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On the importance of π–π interactions in the structural stability of phycocyanins

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Abstract: The influences of π–π interactions in phycocyanin proteins and their environmental preferences were analyzed. The observations indicate that the majority of the aromatic residues in phycocyanin proteins are involved in π–π interactions. Phenylalanine (Phe) and tyrosine (Tyr) residues were found to be involved in π–π interactions much more frequently than tryptophan (Trp) or histidine (His). Similarly, the Phe–Phe and Tyr–Tyr π–π interacting pair had the highest frequency of occurrence. In addition to π-π interactions, the aromatic residues also form π-networks in phycocyanins. The π–π interactions are most favourable at the pair distance range of 5.5–7 Å, with a clear preference for T-shaped ring arrangements. Using *ab initio* calculations, we observed that most of the π–π interactions possess energy from 0 to −10 kJ mol^{-1}. Stabilization centres for these proteins showed that all residues found in π–π interactions are important in locating one or more such centres. π–π interacting residues are evolutionary conserved. The results obtained from this study will be beneficial in further understanding the structural stability and eventual development of protein engineering of phycocyanins.

Keywords: phycobiliproteins; aromatic interactions; stabilization centers; amino acid conservation; *ab initio* study.

INTRODUCTION

Phycobiliproteins (PBPs) are a family of water-soluble, intensely fluorescent holoproteins consisting of apoprotein and covalently bound linear tetrapyrrole chromophores called phycobilins that function as components in the photosynthetic apparatus of cyanobacteria and certain algae.[1] These organisms have been major contributors to the evolution of oxygen and the absorption of carbon diox-

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ide from the atmosphere.[2] Most common PBPs, differing in their protein structure, phycobilin content attached to conserved cysteine residues, absorbance, and fluorescent properties, are phycoerythrins, with phycoerythrobilin as red chromophore and phycocyanins (C-phycocyanin and allophycocyanin) with blue-purple phycocyanobilin chromophore. They are efficiently used in various sectors, *e.g.*, as colourants in the food and cosmetics industries and pharmaceuticals.[3] In general, the stability of phycocyanin aggregates depends on their origin, amino acid composition, light, pH, temperature and some exogenous substances.[4] Interestingly, molecular forces (predominantly non-covalent interactions) responsible for the observed differences in thermal and chemical stability of different phycocyanin complexes are not entirely understood.[5] Understanding the nature of non-covalent interactions is thus extremely important to see what causes these variations in the properties.

Interaction between the arene systems (π–π) has been recognized as a key stabilizing force in supramolecular chemistry, drug design, biochemistry, crystal engineering, and molecular science.[6-10] The nature of π–π interaction was primarily thought to be dispersive with notable electrostatic contribution depending on the system in question.[11] Although π–π interactions are accepted as weak, they still play an important role in the folding and the thermal stability of proteins.[12,13] The calculated π–π interaction energies of the parallel, edge–face (T--shaped) and offset stacked are –6.19, –10.29 and –10.38 kJ mol^{-1}, respectively,[14] and the major source of attraction are not short-range (such as charge--transfer), but long-range interactions (quadrupole–quadrupole electrostatic and dispersion).[15] Aromatic residues show a high tendency towards forming clusters beyond the dimer, significantly influencing protein folding, structure and stability.[7,16]

The presented study expands on our previous work on the non-covalent interactions and cation–π interactions of phycocyanin crystal structures by analyzing the same protein group with respect to π–π interactions to better understand their stabilizing role.[17,18] We have focused our study on the phycocyanin interfaces and therefore the π–π interactions within a protein are not considered. Obtained results might contribute to the understanding of the structural stability of this class of evolutionary essential proteins with increased practical application and future designs of novel protein–bioactive compound interactions.

EXPERIMENTAL

Dataset

For this study, we used the Protein Data Bank (PDB), accessed on June 14th, 2022, at that moment, listing 191,308 resolved structures.[19] The selection criteria for phycocyanins to be included in the dataset were as follows: 1) structures of proteins containing phycocyanin alpha or beta subunit domain (SCOP Classification, version 1.75)[20] were accepted; 2) theoretical model structures and NMR structures were not included (these structures were not accepted as it was difficult to define the accuracy of the ensemble of structures in terms of dis-

placement that was directly comparable to the X-ray diffraction studies); 3) only crystal structures with the resolution of 2.0 Å or better and a crystallographic *R*-factor of 25.0 % or lower were accepted; 4) we included only representatives having at least 30 % sequence identity. After assembling the dataset, several structures containing ligands and mutant amino acids were rejected, leaving 20 proteins that were actually used as the dataset in our analysis. Hydrogen atoms were added and optimized, where needed, using the program REDUCE,[21] with default settings. REDUCE software adds hydrogen atoms to protein and/or DNA structures in standardized geometry, optimizing them to the orientations of OH, SH, NH_3^+, Met (methionine) methyls, Asn (asparagine) and Gln (glutamine) sidechain amides and His rings. The software determines the best hydrogen positions by selecting the best overall score from all of the possible combinations, taking into account single scores assigned for each individual residue and for groups containing movable protons partitioned in closed sets of local interacting networks. The PDB IDs of selected protein chain structures were as follows: 1all, 1b33, 1cpc, 1f99, 1gh0, 1jbo, 1kn1, 1phn, 2bv8, 2vjt, 2vml, 3dbj, 3o18, 4f0u, 4l1e, 4lm6, 4lms, 4po5, 4rmp and 4yjj.

π–π Interaction analysis

A computer program Discovery Studio Visualizer 2020[22] was used for the calculation of various types π–π interactions and their geometrical features with default settings (Fig. 1). π–π interactions are determined following the methodology of McGaughey.[23] This method finds stacked and staggered π–π interactions by performing the following tests: 1) the distance between the centroid of each pair of π rings is determined to find those which fall within the π–π centroid (R_{cen}) cutoff distance (7 Å by default). For these, an atom from each ring should be within the closest atom distance (R_{clo}) cutoff distance (default 7 Å). The angle θ between the normal of one or both rings and the centroid–centroid vector must fall between 0°, and ± the theta angle cutoff (default 90°), and the angle λ between the normal to each ring must fall between 0° and ± the lambda angle cutoff (default 90°) or greater. The aromatic systems include the aromatic side chains of the residues Trp, Tyr, Phe, and His. However, as His can act either as a cation or as an aromatic moiety depending on its protonation state, in our study, REDUCE software did not suggest protonated form presence in our set of proteins.

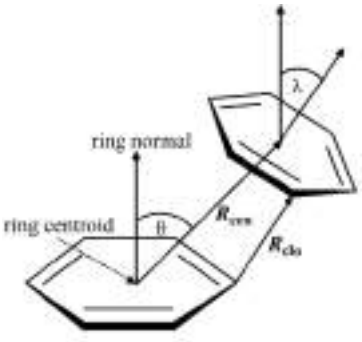

Fig. 1. Parameters for π–π interactions: R_{cen}, the distance between the centroid of each pair of π rings; R_{clo}, the distance between the closest atom of each π ring; θ, the angle between the normal of one or both rings and the centroid–centroid vector; λ, the angle between the normal to each ring.

Computation of π–π interaction energy

To apply *ab initio* methods in determining the energies of π–π pairs on the desired level of theory, with a sufficient level of accuracy and still in the satisfactory time frame, calculations were performed on the structurally reduced model systems: phenylalanine was simpli-

fied to toluene (**1**), histidine to 5-methyl-1*H*-imidazole (**2**), tryptophan to 3-methyl-1*H*-indole (**3**) and tyrosine was reduced to 4-methylphenol (**4**), Fig. 2.[24]

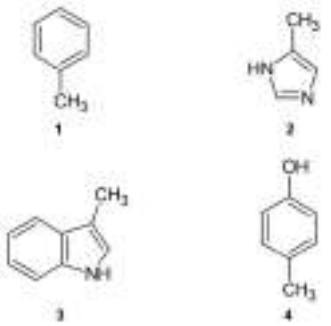

Fig. 2. Structurally reduced structures used for calculations of π–π interaction energy. **1** instead of Phe; **2** instead of His; **3** instead of Trp; **4** instead of Tyr.

Using reduced model systems in calculations of a specific intramolecular interaction in large systems is well known and already proven methodology,[25] producing accurate enough results and still significantly reducing computation times and strength needed. More extensive models, like whole amino acids or parts of the protein chain, will unnecessarily complicate calculations and probably even bring in errors. Numerous interaction mechanisms are possible in a larger protein structure, and a single binding energy computation cannot always correctly determine which of these interactions are present and to what amount they contribute to overall stabilization. As a result, it is difficult to separate the involvement of the π–π interaction and their energy contributions from the interacting pair residues involved in other noncovalent interactions.

Ab initio calculations were performed using Jaguar from Schrödinger Suite 2018-1,[26] using the LMP2 method with triple zeta Dunning's correlation consistent basis set[27] and ++ diffuse functions.[28] All calculations were performed in a vacuum. The LMP2 method applied to the study of π–π interactions, showed to be considerably faster than the MP2 method. Contrary to that, the calculated interaction energies and equilibrium distances were almost identical for both methods.[26] Several authors found that LMP2 represents an excellent method for calculating interaction energies in proteins.[29,30] Sometimes calculation results can be influenced mainly by BSSE, and taking that into account is mandatory for correct results, making the calculation times significantly longer. Local correlation methods (such as LMP2) not only reduce the cost of the calculations but the local Møller–Plesset second-order method LMP2 is well known for reducing intramolecular BSSE.[31-33]

Geometries of interacting structures were optimized using the LMP2/cc-pVTZ(-f)++ level of theory, and their single point energies were calculated at LMP2/cc-pVTZ++ level. We used a slightly smaller basis set for optimization than for SP calculations because the calculated geometries are almost identical as the ones produced with a larger basis set, and the calculations are more suitable for our equipment and almost twofold faster. The optimized geometries were placed in space to match corresponding complexes by superimposing heavy atoms onto their respective coordinates from the crystal structures. Then the energies of dimeric structures produced that way were calculated. The π–π interaction energies in dimers (π–π pairs) were calculated as the difference between the energy of the complex and the sum of the energies of the monomers in their optimized geometries.

As mentioned earlier, the energies in this work were calculated in the gas phase. When observing *in vivo* processes, we can expect that the water molecules and other atoms and

groups from the protein structure could be present in the vicinity, influencing the binding process. To correctly describe the binding, one must be well aware of the role of solvent in the complete process of binding to the proteins. To accurately depict the enthalpy of binding and calculate the interacting energy of bonded structures, high-level quantum mechanical calculations with extended basis sets, including a large number of atoms both in protein and ligand as well, together with water molecules, would be needed. But for a complete understanding of biological complexes and their behaviour, the free-energy changes (ΔG) have to be calculated using some statistical mechanics method.[34,35] However, this will exceed the main goal of this article, which is to point out the possible contribution and significance of the energies of π–π interactions to stability and orientation in protein complexes. Nevertheless, in the description of the complete biomolecular process of binding, accompanying entropies and solvation–desolvation processes are important and can be a dominant factor in the formation of complexes.

At this moment, our main focus was on the possible influence of the energy profile of π–π interactions on protein complexes. Therefore, we selected already-known structures of protein complexes and attempted to calculate energy contributions that originated just from specific π–π interactions whenever it was possible. The results relate only to gas-phase complexes, and the role of the solvent was disregarded. It should, however, be mentioned that the interactions inside the biomacromolecules correspond merely to the gas-phase model, and the gas-phase interactions thus play a vital role.[36]

Computation of stabilization centres

Stabilization centres (SC) are defined as the clusters of residues making cooperative, noncovalent long-range interactions.[37] Measured as individual interactions, stabilization forces resulting from noncovalent long-range interactions are not very strong. Still, since they are cooperative by their nature, in regions where they act in a group of SC, they could play an important role in maintaining the overall stability of protein structures. To analyse the SC of interaction-forming residues, we used the SCide program.[38] The criteria SCide uses for determining SC are as follows: 1) two residues are in contact if there is at least one heavy atom––atom distance smaller than the sum of their van der Waals radii plus 1 Å; 2) a contact is recognized as a "long-range" interaction if the interacting residues are at least ten amino acids apart; 3) two residues form stabilization centres if they are in long-range interaction and if it is possible to select one–one residue from both flanking tetrapeptides of these two residues that make at least seven contacts between these two triplets.[38]

Computation of conservation of amino acid residues

The conservation of amino acid residues in each protein was computed using the ConSurf server.[39] This server computes the conservation based on the comparison of the sequence of the given PDB chain with the proteins deposited in Swiss–Prot database.[40] It identifies ones that are homologous to the PDB sequence. The number of PSI–BLAST iterations and the *E*-value cutoff used in all similarity searches were 1 and 0.001, respectively. All the evolutionary sequences related to each of the proteins in the dataset were used in the subsequent multiple alignments. The residues were classified into nine categories based on these protein sequence alignments. Residues with a score of 1 are considered highly variable, and residues with a score of 9 are considered highly conserved.

RESULTS AND DISCUSSION

In this work, we studied the role of π–π interactions in the interfaces of phycocyanin proteins and their environmental preferences. We performed computat-

ional analysis of the 20 X-ray structures of phycocyanin-containing proteins and summarized π–π interactions to better understand the high stability of phycocyanin oligomers. Also, the relative preference of π–π interacting amino acids in interfaces, interaction geometries, and energetic contribution of π–π interactions, stabilization centres, and conservation score of amino acid residues were analysed.

Preference of aromatic residues for forming π–π interactions

We have analysed the frequency of occurrence of aromatic amino acid residues which are involved in π–π interactions. The results are given in Table 1. There are 158 π–π interactions in phycocyanin proteins in the data set we used. It is interesting to note that there is an average of 8 interactions per protein interface. We observed that in these proteins, Phe and Tyr have a higher occurrence than His and Trp. However, many amino acids are found in phycocyanin interfaces very rarely. Less than 1 % of the His and Trp residues in our database are in phycocyanin interfaces.[17] Considering the benzene ring in Phe and Tyr residues, the greater electro negativity of sp^2 C relative to H produces substantial C^{-}–H^{+} dipole. The C–H dipole accounts well for π–π interaction in phenylalanine.[41] In tyrosine, the hydroxyl group in the ortho position on the benzene ring increases the π-stacking by withdrawing the π-electron density from the substituted benzene, reducing the electrostatic repulsion with other the benzene ring.[42] We compared the occurrence of interacting pairs to find the preference for phycocyanin proteins (Table I). When homo-pairs of aromatic side chains are considered, the

TABLE I. Frequency of occurrence of π–π interaction-forming residues in active centers of phycocyanin proteins

Residue	Number[a]	Occurrence, %[b]
His	12	3.80
Phe	144	45.57
Trp	5	1.58
Tyr	155	49.05
Total	316	100
Interacting pair		
His–His	–	–
His–Phe	6	3.80
His–Trp	–	–
His–Tyr	6	3.80
Phe–Phe	63	39.87
Phe–Trp	–	–
Phe–Tyr	12	7.59
Trp–Trp	–	–
Trp–Tyr	5	3.16
Tyr–Tyr	66	41.78
Total	158	100

[a]The number of times a particular amino acid occurs in an appropriate interaction; [b]percent of amino acid occurs in an appropriate interaction

highest percentage of interactions is observed between Phe–Phe and Tyr–Tyr residues. Among the hetero-pairs, the occurrences of the Phe–Tyr pair are more frequent than other interacting pairs. Hence, these interaction pairs may be quite important in the structural stability of phycocyanin proteins.

We have also analysed the multiple π–π interactions (π-networks) in phycocyanin proteins. These π-networks might add more stability and play an important role in understanding the 3D structure of proteins.[43] The analysis showed that about 75 % of the total π–π interactions in the dataset are involved in the formation of multiple π interactions. The connectivity of the π-ring is found to increase along the length of a network from 2π to 7π. A large π-network can enhance the stability of a protein conformation and can have a considerable influence on protein–ligand interactions. It has also been shown that the addition of an aromatic pair on the protein surface increases its stability.[44] An illustrative example of a typical 7π-network of allophycocyanin B from *Synechocystis* PCC [PDB ID 4PO5] is shown in Fig. 3.

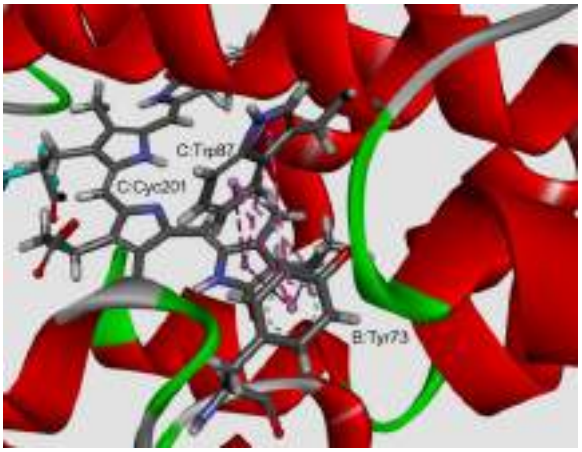

Fig. 3. The view of Trp–Tyr interacting pair and 7π network in allophycocyanin B from *Synechocystis* PCC [PDB ID 4PO5]. The interactions are marked with pink dashed lines.

Interaction geometries and energetic contribution of π–π interactions

The native structure is the compromise of many noncovalent interactions existing in proteins, and the geometrical features relating to the two residue-types are expected to be rather broad. However, based on the distribution of interplanar angles, it was suggested that there is nonrandomness in the packing of side chains.[45] On the basis of the orientation of the aromatic rings, the π–π interactions between two aromatic species have been broadly classified into three categories: edge to face (T-shaped), parallel displaced, and parallel stacked.[46] For example, McGaughey *et al.* analysed 505 proteins and determined that an offset parallel-

-stacked conformation was, on average, 4.18 kJ mol^{-1} more stabilizing than a T--shaped geometry.[23,47] The frequency distribution of the distance and angle parameters of π–π interacting pairs are analysed. These results are shown in Fig. 4. The π–π interacting pairs are most favourable in the distance range (R_{cen}) of 5.5––7 Å (Fig. 4a). At separation distances below 5 Å, aromatic pairs are rarely observed, a result of obvious physical constraints. The distribution of R_{clo} for π–π interactions was found to be a narrow peak at 4 Å (Fig. 4b), which is the optimal average distance between two aromatic rings in a T-shaped orientation. This is because T-shaped orientations have a shorter R_{clo} than parallel orientations. Regardless of the angle, the aromatic side chains orient in a fashion to minimize R_{clo} between the two rings and thus maximize the van der Waals attraction. The normal of one or both rings and the centroid–centroid vector (θ) was found to be bimodal with a prominent minimum between 40 and 60° (Fig. 4c) and it nearly equally prefers apical and equatorial ring orientations. Considering the plane––plane angle (λ), the angles were distributed between all angles (0–90° range) (Fig. 4d). While axial aromatic pairs (λ > 50°) are more frequent, there were a few interactions with angles below 40° (shows coplanarity), possibly to maximize π stacking and packing.[23] Overall, the preferred orientations are quite sim-

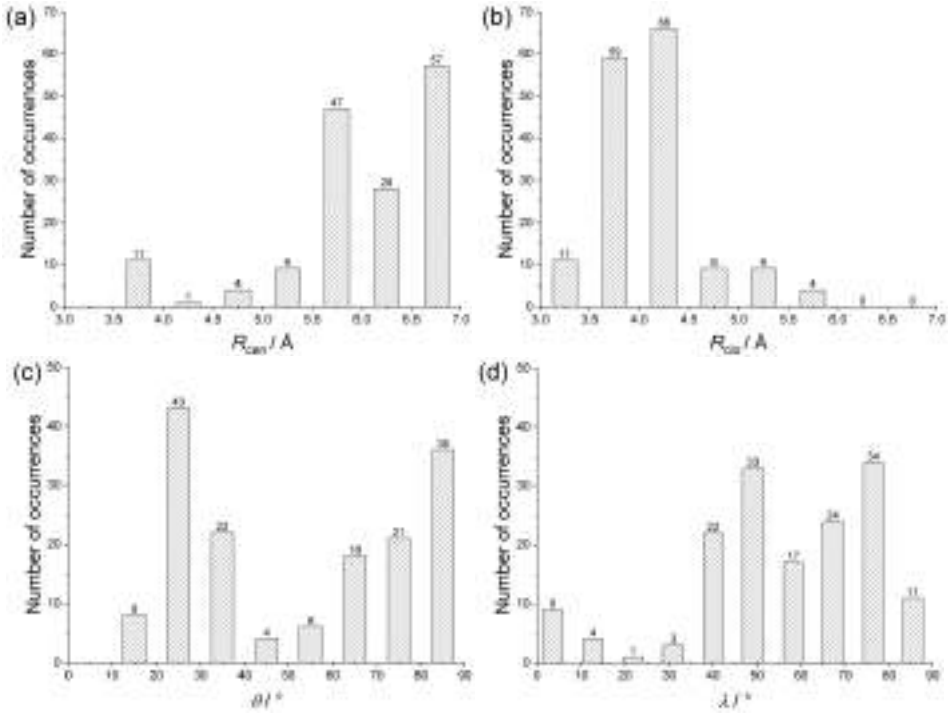

Fig. 4. Interaction geometries of π–π interactions in phycocyanins: a) R_{cen} distance distribution, b) R_{clo} distance distribution c) θ angle distribution, d) λ angle distribution.

ilar to those found with aromatic–aromatic interactions,[48] and the T-shaped orientation is observed. The geometries observed in abundance are not necessarily the ones that have the highest interaction energy between the two moieties in a pair, but the ones that can provide the maximum overall stability to the protein structure by the optimum use of all π interactions.

The quantification of non-covalent interactions is of great importance for a rational approach to biological systems, including protein structure and function, antibody binding, or drug design, as well as for the further development of supramolecular chemistry.[49] Therefore, the energetic contributions of residues involved in π–π interactions were computed using *ab initio* calculations at the LMP2 level. Within a large protein structure, numerous interactions are possible, and sometimes it is not easy to parse out the role of the π–π interaction in their energetics by a simple calculation. Therefore, the interacting pair residues participating in other non-covalent interactions were not analysed. The results for π–π interacting pairs are presented in Fig. 5. The energies calculated for many of the π–π interactions are substantially stabilizing, with 10 % of the total showing positive (repulsive) interaction energies. The repulsive nature of those interactions emerges from the unfavourable geometries of π–π interactions in the crystal structures and is usually counterbalanced by other stronger interactions (salt bridge, H-bonding, or similar).[24] Namely, we mentioned earlier that, when examined under isolated conditions, this type of interaction is considered unfavourable, but similar to other potentially unfavourable interactions, their influence can be compensated by other interactions from the rest of the polypeptide chain. In our database, it was found that π–π interactions showed energy less than −20 kJ mol^{-1}, and most of them have energy in the range 0 to −10 kJ mol^{-1}. The energies associated with π–π interactions may be important contributors to the overall protein stability. It should also be taken into account in supramolecular chemistry and protein engineering fields.[43]

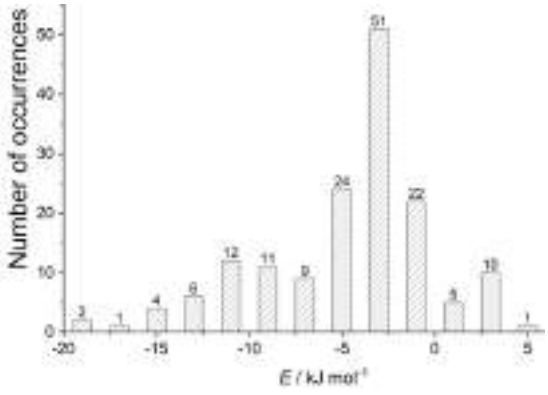

Fig. 5. Interaction energies of π–π interactions in phycocyanins.

The results of our *ab initio* calculations of optimized structures showed that the strongest attractive π–π interaction (−18.58 kJ mol^{-1}) exists between A:Tyr65–I:Tyr65 pair in the monoclinic structure of phycocyanin from *Gloeobacter violaceus* (PDB ID 2vml; Fig. 6).

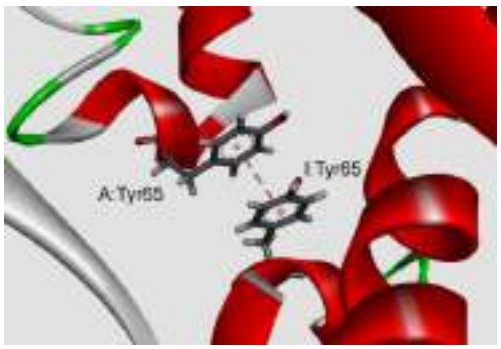

Fig. 6. Details of the strongest attractive π–π interaction of phycocyanin from *Gloeobacter violaceus* (PDB ID 2vml). The interaction is marked with a pink dashed line: A:Tyr65 – I:Tyr65; R_{cen} = 4.00 Å, R_{clo} = 3.55 Å, θ = 24.85°, λ = 5.53°, E = = –18.58 kJ mol^{-1}.

Stabilization centers and conservation of amino acid residues

The unit of stabilization centre is one pair of interacting residues that are far enough in the primary structure and the interactions of which are also supported by other interactions formed by residues located in their vicinity in the primary structure.[37] We have computed the stabilization centre for all π–π interaction forming residues in phycocyanins. Considering the whole data set, 41.4 % of all stabilizing residues are involved in building π–π interactions. It was interesting to note that all residues involved in π–π interactions were included in at least one stabilization centre. These observations strongly reveal that these residues may contribute significantly to the structural stability of these proteins in addition to participating in π–π interactions.

The level of evolutionary conservation was often used as an indicator of the importance of certain positions in maintaining the protein's structure and/or function.[50] Conservation score is a useful parameter for the identification of conserved residues in a protein sequence based on the phylogenetic relations between homologous sequences. Considering the conservation score of π-interacting residues, we found several residues with a conservation score of 9, and there are 57.8 % of residues with a conservation score higher or equal to 6. Our results assumed that most of the residues involved in π–π interactions are evolutionarily conserved. Therefore, we believe that π–π interacting residues have an additional role in maintaining the structure and function of phycocyanin proteins.

CONCLUSION

Even though many studies are done on the molecular aspects, there are no reports on the systematic analysis of π–π interactions in phycocyanin proteins. In the present study, the analysis of the role of π–π interactions in phycocyanin

proteins indicate that most of the aromatic residues are involved in π–π interactions and contribute significantly to the structural stability of these proteins. Considering the individual contribution of the aromatic residues towards π–π interactions, Phe and Tyr residues are found to have exceeded the other two aromatic amino acids. We compared the occurrence of interacting pairs to find the preference for phycocyanin proteins. The Phe–Phe and Tyr–Tyr pairs have a higher frequency of occurrence than other pairs. Furthermore, the multiple interaction patterns found in the present study indicate that around 75 % of the total interacting residues participate in multiple π–π interactions. We also find that all these interacting pairs are favourable in the distance range of 5.5–7 Å. Considering the angle distribution, effective π–π interactions can be realized above a wider area above the π ring, indicating a clear overall preference for T-shaped rings arrangements. The *ab initio* calculations of the optimized structures of interacting π–π pairs showed that favourable energy interactions were less than –20 kJ mol^{-1}, while most of them have energy from 0 to –10 kJ mol^{-1}. A significant percentage of the π–π interacting residues also are located as stabilization centres and thus might provide additional stability to these proteins. The conservation patterns in the present study indicate that more than half of the residues involved in these interactions are evolutionarily conserved. These results were comparable with our earlier observations in protein–porphyrin complexes and superoxide dismutases and show that the fundamental property of π–π interactions, namely non-randomness in the packing of side chains, holds by and large for all categories in macromolecular structures.

In conclusion, the observations obtained in this study identify π–π interactions and structural motifs that contribute to stabilizing the increasingly used phycocyanin proteins, are relevant to the understanding of structure-function relationships, and are helpful to the efforts made to design and engineer protein–protein complexes.

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И З В О Д

О ЗНАЧАЈУ π–π ИНТЕРАКЦИЈА У СТРУКТУРНОЈ СТАБИЛНОСТИ ФИКОЦИЈАНИНА

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Анализирани су утицаји π–π интеракција у протеинима фикоцијанинима и њихове преференције ка окружењу. Запажања показују да је већина ароматичних остатака у протеинима фикоцијанинима укључена у π–π интеракције. Утврђено је да су остаци фенилаланина (Phe) и тирозина (Tyr) много чешће укључени у π–π интеракције него триптофана (Trp) или хистидина (His). Слично томе, интерагујући π–π парови Phe–Phe и

Tyr–Tyr имали су највећу учесталост појављивања. Додатно, ароматични остаци такође стварају π-мреже у фикоцијанинима. π–π интеракције су најповољније у распону дистанци парова од 5,5–7 Å, с јасном склоношћу за распоред прстенова у облику слова T. Користећи *ab initio* прорачуне, приметили смо да већина π–π интеракција има енергију у распону од 0 до –10 kJ mol^{-1}. Стабилизациони центри ових протеина показали су да су сви остаци пронађени у π–π интеракцијама важни у лоцирању једног или више таквих центара. π–π интеракциони остаци су еволутивно конзервирани. Резултати добивени овом студијом биће од користи у даљем разумевању структурне стабилности и евентуалном развоју протеинског инжењеринга фикоцијанина.

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