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ORIGINAL ARTICLE

Non-canonical interactions of porphyrins in porphyrin-containing proteins

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Abstract In this study we have described the non-canonical interactions between the porphyrin ring and the protein part of porphyrin-containing proteins to better understand their stabilizing role. The analysis reported in this study shows that the predominant type of non-canonical interactions at porphyrins are CH···O and CH···N interactions, with a small percentage of CH···π and non-canonical interactions involving sulfur atoms. The majority of non-canonical interactions are formed from side-chains of charged and polar amino acids, whereas backbone groups are not frequently involved. The main-chain non-canonical interactions might be slightly more linear than the side-chain interactions, and they have somewhat shorter median distances. The analysis, performed in this study, shows that about 44% of the total interactions in the dataset are involved in the formation of multiple (furcated) non-canonical interactions. The high number of porphyrin–water interactions show importance of the inclusion of solvent in protein–ligand interaction studies. Furthermore, in the present study we have observed that stabilization centers are composed predominantly from nonpolar amino acid residues. Amino acids deployed in the environment of porphyrin rings are deposited in helices and coils. The results from this study might be used for structure-based porphyrin protein prediction and as scaffolds for future porphyrin-containing protein design.

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Introduction

Porphyrins are heterocyclic macrocycles composed of four modified pyrrole subunits interconnected at their α carbon atoms via methine bridges. In addition, porphyrins are aromatic conjugate acids of ligands that bind metal to form complexes. Some iron-containing porphyrins are called hemes (Rothemund 1935, 1936). Porphyrin-containing proteins are involved in many different processes in living organisms, including oxygen binding, electron transfer, signaling function, and catalysis. For example, porphyrin-containing proteins are constituents of photosynthetic reaction centres. A light-harvesting antenna complex is a complex of subunit proteins that may be part of a larger supercomplex of a photosystem, the functional unit in photosynthesis. It is used by plants and photosynthetic bacteria to collect more of the incoming light than could be captured by the photosynthetic reaction centre alone using resonance energy transfer (Karrasch et al. 1995; McDermott et al. 1995; Papiz et al. 2003; Roszak et al. 2003; Bahatyrova et al. 2004).

Hydrogen bonding plays a key role in the structure and function of proteins, including features such as protein folding, local architecture, protein–ligand recognition, enzymatic activity, protein hydration, and molecular dynamics (Jeffrey and Saenger 1991; Sarkhel and Desiraju 2004; Panigrahi and Desiraju 2007). The hydrogen bonds are manifested in a variety of strengths and geometries. It is well known that molecular frameworks are determined by covalent bonds with the typical energy of 100–200 kcal/mol depending on the extent of unsaturation in the bonds. The bonding energies of hydrogen bonds are lower than

energies of covalent interactions (Bartlett et al. 2002; Steiner 2002; Sarkhel and Desiraju 2004; Panigrahi and Desiraju 2007). Accordingly, hydrogen bonds like O–H···O, N–H···O, O–H···N and N–H···N may be considered to be strong, whereas interactions like C–H···O, C–H···N, C–H···S, O–H···π, N–H···π and C–H···π are weak (also known as non-canonical interactions) (Tiwari and Panigrahi 2007). The existence of non-canonical interactions was well documented very early, but their importance was not immediately appreciated (McPhail and Sim 1965). In recent years, the importance of weaker interactions in various processes among which are stabilization of secondary structure elements, collagen, DNA recognition, enzymatic action and protein–protein complexes have been highly recognized (Armstrong et al. 1993; Parkinson et al. 1996; Desiraju and Steiner 1999; Kryger et al. 1999; Panigrahi 2008; Panigrahi and Desiraju 2007).

Theoretical ab initio calculations have also been performed, showing that the energy of these non-canonical interactions is lower than the energy of strong hydrogen bonds. For instance, the energy of NH···π interaction is lower than the energy of a conventional hydrogen bond (O/N···O=C). Based on the literature data, N–H···π interactions may contribute a maximum of 3.5 kcal/mol, whereas regular hydrogen bonds may contribute up to 5.5 kcal/mol (Steiner and Koellner 2001). Although the energies of non-canonical interactions are several orders of magnitude weaker than typical covalent bonds or ionic interactions, they are, however, qualitatively very important. The stabilities of protein–ligand complexes, protein oligomers, complexes of proteins with cofactors are determined by the delicate balance between a variety of weak and strong non-covalent interactions. Weak interactions have a modest individual influence on chemical structures; however, their cumulative effect can be profound and has a large influence on the conformational stability of a biomolecule (Desiraju and Steiner 1999; Hu et al. 2002; Chakkaravarthi et al. 2006). The non-canonical interactions have been shown to be of much greater importance than previously thought (Senes et al. 2001; Babu et al. 2002; Panigrahi 2008).

Studies of the metal center in heme proteins and model systems have shown that many factors, including noncovalent interactions, may play important roles in the properties of these metalloproteins (Banci et al. 2002; Iakovleva et al. 2002; Fabian et al. 2004a, b; Walker 2004). The importance of XH/π interactions in proteins (Brandl et al. 2001; Steiner and Koellner 2001; Anand et al. 2008; Stojanović et al. 2011) and CH/π interactions of aromatic residues with porphyrin in hemoproteins (Liu et al. 1999) was shown. It was demonstrated that the noncovalent interactions with the propionic groups of porphyrins are very important for the orientations of the imidazoles and that the conformations of the propionic groups have strong influence on these interactions (Zarić et al. 2001; Galstyan et al. 2005). Searching structures of porphyrin-containing proteins from the Protein Data Bank, it was revealed that the π-system of every porphyrin ring is involved in CH/π interactions and most of the porphyrins are making several interactions (Medaković et al. 2004). We have previously showed that XH/π interactions are involved in interactions of every porphyrin ring with surrounding amino acids and that these amino acids are highly conserved, demonstrating that XH/π interactions play important roles in the stability of porphyrin interactions with the protein part (Stojanović et al. 2007). Furthermore, we have studied strong hydrogen bonds and hydrophobic interactions of porphyrins in porphyrin-containing proteins (Stojanović and Zarić 2009). In this study, we have extended the analysis of interactions between the porphyrin ring and protein part of porphyrin-containing proteins on all the non-canonical interactions in order to better understand their stabilizing role. Results from this study might be used for structure-based porphyrin protein prediction and as scaffolds for future porphyrin-containing protein design.

Methods

Dataset

For this study we have used the PDB Select May 2010 list of nonredundant protein chains (25% threshold list, 4,869 protein chains and 712,424 amino acid residues) (Griep and Hobohm 2010). Included were chains with mutual sequence similarity of <25%. The following criteria were employed to assemble the set: (1) no theoretical model structures and no NMR structures were accepted, (2) only crystal structures with the resolution of 2.0 Å or better and a crystallographic R-factor of 25.0% or lower were accepted, and (3) crystal structures containing porphyrin were accepted. Using these criteria, we created a dataset of 74 porphyrin-containing proteins. The PDB IDs are as follows: 1A6M, 1B0B, 1BVY, 1C75, 1CG5, 1CXY, 1DM1, 1E29, 1E85, 1ECA, 1EW0, 1FT5, 1GQ1, 1GU2, 1GWE, 1H97, 1IT2, 1J0P, 1JFB, 1JMX, 1KQF, 1M1Q, 1M70, 1MJ4, 1OFW, 1OJ6, 1PA2, 1RWJ, 1V9Y, 1W2L, 1X3K, 1X3X, 1X8Q, 1YCC, 256B, 2BH4, 2BK9, 2BKM, 2BLF, 2C1D, 2CE0, 2CIW, 2CY3, 2CZS, 2GHC, 2GKM, 2H88, 2HBG, 2IJ2, 2IMQ, 2NW8, 2OLP, 2P0B, 2VEB, 2W72, 2WTG, 2WY4, 2Z6F, 2ZS0, 2ZXY, 3A9F, 3B47, 3B98, 3BNJ, 3BXU, 3CP5, 3CU4, 3CX5, 3DR0, 3EGW, 3FO3, 3G46, 3M5Q, and 451C.

Non-canonical interaction analysis

If not already present, all hydrogen atoms were added using the program Discovery Studio Visualizer 3.0 (Accelrys

2011). The H-atom positions were then refined, keeping the position of the non-H atoms fixed, using the MMFF94 force field (Halgren 1996). All the optimized structures were exported to the hydrogen bond analysis tool (HBAT) for calculation of various types of non-canonical interactions and their properties (Tiwari and Panigrahi 2007) with default settings. The positions and geometry of donor and acceptor atoms are shown in Fig. 1. The used criteria for C−H···A interactions are d (H···A) $\leq$3.0 Å and θ (C−H···A) $\geq$90°. Parameters are d, distance between the H atom and the A (acceptor, A=N, O, S) atom; θ, defined as the angle between the C–H bond and the center of the acceptor atom. The criteria for XH···π interactions are P1 $\leq$ 5.0 Å, P2 $\leq$ 4.0 Å, P3 $\geq$ 90°, P4 $\leq$ 40°. The donor group is represented as X–H (X=N, O, C) and the acceptor is the π system. The distances are usually measured from the centroid (M), i.e., center of the π ring. P1 and P2 are distances from X and H, respectively, to M. P3 is the angle between vectors X–H and H–M while P4 is the angle between the XM and MN. Here, N is the normal axis to the centre of the π ring.

The program HBAT was used for statistical analysis, providing distance–angle distributions as well as furcation and porphyrin–water interactions.

Computation of stabilization centres

Stabilization centres are the clusters of residues that make cooperative, non-covalent and long-range interactions (Dosztanyi et al. 1997). Thus, they are likely to play an important role in maintaining the stability of protein structures. Residues can be considered as parts of stabilization centers if they are involved in medium- or long-range interactions and if two supporting residues can be selected from their C and N terminal flanking tetrapeptides, which together with the central residues form at least seven out of the nine possible contacts. We have used the online server, available at http://www.enzim.hu/scide (Dosztanyi et al. 2003), to analyze the stabilization centers of interaction-forming residues. This server defines the stabilization center based on the following criteria: (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 "long-range" interaction if the interacting residues are at least ten amino acids apart. (3) Two residues are forming a stabilization center if they are in long-range interaction, and if it is possible to select one–one residues from both flanking tetrapeptides of these two residues that make at least seven contacts between these two triplets (Dosztanyi et al. 2003).

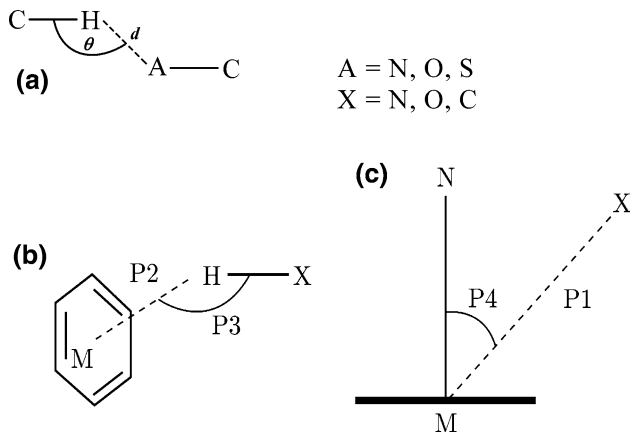

Fig. 1 Parameters for non-canonical interactions: C−H···A interactions (**a**) and XH···π interactions (**b**), (**c**)

Secondary structure and solvent accessibility studies

Secondary structure and solvent accessibility of the amino acid residues were among the key factors that were essential for understanding the environmental and structure–function relationship of proteins. Hence, a systematic analysis of each non-canonical interaction forming a residue was performed based on its location in different secondary structures of porphyrin-containing proteins and their solvent accessibility. We used the program DSSP (Kabsch and Sander 1983) to obtain the information about secondary structures and solvent accessibility. The secondary structures have been classified into alpha helix, beta turn, beta strand, and coil as suggested by the DSSP output. Solvent accessibility is the ratio between the solvent accessible surface area of a residue in a 3D structure and in an extended tripeptide conformation. Solvent accessibility was divided into three classes: buried, partially buried, and exposed, indicating, respectively; the least, moderate and high accessibility of the amino acid residues to the solvent.

Figures 2 and 4 were prepared using the program Discovery Studio Visualizer 3.0 (Accelrys 2011).

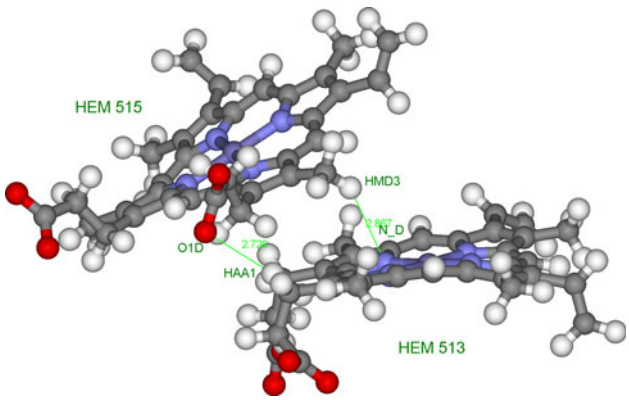

Fig. 2 Details of the interactions linking the porphyrins of the cytochrome c-552 from *Wolinella succinogenes* (PDB ID code 3BNJ). The CH···O and CH···N interactions are marked with *green lines* (HEM513 (CAA–HAA1)···HEM515 (O1D)); (HEM515 (CMD–HMD3)···HEM513 (N_D)) (color figure online)

Results and discussion

Much of the current research involving porphyrin-containing proteins concentrates on understanding the details of their biological function and how this relates to the protein structure. However, another area of active research is the design of new proteins binding porphyrin groups and related ligands. Porphyrin-containing proteins are being developed as novel biomolecules with non-linear optical properties, as molecules that self-assemble at an interface. These provide an ordered material with electron transfer properties, and as ''smart'' materials that respond to stimuli such as changes in pH, ionic strength or redox environment (Huang et al. 2004; Koder and Dutton 2006; Ueno et al. 2006; Zou et al. 2007; Lehmann and Saven 2008; Ramanavicius and Ramanaviciene 2009). The design and development of such porphyrin-containing proteins with specific structural and functional properties requires a firm understanding of the porphyrin group and its binding sites in proteins. One strategy for obtaining such an understanding is through the systematic study of porphyrin protein structures currently available. We have concentrated particularly on identifying the characteristics of the binding pockets that recognize and bind porphyrin molecules, as well as the features that enable porphyrin groups to perform non-canonical interactions. We have studied (1) Distribution of non-canonical interactions (the number and nature of porphyrin–protein interactions; amino acid composition of porphyrin environment and forming interactions), (2) Geometric characteristics of interactions (lengths, angles and furcation), (3) Porphyrin–water interactions, (4) Stabilization center residues, and (5) Secondary structure preferences and solvent accessibility of amino acids.

In the nonredundant database of the PDB with 4,869 protein chains, we found 74 proteins with porphyrin. The analyzed protein set contains 193 porphyrins due to the fact that some of the proteins have more than one porphyrin ring.Using the geometrical criteria described in the "Methods" section we have noticed that all of the porphyrins are involved in non-canonical interactions.

Distribution of non-canonical interactions

The percentage contribution of various types of non-canonical interactions and different amino acids in a particular type of non-canonical interaction in the total porphyrin-containing proteins in our dataset is shown in Table 1. The interaction abbreviation consists of three parts: interaction type, donor, and acceptor. B stands for backbone, S is side chain, L is ligand (porphyrin ring), D is donor, and A is acceptor. For example, {CHO BD SA} denotes a CH···O interaction involving a backbone CH donor and a side-chain O-atom acceptor.

The distribution of non-canonical interactions, as shown in Table 1, based on data in porphyrin-containing proteins in our dataset, totalling 3,204 interactions. The present dataset contains 193 porphyrins. Thus, on average, every porphyrin forms 17 non-canonical interactions with the protein part. However, since these interactions can occur more frequently than regular hydrogen bonds (Stojanović and Zarić 2009), they may well contribute to the protein's stability to the same extent as standard hydrogen bonds. The population of non-canonical interactions from the backbone and the side-chain donor is 4.7 and 45.3%, respectively, while for interactions from the backbone and the side-chain acceptor is 23.5 and 26.0%, respectively. In the case of donor groups, the backbone groups are the less frequently involved, because their atoms are not as accessible as the side-chain atoms and also because the backbone groups are involved in intrachain CH···O interactions to a substantial extent. The population of interactions from the ligand donor and acceptor is 50.0 and 50.5%, respectively.

The CH···O interactions are the most frequently involved (53.9%), followed by CH···N interactions (32.9 %), CH···S interactions (8.1%), and CH···π interactions (5.1%). These results were similar to those observed with protein–ligand complexes of kinases (Panigrahi and Desiraju 2007; Panigrahi 2008) where the CH···O interactions are the predominant type. It is interesting to note that, our results were somewhat different where the contribution to CH···O interactions was equal from ligand-donor and ligand-acceptor groups. This is probably due to the fact that the common porphyrin substituents (carboxymethyl, carboxyethyl, methyl and vinyl groups) are capable of making donor/acceptor CH···O interactions. The higher percentage of CH···O interactions may be explained in terms of the larger abundance of CH groups in the protein part, and therefore, many investigations of CH···O interactions focus on the CH groups as donors (Novoa and Mota 1997). Furthermore, we have observed a small percentage of interactions involving sulfur atoms (8.1%). The contribution from interactions with π-acceptors was found only in LD-SA CH···π interactions (5.1%). The lack of NH···π and OH···π interactions is probably a consequence of the large distance of these groups from aromatic amino acids.

It was previously recognized that heme-contacting residues other than the histidine ligands are important for heme binding (Robertson et al. 1994). We have previously showed that the majority of hydrogen bonds are formed from side chains of charged and polar amino acids, whereas backbone groups are not frequently involved because of lacking charge and lower accessibility of their atoms (Stojanović and Zarić 2009). The involvement of backbone atoms in hydrogen bonds necessary to form secondary structure elements also makes those atoms less available

Table 1 Distribution of analyzed non-canonical interaction types and percentage contribution of different amino acids in a particular type of non-canonical interactions between the porphyrin ring and protein part

	CH···O				CH···N				CH···S	CH···π
Type of interaction	BD-LA	LD-BA	SD-LA	LD-SA	BD-LA	LD-BA	SD-LA	LD-SA	LD-SA	LD-SA
%	4.4	20.0	22.5	6.8	0.3	3.4	22.9	6.1	8.0	5.1
Amino acid	Don (B-L)	Acc (L-B)	Don (S-L)	Acc (L-S)	Don (B-L)	Acc (L-B)	Don (S-L)	Acc (L-S)	Acc (L-S)	Acc (L-S)
Occurrence of amino acid (%)										
Nonpolar										
Gly (7.6)	18.7	9.0	–	–	88.9	13.2	0.1	–	–	–
Ala (9.5)	13.7	9.3	3.8	–	–	8.5	0.4	–	–	–
Val (6.2)	5.0	5.6	5.3	–	–	3.8	1.9	–	–	–
Leu (8.3)	2.9	5.7	7.8	–	–	4.7	1.5	–	–	–
Ile (4.1)	–	3.1	4.3	–	–	1.9	0.7	–	–	–
Met (2.5)	2.9	1.8	4.9	–	–	3.8	12.4	–	16	–
Pro (5.0)	10.8	7.1	5.0	–	–	0.9	1.2	–	–	–
Phe (4.3)	1.4	3.6	7.7	–	–	5.7	1.9	–	–	37.4
Trp (1.5)	–	0.8	1.1	0.5	–	–	0.4	5.8	–	42.4
Polar										
Ser (5.7)	10.1	8.0	4.2	16.8	–	3.8	0.1	–	–	–
Cys (1.6)	–	4.0	–	–		40.6	1.5	–	84	–
Thr (5.7)	5.8	3.4	3.1	23.4	–	2.8	–	–	–	–
Asn (4.3)	0.7	4.9	1.5	8.4	–	2.8	–	6.3	–	–
Gln (4.0)	–	4.8	3.1	17.3	–	–	–	11.5	–	–
Tyr (3.0)	2.9	3.6	9.4	22.9	11.1	–	0.3	–	–	7.4
Charged										
Lys (6.7)	10.1	6.6	20.4	–	–	3.8	1.4	6.3	–	–
Arg (4.3)	5.8	9.4	8.0	–	–	2.8	0.7	42	–	–
His (3.2)	5.0	3.4	7.3	–	–	0.9	75.4	28	–	12.8
Asp (6.2)	2.2	2.6	1.5	5.1	–	–	–	–	–	–
Glu (6.2)	2.2	3.2	1.5	5.6	–	–	–	–	–	–

Don donor, *Acc* acceptor, *L* ligand

for interactions with ligands. Table 1 represents percentage contribution of various amino acids, in particular non-canonical interaction. In CH···O (B-L) interaction type all amino acids except Ile, Trp, Cys, and Gln serve with their backbones as donors, with majority of Gly, after which follow Ser and Ala. The amino acid residue glycine, which generally induces greater flexibility in the protein chain, seems to have greater apparent involvement in the interaction as indicated by its percentage contribution. This could be attributed to the greater probability of the glycine residue to be the donor because of the presence of two α protons opposed to one in all the other amino acid residues. Moreover, we have found that in this type of interaction, Asp is defined as the hydrogen donor. It is somewhat unexpected since the side chains of Asp are usually negatively charged. Concerning CH···O (L-B) interactions, all amino acids serve as acceptors, with Gly and Ala beeing the most represented. In CH···O (L-S) interactions a few amino acids play roles of acceptors, and among them the dominant with higher percentages than others are Thr and Tyr. Donors in CH···O (S-L) are almost all amino acids and their side chains play the role in establishing interactions with similar percentages.

In the group of CH···N (B-L) interactions there are only two donors: Gly represented by 88.9% and Tyr with 11.1%. In CH···N (L-B) interactions most of the amino acids serve as acceptors and among them with high share, Cys, Gly, and Ala. In CH···N (L-S) interactions, six amino acids are with their side chains involved in building of interactions (Arg, Asn, Gln, His, Lys and Trp). Among them the most presented is Arg. This finding is in accordance with previous studies (Stojanović and Zarić 2009) where we have shown that side chains of the positively charged amino acids (Arg, His, Lys) form hydrogen bonds. There are only two amino acids which appear as acceptors in CH···S (L-S) interactions and these are Cys and Met.

We have previously reported (Stojanović et al. 2007) that every porphyrin is involved in XH···π interactions, where CH and NH groups are hydrogen-atom donors, and the number of CH···π interactions is much higher than the number of NH···π interactions. However, in analyzing proteins of the present data base, we have found only CH···π interactions, probably due to the larger abundance of CH groups. In CH···π interactions, the main acceptors are Trp, Phe, His, and Tyr represented by lower percentage.

The frequency occurrence analysis of porphyrin ring atoms included in the formation of non-canonical interactions shows that most of the acceptors are carbonyl oxygen atoms (O1A, O1D and O2D) of propionate groups of porphyrins, while oxygen atoms of acetyl groups form a much smaller number of non-canonical interactions. Most of the donors are various carbonyl atoms bound to pyrrole rings of porphyrin, with the exception of CH···N interactions where all acceptors are nitrogens from pyrrole groups where the least represented is the nitrogen atom from the pyrrole B ring. Thus, our analysis indicates that the contribution of amino acids toward a particular non-canonical interaction is specific in porphyrin-containing proteins.

It is very interesting that in the proteins that contain more than one porphyrin, the CH groups at side chains of porphyrin can be involved in the CH···O and CH···N interactions with another porphyrin in the protein. We have found 17 (0.5%) of those interactions. It is likely that these interactions contribute significantly to the overall stability of porphyrin rings. Two iron-porphyrins from the binding pocket of the cytochrome c-552 from *Wolinella succinogenes* (PDB ID code 3BNJ) are shown in Fig. 2. There is one CH···O interaction between porphyrins (HEM513 (CAA–HAA1)···HEM515 (O1D)) with distance of 2.73 Å and one CH···N interaction (HEM515 (CMD–HMD 3)···HEM513 (N_D)) with a distance of 2.87 Å.

Comparing the percentage of amino acid occurrences of the analyzed dataset and percentage contribution of different amino acids in a particular type of non-canonical interaction between the porphyrin ring and protein part (Table 1) suggests that the high percentage of interaction for a certain amino acid is indeed due to the frequent occurrence of this interaction in the first place, nature of amino acid and its capability of forming an interaction. For example, Trp is represented by a low percentage of occurrence (1.46%), whereas, in a certain type of interaction CH···π (LD-SA), is represented by a high percentage as acceptor amino acid (42.4%). Also, some charged amino acids (Lys, Asp, Glu) are represented by similar percentages of occurrences, while Lys is involved with higher percentages in the same type of interaction, which reflects that other factors than simply a high content of amino acid are required for higher involvement in interaction: such as the type of side chain, location, and whether the amino acid is located near the porphyrin ring or not.

Non-canonical interaction geometry: lengths, angles, and furcation

The interaction geometry of the most abundant type of non-canonical interactions (CH···O) in the total porphyrin-containing proteins in our dataset is shown in Fig. 3.

The CH···O interactions include {CHO LD BA}, {CHO LD SA}, {CHO BD LA}, and {CHO SD LA}. For {CHO BD LA} the angle distribution has two distinct maxima at 115° and 140° with a narrow range of linearity. The metrics of the other CH···O interactions are surprisingly consistent. Also, it is similar for {CHO LD BA; CHO LD SA and CHO SD LA}, the maxima being still around 110–120° and 140–160° with variable geometry. Deviations from linearity of the non-canonical interactions may be due to the lesser freedom of the donor groups to orientate appropriately toward the acceptor. Also, the rotation of the interactions can offset the entropy loss that is associated with protein–ligand binding. In both cases, the lower angle maxima distribution corresponds generally to multifurcated geometries (Panigrahi and Desiraju 2007).

The median CH···O distances, d, in all the above cases are <3.0 Å. For {CHO BD LA}, d is 2.7 Å. For other CH···O interaction types, the median distances are 2.8–2.9 Å.

The inverse length–angle correlations are also well behaved in all of these cases. To summarize, the backbone CH···O interactions {CHO BD LA}, might be slightly more linear than the side-chain interactions, and they have somewhat shorter median distances.

The geometries for other interactions observed in the porphyrin-containing proteins (data not shown) are consistent and fall within acceptable limits. For backbone interactions, the median distances, d, are less than 2.7 Å. The angular distributions for backbone interactions are similar with maxima in the range of 110–120° and 140–160°. Backbone interactions show better linearity and shorter distances compared with side-chain interactions. The side-chain interactions have variable geometry.

These observations are reassuring and show that the fundamental property of non-canonical interactions, namely linearity, holds by and large for all categories in macromolecular structures (Panigrahi 2008; Panigrahi and Desiraju 2007).

Hydrogen bond furcation is a ubiquitous phenomenon in macromolecular structures. A donor can interact with several acceptors simultaneously or an acceptor can interact simultaneously with many donors. This type of interaction is marked as furcation. The role of interactions in recognition of the ligand by the protein has been studied

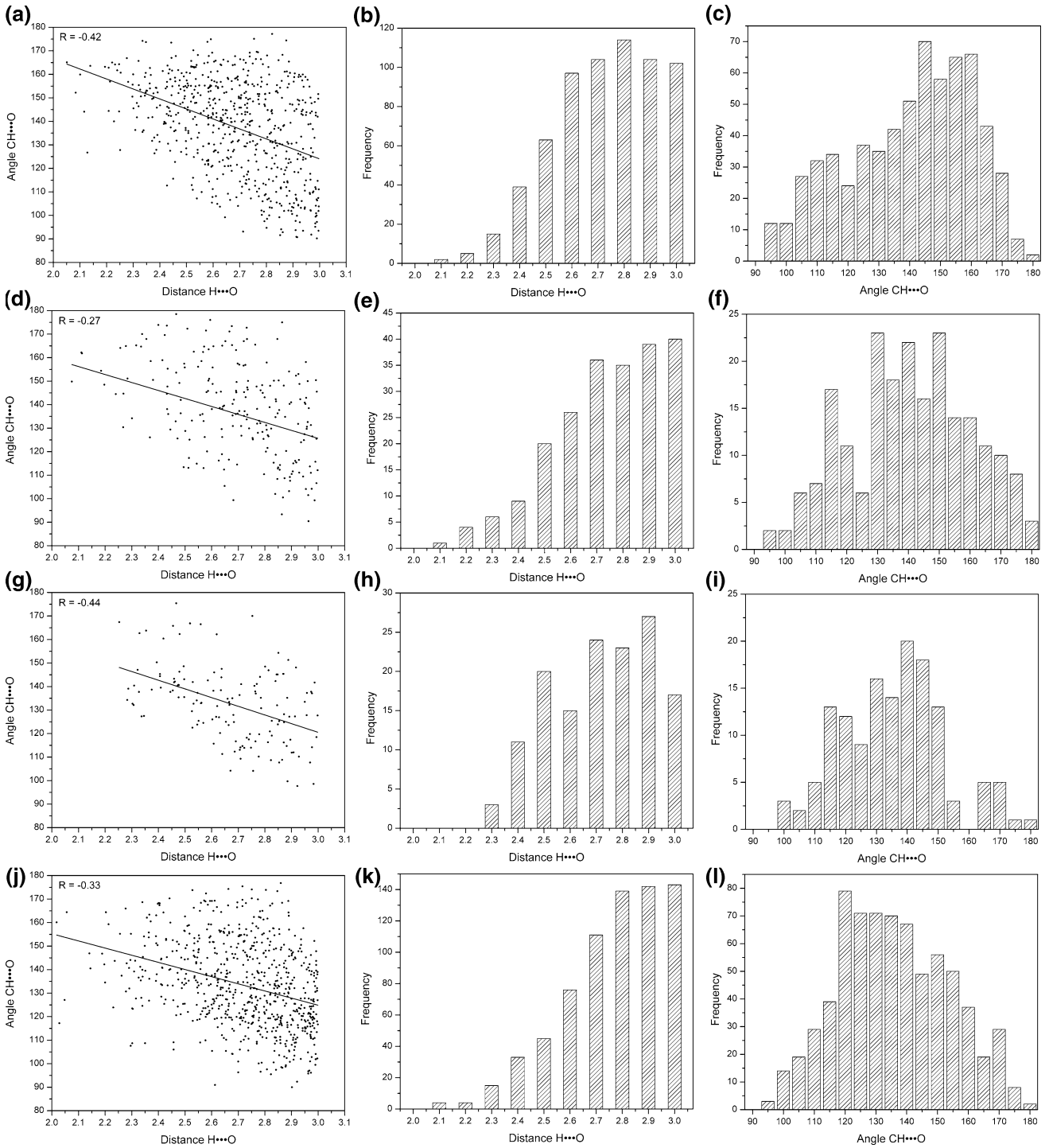

Fig. 3 Non-canonical interaction geometry for {CHO LD BA} (**a–c**), {CHO LD SA} (**d–f**), {CHO BD LA} (**g–i**), and {CHO SD LA} (**j–l**). In each case the inverse length-angle scatterplot is followed by histograms of distances and angular distributions

and the donor and acceptor furcation has been analyzed in detail (Sarkhel and Desiraju 2004; Panigrahi and Desiraju 2007). In our analysis, we have investigated the cases of donor (approach of many acceptors towards a donor) and acceptor (approach of many donors towards an acceptor) multifurcated non-canonical interactions of porphyrin rings. The details of donor and acceptor furcation are presented in Table 2.

Table 2 shows that the total number of furcated non-canonical interactions is 1,416. This corresponds to an average level of furcation of 7.3 interactions to each porphyrin ring (193) in the active site. The level of furcation

Table 2 Non-canonical interactions for porphyrin rings at various levels of donor and acceptor furcation

Furcation level	Furcated acceptor			Furcated donor		
	CH···O	CH···N	CH···S	CH···O	CH···N	CH···S
Bifurcated	338	251	–	282	82	86
Trifurcated	104	14	–	34	22	10
Tetrafurcated	48	27	–	36	27	5
Pentafurcated	12	–	–	7	2	2
Hexafurcated	14	–	–	13	–	–
Total	516	292	–	372	133	103
Non-furcated	346	451	–	487	171	153

ranges from bifurcated to hexafurcated. The most frequently observed furcated interactions in this study involve bifurcated and trifurcated acceptors. With an increase in furcation level, the numbers of interactions decrease. The frequency of furcated acceptors is more than that of furcated donors. This is in accordance with the fact that there are more acceptor atoms in ligands than donor atoms, and this might be due to steric reasons. We emphasize that CH···O interactions are more common than the others in the furcated geometries (Table 2). There is a lack of CH···S furcated acceptor interactions, because porphyrin rings have no thiol groups. The furcation level of non-canonical interactions is much higher than that of strong hydrogen bonds in porhyrin-containing proteins (Stojanović et al. 2007).

The majority of furcated interactions exhibit longer (H···A) distances (2.8–3.0 Å) than the simple non-furcated interactions (2.6–2.7 Å) as expected (Panigrahi and Desiraju 2007).

The analysis shows that about 44% of the total interactions in the dataset are involved in the formation of multiple non-canonical interactions. This conveys that furcation is an inherent characteristic of macromolecular crystal structures.

The binding pocket for the C subunit of ubiquinol-cytochrome c oxidoreductase from bovine, as an illustrative example for CH···O trifurcation, is shown in Fig. 4. An example of an acceptor trifurcated interaction is the atom O2D from propionate group of the porphyrin ring, interacting with Phe109, Ser205 and Asn206.

Porphyrin–water interactions

The hydrogen bonding capacity of water makes it easy to interact with protein, ligand or neighboring water molecules. Oxygen atoms of porphyrin might form hydrogen bonds with molecules of water, and water can be the bridge towards the amino acid residues. It is known that water networks in protein interfaces can complement direct interactions contributing significantly to molecular recognition, function, and stability of protein association (Ben-Naim et al. 1990; Olkhova et al. 2007; Teyra and Pisabarro 2007).

The total number of non-canonical interactions formed by water (as donor or acceptor) of the 193 porphyrins and surrounding amino acids under consideration is 1,732. Thus, on average, this is nearly nine such interactions for each porphyrin ring. We found out that all of the porphyrins are involved in the porphyrin–water interactions. Some of the porphyrins have only one porphyrin–water interaction (the structures with PBD ID code 1C75, 1CG5, 1E29, 1V9Y, 1W2l, 1YCC, 2CE0, 3M5Q), while most of the porphyrins have several interactions. Some of the porphyrins have up to 20 interactions (e.g., PBD ID code 1JFB, porphyrin HEM 501 have 28 interactions). Among the ligand (donor)–water (acceptor) interactions, {CHO LD WA} interactions constitute as many as 57%, while {CHN LD WA} account for 39%. A small number (4%) of these interactions are of the type {CHS LD WA}. In ligand–water interactions, we find that the {CHO LA WD} interactions constitute as much as 68% of the total number of ligand (acceptor)–water (donor) interactions, while the

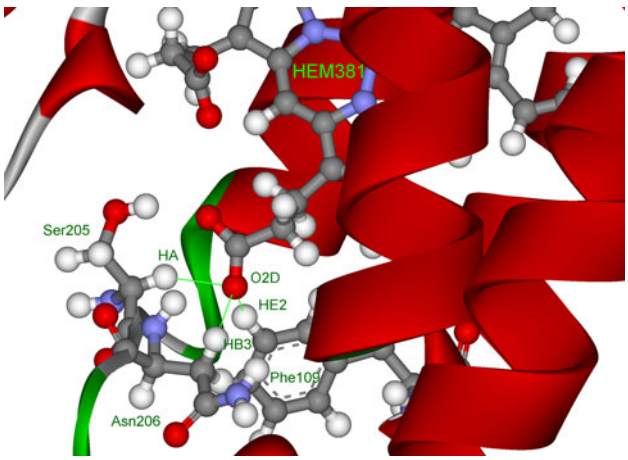

Fig. 4 Details of the CH···O trifurcated interaction for the HEM381 of ubiquinol-cytochrome c oxidoreductase from bovine (PDB ID code 1BE3). The trifurcated interactions are marked with *green lines* (Phe109 (CE2-HE2)···HEM381 (O2D)); (Ser205 (CA-HA)···HEM381 (O2D)); (Asn206 (CB-HB3)···HEM381 (O2D)) (color figure online)

{CHN LA WD} account for only 32%. The high number of CH···O interactions could follow from the fact that porphyrins bind to the protein mainly via these interactions. The large number of porphyrin–water interactions show importance of the inclusion of solvent in protein–ligand interaction studies.

Stabilization center residues (SC): amino acid composition

Stabilization centers are composed of certain clusters of residues, involved in cooperative long range interaction of proteins that regulate flexibility, rigidity, and stability of protein structures. Stabilization centers are important in regulating the turnover of certain proteins by preventing their decay with their cooperative long-range interactions. The most frequent stabilization center residues are usually found at buried positions and have a hydrophobic or aromatic side chain, but some polar or charged residues also play an important role in the stabilization. The stabilization centers show significant difference in the composition and in the type of linked secondary structural elements, when compared with the rest of the residues. The performed structural and sequential conservation analysis showed the higher conservation of stabilization centers over protein families (Dosztanyi et al. 1997; Magyar et al. 2005).

If we consider amino acid composition of stabilizing centers of porphyrin-containing proteins we are able to notice differences in the composition of nonpolar (52%), polar (25.6%), and charged (22.4%) amino acid residues (Fig. 5). Amino acids Ser, Thr, Asn, Gln, and Tyr are more represented than Cys which contributes with 2–3 fold less

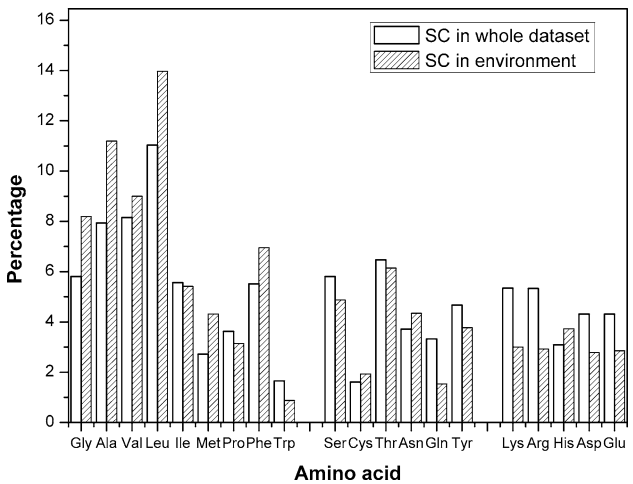

Fig. 5 Amino acid composition of stabilizing centers (*white caps* on the chart) and amino acid composition of stabilizing centers which are part of porphyrin environment (*shaded caps* on the chart) of porphyrin-containing proteins

than other polar amino acids. A similar distribution of amino acids which are part of SC has been previously reported (Dosztanyi et al. 1997). Namely, it is well known (Dosztanyi et al. 1997) that nonpolar amino acids are predominant in SC, especially Ile and Val. In the analyzed sets of proteins, the most represented nonpolar amino acid is Leu with 11%. This finding can be due to the fact that all analyzed proteins are composed of helices and strands and Leu is a strong helix former, whereas Ile and Val are indifferent or weaker formers of helices than Leu. This is in agreement with a certain extent with the previous study of Dosztanyi et al. (1997).

Amino acids which are characterized as SC residues and which are at the same time part of the porphyrin environment have the following distribution of different types of amino acids: 63.1% of nonpolar, 21.6% of polar, and 15.3% of charged amino acid residues (Fig. 5). Markedly, more represented compared with other nonpolar amino acids is Leu (13%) followed by Ala (11%) and Val (9%). A similar distribution is observed in the composition of stabilizing centers. In the group of polar amino acids, Thr and Ser are slightly more abundant (∼4–6%). Among charged amino acids, His (3.7%) is slightly more abundant than other amino acids of this type, which are represented by similar percentages (∼2.8–3%). Interestingly, His was the least abundant among charged amino acids which are stabilizing centers of the whole data set (Fig. 5 white caps). This could be explained by the fact that His is involved in various strong and weak hydrogen bonds with porphyrin ring and is located in the environment.

In addition, we have computed the stabilization centers for all non-canonical interaction forming residues in porphyrin-containing proteins. Table 3 shows the percentage contribution of the individual amino acid residue which is part of the stabilizing center involved in non-canonical interactions with porphyrin ring.

However, considering the whole data set of 6,401 stabilizing residues, 171 (2.7%) are involved in building non-canonical interactions with the porphyrin ring. These data suggest that the amino acids that are part of stabilization centers are not involved in the building of interactions with the porphyrin ring, but they are involved in the stabilization of surrounding structure and overall structure of protein.

Most of amino acids which are SC residues are present with around 2%, except His (19.19%), Arg (5.57%), Phe (4.25%), and Cys (3.88%). It may be observed that the highest contribution comes from charged and polar amino acids. Interestingly, these residues are not abundant amino acids at porphyrin-containing proteins (Fig. 5), suggesting that these amino acids when part of SC are at porphyrin environment involved in the building of non-canonical interactions. These observations strongly reveal that these residues can contribute significantly to the structural

Table 3 Involvement of stabilizing center residues in non-canonical interactions of porphyrin-containing proteins

Amino acid	Total number of SC residues	Total number of SC residues involved in NCI	% of SC residues involved in NCI
Nonpolar			
Gly	372	2	0.54
Ala	508	12	2.36
Val	522	6	1.15
Leu	706	8	1.13
Ile	356	3	0.84
Met	174	6	3.44
Pro	232	5	2.15
Phe	353	15	4.25
Trp	106	2	1.89
Polar			
Ser	372	9	2.41
Cys	103	4	3.88
Thr	414	6	1.45
Asn	238	7	2.94
Gln	213	5	2.35
Tyr	299	11	3.68
Charged			
Lys	342	10	2.92
Arg	341	19	5.57
His	198	38	19.19
Asp	276	1	0.36
Glu	276	2	0.72

stability of these proteins in addition to participating in non-canonical interactions.

Occurrences of amino acids in stabilization centers are similar to occurrences of amino acids in proteins which exist in nature (Magrane et al. 2011). Despite the fact we cannot draw a simple conclusion that the high occurrence of certain amino acids in stabilization centers is simply due to their higher occurrence in proteins, because the stabilizing centers of proteins are defined by terms other than just the amino acid composition of proteins. Stabilization centers in proteins are important for maintaining the folded protein structure, because breaking of the interaction in stabilization centers is the rate-limiting step in the unfolding process. We can say to a certain extent that a higher occurrence of certain amino acids in stabilization centers coincide with their higher occurrence in nature. In composition and formation of stabilization centers, an important role is played by folds of proteins and interactions, as well as amino acids which are involved in the stabilization of tertiary structure of protein.

Secondary structure preferences and solvent accessibility of amino acids

The occurrence of these weak interactions has been observed at the terminus of the secondary structural units, in particular α-helix and β-sheet (Fabiola et al. 1997; Babu et al. 2002). These interactions have been proposed to have a definitive role in stabilizing these secondary structural scaffolds of proteins. The propensity of the amino acid residues to favor a particular conformation is well described. Such a conformational preference is not only dependent on the amino acid alone, but also on the local amino acid sequence (Chakkaravarthi et al. 2006).

In order to obtain the preference and pattern of each non-canonical interaction-forming residue in porphyrin-containing proteins, we conducted a systematic analysis based on their location in different secondary structures and their solvent accessibility. Thus, we have analyzed amino acid secondary structure preferences for the whole data set of 74 proteins. In all analyzed proteins, only two types of secondary structures were present: helices and coils. Nonpolar amino acids were represented by 49.9%, whereas polar and charged with 24.4% and 25.7%, respectively. It is found that most of the non-canonical interactions between the amino acid residues and porphyrin ring prefer the secondary structure of alpha helical segments. We found that Leu, Pro, Thr, and Lys preferred to be coiled, while other amino acid residues preferred helix conformation. These data are consistent with the information that 65% of amino acids are in helices, and 35% amino acids from the whole composition are in coils. It was interesting to observe that a significant percentage of Ser and Arg residues favored coil and helix conformation. From this observation, we infer that Ser and Arg residues might stabilize coils and helices by bonding in porphyrin-containing proteins. The acceptor π residues (Phe, Tyr, Trp, His) preferred to be in helix. This analysis indicates that the non-canonical interactions do not occur at random but have residue-specific preference for a particular secondary structure.

We found that most of the polar amino acid residues involved in non-canonical interactions were solvent exposed and most of the non-polar residues involved in non-canonical interactions were excluded from the solvent. Hence, the polar residues might contribute significantly to the stability of porphyrin-containing proteins. Aromatic residues are in principle nonpolar residues and tend to be buried. Therefore, this analysis indicates that the majority of the amino acid residues prefer to involve in non-canonical interactions only when they are excluded from the solvent, especially when the interaction involves main-chain atoms.

Conclusions

The presented study expands on our previous work on the hydrogen bonds, XH/π and hydrophobic interactions of porphyrins in porphyrin-containing proteins by analyzing the same protein group with respect to non-canonical interactions in order to better understand their stabilizing role.

Our analysis presented in this article shows that predominant types of non-canonical interactions at porphyrins are CH···O, and CH···N interactions, represented by 53.9 and 32.9%, respectively. Other interactions involving π acceptors are also important in the protein–porphyrin interface. We have found a small percentage of CH···π interactions and non-canonical interactions involving sulfur atoms. On average, there are 17 non-canonical interactions per porphyrin ring. The majority of non-canonical interactions are formed from side chains of charged and polar amino acids, whereas backbone groups are not frequently involved. Residues like Gly, Phe, Cys, Arg, and His frequently accept non-canonical interactions from the porphyrin. The Gly residue is smaller in size and has greater flexibility and participates well in both acceptor and donor interactions. In this respect, Gly frequently interacts with the ligand. The porphyrin donor capacity of Ser and Lys is noteworthy. The main-chain non-canonical interactions might be slightly more linear than the side-chain interactions and they have somewhat shorter median distances. The analysis shows that about 44% of the total interactions in the dataset are involved in the formation of multiple (furcated) non-canonical interactions. Furcated interactions are manifested by both donors and acceptors. Acceptor furcation is more common than donor furcation. The majority of furcated interactions exhibit longer d (H··· Acceeptor) distances when compared with simple non-furcated interactions. The high number of porphyrin–water interactions indicates the importance of including the solvent in protein–ligand interaction studies. Furthermore, in the present study we have observed that stabilization centers are composed predominantly of nonpolar amino acid residues. Comparison of stabilizing amino acid residues with amino acids which build non-canonical interactions with the porphyrin ring shows that certain amino acids such as His, Arg, Phe, and Cys, when part of the stabilizing center are by percentage highly involved in the building of non-canonical interactions. Amino acids which are deployed in the environment of porphyrin rings are deposited in helices and coils. It is found that most of the non-canonical interactions between the amino acid residues and porphyrin ring prefer the secondary structure of alpha helical segments. We found that most of the polar amino acid residues involved in non-canonical interactions were solvent exposed and most of the non-polar residues involved in non-canonical interactions were excluded from the solvent.

In conclusion, observations obtained in this study identify non-canonical interactions and structural motifs that contribute to the stabilization of porphyrin ring by proteins, relevant to the understanding of structure–function relationships in porphyrin-containing proteins, and useful to the efforts made to design proteins able to incorporate this versatile and ubiquitous prosthetic group.

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