used the geometric criteria of separation 4–6.5 Å between the centroids of aromatic ring of interacting residues to predict π - π interactions. All significant π - π interactions, where the aromatic residues (Phe, Tyr and Trp) donate π electron density with other acceptor aromatic residues were identified. We did not consider C-H... π interactions for our analysis in the present study, because in C-H... π interactions the donor residues were not always π -residues. The π - π interaction types were represented by a two letter code S and S5. S represented the side-chain (6 membered aromatic

ring) of Phe, Tyr and Trp. S5 represented the side-chain (5 membered ring) in Trp. Even though His has a 5 membered aromatic ring, it was not considered as the protonation state of histidine depended on the pH and local environment of residues. It may act as either cation or π residue, and can change the preferred relative orientations of the π - π interaction.

2.3. Location of π - π interaction forming residues based on secondary structure and solvent accessibility

Based on the location of π - π interaction forming residues in different secondary structures of eNOS and nNOS proteins and their solvent accessibility, a systematic analysis was undertaken. We obtained the information about secondary structures from PDB [33] and identified solvent accessibility of the proteins using the program ASAView [43].

2.4. Computation of conservation of amino acid residues

Conservation scores of π - π interacting residues were computed using ConSurf [44]. The program employed PSI-BLAST for similarity search. All the sequences that were evolutionary related with each one of the proteins in the data set were used in the subsequent multiple alignments. Based on these protein sequence alignments the residues were classified into nine categories from highly variable to highly conserved. Residues with a score of 1 were considered highly variable and residues with a score of 9 were considered highly conserved.

2.5. Computation of stabilization centers

Stabilization centers were clusters of residues that are involved in long range interactions [45]. Two residues were considered to form a long range contact, if they were at least four amino acid apart in the sequence and lie within 8 Å radius limit in the three dimensional space. We used the SCode program for predicting stabilization centers among π - π interacting residues [45].

2.6. Sequential distances

Sequential distances were calculated by employing standard accepted methodologies reported earlier [22,46–50].

3. Results and discussion

3.1. π - π Interactions

The π - π interactions in the eNOS and nNOS proteins investigated are shown in Fig. 1. We find that 95% of interactions are side-chain to side-chain interactions in both eNOS and nNOS proteins. In nNOS, 5% of interactions are side-chain five member aromatic ring to side-chain interactions. In eNOS proteins, 1% of interaction is side-chain to side-chain five member aromatic ring and remaining 4% are side-chain five member aromatic ring to side-chain interactions. The major contribution to π - π interactions are mainly from side-chain to side-chain interactions and the contribution of side-chain to side-chain five member aromatic ring π - π interactions are very minimal. In the data set, there is an average of 19 π - π interactions per protein in eNOS and 21 π - π interactions per protein in nNOS. There is an average of one significant π - π interaction for every 20 amino acid residues in nNOS proteins, whereas it is for 22 residues in eNOS proteins.

There are 65 Phe, 53 Tyr, 48 Trp and in total 166 π residues in eNOS. There are 120 Phe, 102 Tyr, 65 Trp and in total 287 π residues in nNOS. There are 76 π - π interactions in eNOS and 124

Table 1
PDB structures employed for the analysis of π - π interactions.

PDB ID	Chain	Source	Resolution (Å)	Number of π - π interactions
eNOS				
1D0C [34]	A	Bovine	1.65	19
1M9J [32]	A	Human	2.43	20
1P6M [35]	A	Bovine	2.27	18
1RS8 [36]	A	Bovine	2.30	19
nNOS				
1F20 [37]	A	Rat	1.90	12
1K2R [38]	A	Rat	2.15	22
1OM4 [38]	A	Rat	1.75	24
1P6I [39]	A	Rat	1.90	23
1QW6 [40]	A	Rat	2.10	23
1TLL [41]	A	Rat	2.30	20

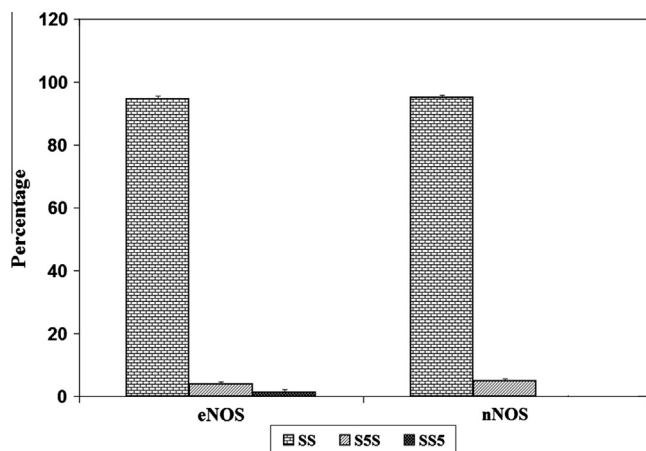


Fig. 1. Types of π - π interactions in eNOS and nNOS proteins.

interactions in nNOS in the whole data set. It is interesting to observe that, there is an average of one significant π - π interaction for almost 2 aromatic amino acid residues. Despite their lesser natural occurrences in both eNOS and nNOS, we consider the higher number of π - π interactions to be important in the structural stability context in these proteins.

3.2. Preference of aromatic residues for forming π - π interaction in eNOS and nNOS proteins

The preferences of amino acid residues that are involved in π - π interactions are analyzed. We observe that in both eNOS and nNOS proteins, the occurrence of Tyr are more than Phe and Trp residues in π - π interactions. The occurrence of Phe, Tyr and Trp residues in eNOS proteins are 36%, 42% and 22% respectively. In tyrosine, the ability of hydroxyl group to act as H-bond donor, substantially potentiates the binding ability of phenolic ring [51]. Also, the hydroxyl group increases the π -stacking by withdrawing π -electron density from the substituted benzene and reduces the electrostatic repulsion with other benzene [52]. The occurrence of Phe, Tyr and Trp residues in nNOS proteins are 37%, 38% and 25% respectively. The lowest frequency of involvement in π - π interactions by Trp residue can be explained by the fact that Trp is the least frequently occurring amino acid in any protein [53]. These results are depicted in Fig. 2.

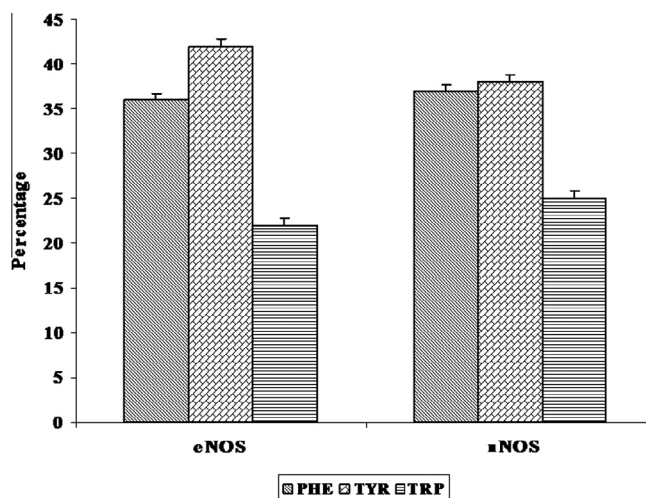


Fig. 2. Preference of π - π interacting residues in eNOS and nNOS proteins.

From these results we infer that Tyr residues have maximum contribution whereas Trp has the lowest occurrence for π - π interaction in both eNOS and nNOS proteins.

3.3. Secondary structure preferences

The systematic analysis of secondary structure preference of each amino acid, which participated in different types of π - π interactions are performed. The preferences correlate with the chemical structure and stereochemistry of the amino acids [54]. The secondary structure preference of each of the amino acids involved in π - π interactions are obtained from PDB. In eNOS proteins, Phe prefer the helix, Tyr and Trp residues prefer strands. These results are represented in Table 2. Because of the aromatic ring, most of these π - π interacting residues in eNOS prefer strand conformation. However, these observations were consistent with the results observed by our group with C-H... π [55] interacting residues in interleukins.

We find that in nNOS proteins, Phe and Tyr residues are found to prefer helix whereas Trp prefer strands. It is interesting to note that, these results were consistent with the results observed with C-H... π interactions in RNA binding proteins [49]. These results are depicted in Table 3.

3.4. Conservation score of interacting residues

Amino acid residues with a conservation score of ≥ 6 are considered to be evolutionary conserved [44]. It is interesting to note that, 67.85% and 68.25% of residues involved in π - π interactions are conserved in eNOS and nNOS proteins respectively, as they have a higher conservation score. The hydrophobic clusters in proteins are conserved within homologous family members [47]. Since most of π - π interacting residues are hydrophobic in nature, they might be conserved. These results are shown in Fig. 3. These results were consistent with our earlier observations in metalloproteins [26].

Thus from these observations, we are able to infer that most of the amino acid residues involved in π - π interactions might be conserved in both eNOS and nNOS proteins. Hence, π - π interacting residues have an additional role in maintaining the structure and function of eNOS and nNOS proteins.

3.5. Stabilization centers

The unit of stabilization center (SC) is one pair of interacting residues that are far enough in the primary structure and whose interactions are also supported by other interactions formed by residues located in their vicinity in the primary structure [56]. We have computed the stabilization center [45] for all π - π interaction forming residues in both eNOS and nNOS proteins. 23%, 14% of π - π interacting residues in eNOS and nNOS proteins respectively have one or more stabilization centers. These results are shown in Fig. 4.

Proteins should have a well balanced stability allowing structural fluctuations and concomitantly ensuring the long-lasting equilibrium structure [57–59]. Residues can be considered part of stabilization centers if they are involved in medium or long-range

Table 2
Secondary structure preferences among π - π interacting residues in eNOS.

	Helix	Strand	Turn
PHE (%)	41.6	34.56	24.1
TYR (%)	32.5	54.3	13.2
TRP (%)	20.35	68.57	11.1

Table 3
Secondary structure preferences among π - π interacting residues in nNOS.

	Helix	Strand	Turn
PHE (%)	41.86	35.97	22.17
TYR (%)	44.23	36.97	18.48
TRP (%)	36.69	43.5	19.8

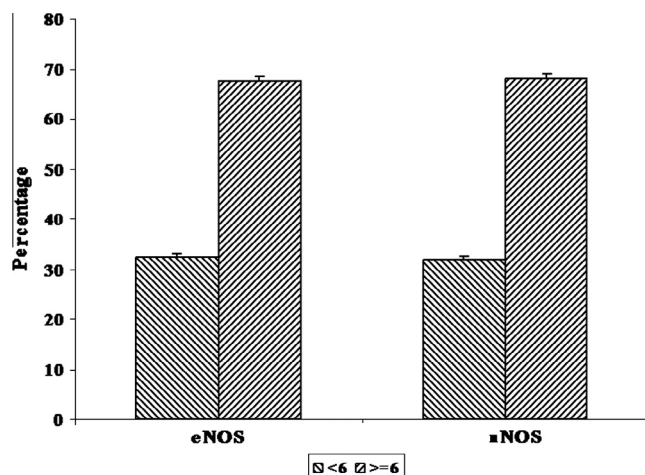


Fig. 3. Conservation score of π - π interacting residues in eNOS and nNOS proteins.

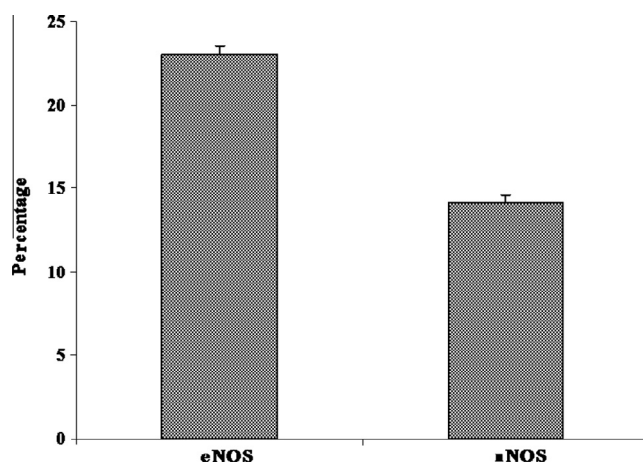


Fig. 4. Stabilization centers of π - π interacting residues in eNOS and nNOS.

interactions and if two supporting residues can be selected from both of their flanking tetra peptides, which together with the central residues form at least seven out of the nine possible contacts. From the observed results, we infer that the percentage of stabilization centers is more in the case of eNOS proteins than nNOS. All these SC residues might contribute additional stability to eNOS and nNOS proteins in addition to their participation in π - π interactions. It is interesting to note that these results are consistent with cation- π interaction studies in RNA binding proteins [22] and comparable to the eNOS and nNOS proteins in which percentage of stabilization centers is more in nNOS than eNOS [28].

3.6. π - π Interacting residues in different ASA ranges

Proteins are the embodiment of the transition from the one-dimensional world of sequences to the three-dimensional world of molecules capable of diverse activities [60]. Secondary structure

and solvent accessibility play a major role to determine the structure and function of proteins. The accessible surface of a molecule is the part of the molecular surface that is exposed to the solvent. Key functional properties of proteins and so-called active amino acid sites strongly correlate with amino acid solvent accessibility or Accessible surface area (ASA) [61,62].

In both eNOS and nNOS proteins, it is observed that majority of π - π interacting residues are preferred to be in the buried region. Hence, these residues stabilize the inner core regions in these proteins. These results are shown in Fig. 5. These predictions were similar to the observations of cation- π interactions in TNF proteins and interleukins [23], as well as comparable to eNOS and nNOS proteins [28].

3.7. Residue pairs involved in π - π interactions

There are six possibilities of π - π interacting pairs namely Phe-Phe, Phe-Tyr, Phe-Trp, Tyr-Tyr, Tyr-Trp, and Trp-Trp pairs. After analyzing π - π interactions in eNOS and nNOS proteins, the highest percentage of interactions are seen between Phe-Tyr pairs. In eNOS, when homo-pairs are considered, 17.11% of interactions are Phe-Phe, 15.79% are Tyr-Tyr and 5.26% are Trp-Trp interactions. The compositions of remaining interacting pairs (F-Y, F-W, and Y-W) towards π - π interactions in eNOS proteins are 27.63%, 10.52% and 23.68% respectively. These results are shown in Fig. 6a.

In nNOS proteins, the highest percentage of interactions is seen between Phe-Tyr residues. Among the homo-pairs, the occurrences of Phe-Phe pair are more than other two pairs. The composition of π - π interacting pairs (F-F, F-Y, F-W, Y-Y, Y-W and W-W) in nNOS proteins are 16.11%, 25.81%, 17.74%, 14.52%, 20.16% and 5.65% respectively. These results are depicted in Fig. 6b.

We have also analyzed the centroid distance between the aromatic rings of π - π interacting pairs. The π - π interacting pairs are most favorable in the distance range of 5–6 Å. These results are shown in Fig. 7. The PyMol view of interacting pair and centroid distance of nNOS protein [PDB ID 1K2R] are shown in Fig. 8.

A larger π -network will add more stability and play an important role in understanding the 3D structure of proteins. So, we analyzed the π - π networks in these eNOS and nNOS proteins. There are π -networks in both eNOS and nNOS proteins. The connectivity of π -ring is found to increase along the length of a network from 2 π to 7 π . We found that 6 π , 7 π are the maximum networks in the data set of eNOS and nNOS proteins respectively. The π -networks of

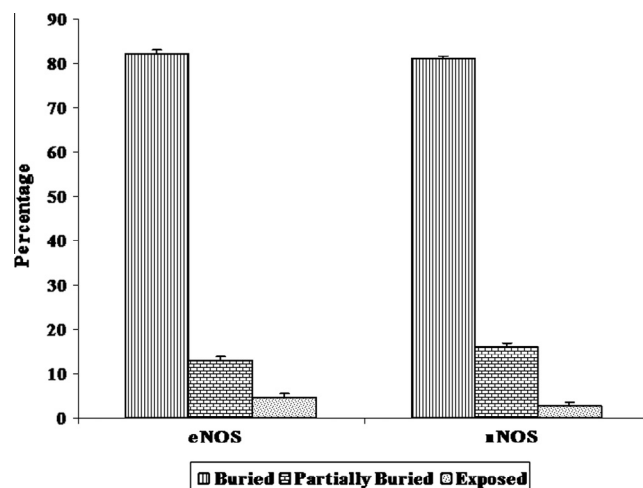


Fig. 5. Solvent accessibility patterns of π - π interacting residues in eNOS and nNOS proteins.

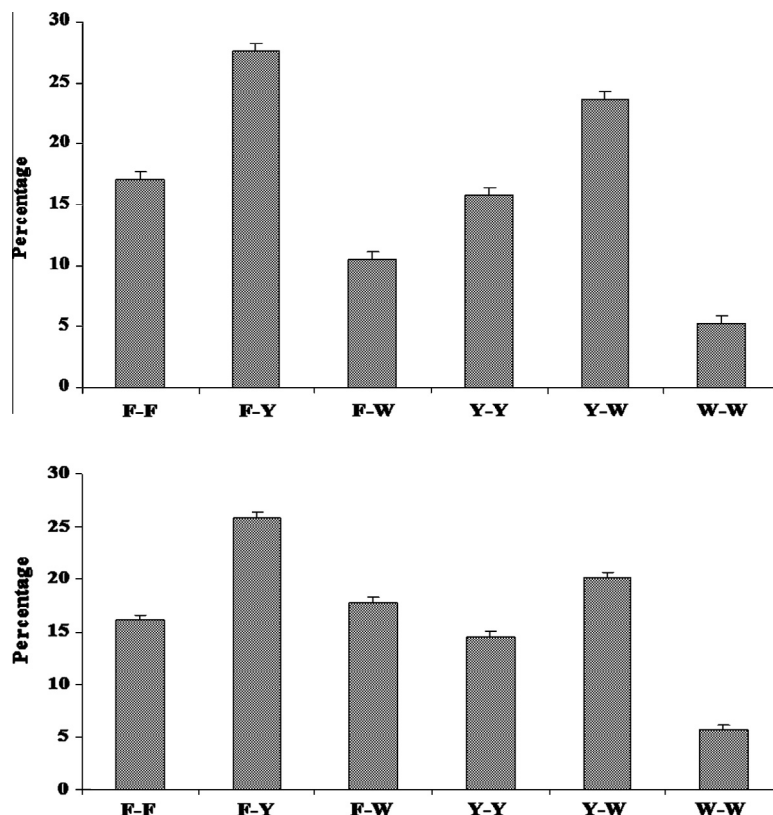


Fig. 6. Interacting pairs in proteins (a) π - π interacting pairs in eNOS and (b) residue pairs involved in π - π interaction in nNOS.

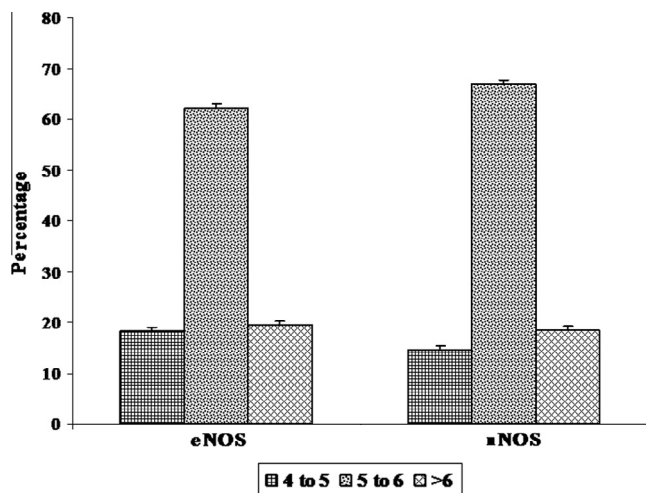


Fig. 7. Preferential distances (in Å) of π - π interacting pairs in eNOS and nNOS proteins.

these eNOS and nNOS proteins are shown in Fig. 9a and b. The PyMol view of π -network in nNOS protein [PDB ID 1K2R] is shown in Fig. 9c.

3.8. Sequential separation of interacting pairs

Long-range interactions play an active role in the stability of protein molecules [48]. Short, medium and long range interactions as a function of percentage of interaction in eNOS and nNOS proteins are studied. It is observed that 85% of eNOS proteins exhibited

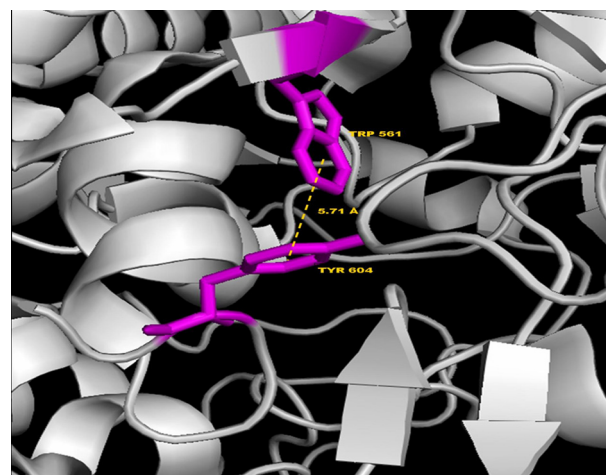


Fig. 8. PyMol view of Tyr-Trp interacting pair in nNOS protein [PDB ID 1K2R].

long range interactions. In nNOS proteins, 80% of interactions are long range interactions. This result revealed that majority of the π - π interactions in eNOS and nNOS proteins are influenced by long range interactions. These results reflect the importance of long-range interactions to the stability of eNOS and nNOS proteins via π - π interactions as reported in other structural classes of globular proteins [56,63–65]. These results are shown in Fig. 10. Long range π - π interactions are the predominant type of interactions in eNOS and nNOS proteins. These results were consistent with the observations in RNA-binding proteins [22] and comparable to C-H \cdots π interaction studies in interleukins [55].

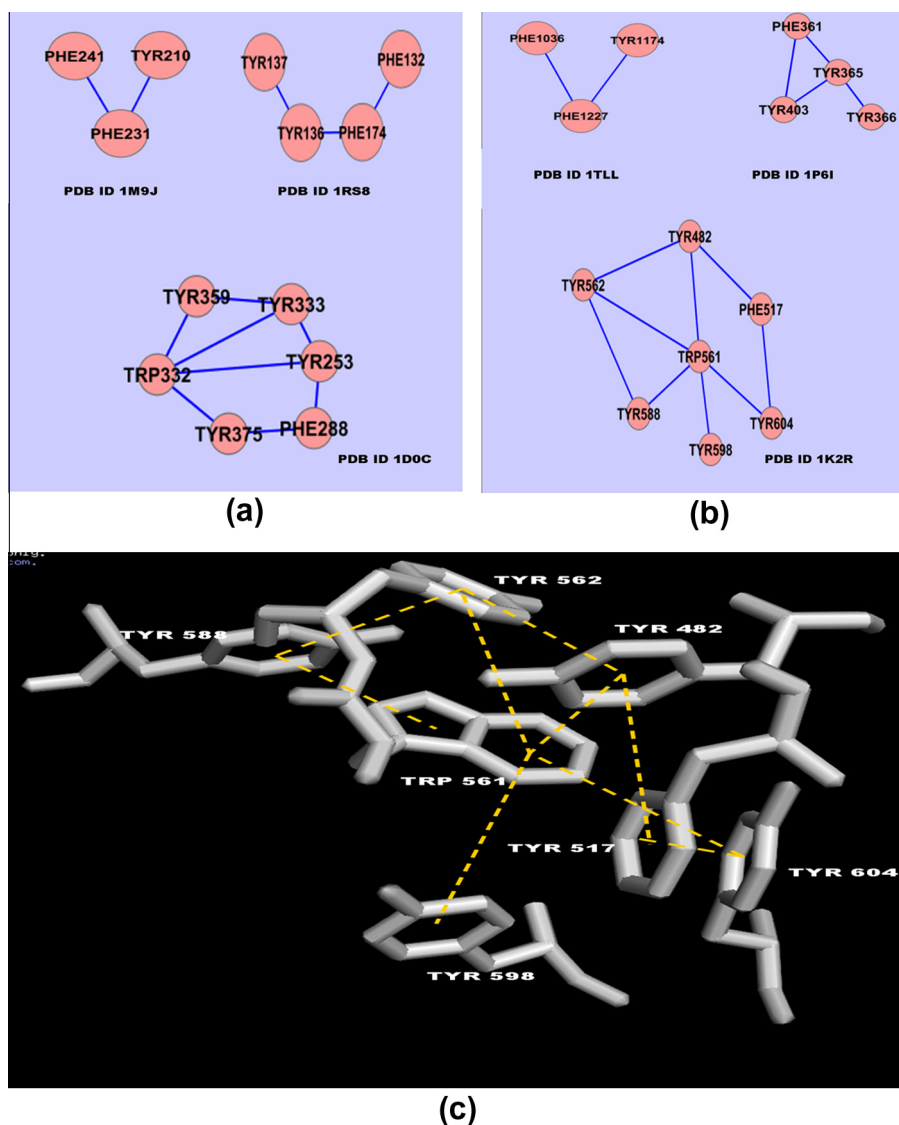


Fig. 9. The 3π , 4π and maximum π networks in (a) eNOS proteins, (b) nNOS proteins and (c) PyMol view of π network in nNOS protein [PDB ID 1K2R].

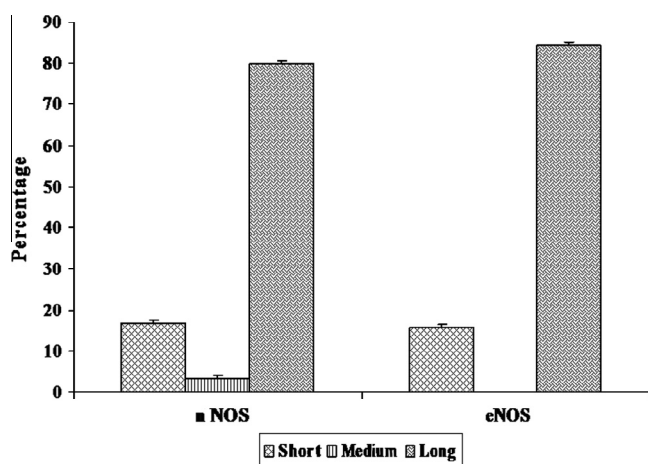


Fig. 10. Sequential distances of π - π interacting pairs in eNOS and nNOS proteins.

4. Conclusion

Based on the results of our analysis on π - π interactions in eNOS and nNOS proteins, we conclude that, there are significant numbers of π - π interactions in these proteins. π - π interactions are slightly higher in nNOS proteins as indicated by their slightly higher occurrences. Among the π - π interacting residues, the participation from Tyr is high and Trp is low in both protein classes studied.

The secondary structure analysis in eNOS proteins among π - π interacting residues suggests that, Phe might stabilize helices, while Tyr and Trp might stabilize strands through π - π interactions. In nNOS proteins, Phe and Tyr might stabilize helices while Trp might stabilize strands through π - π interactions.

Since, most of the π - π interactions are localized in the buried regions, these interactions might be important in stabilizing the inner core regions in these proteins. Most of the π - π interactions are located in long-range contacts and hence, they might play an important role in the global structural stability of these proteins. Majority of the amino acid residues involved in π - π interactions are conserved and they might be important in maintaining the structural stability through these interactions in both eNOS and

nNOS proteins. Significant percentages of π - π interacting residues have one or more stabilization centers. The π - π interacting pairs are most favorable in the distance range of 5–6 Å. There is also a significant number of π -networks in both eNOS and nNOS proteins. On the whole, we infer that π - π interactions and π - π networks play an important role in the structural stability of eNOS and nNOS proteins.

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