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Research Article

A Bioinformatics Approach to the Identification of Conserved and Semi-Conserved Water Molecules in Kinase Domain of hGRK2 Protein

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Abstract

Human G protein-coupled receptor kinase 2 (GRK2) is emerging as a critical hub in cell signalling cascades and also acts as a pharmaceutical target for treating cardiovascular diseases. Based on sequence similarity, human GRKs are classified into three subfamilies: GRK1, GRK2, and GRK4. All GRKs have a central catalytic Ser-Thr Kinase domain having a sequence length of ~270 residues. No detailed computational investigation has yet been reported for evaluating the following information about the kinase domain: (i) identifications of conserved and semi-conserved water molecules (ii) characterization, and effect on salt-bridges in the stabilization of kinase domain and its recognition with other domains. Our previous computational investigations on multiple analyses of crystal structures (bound and unbound conformation of $G_{\beta}G_{\gamma}$) reveal the presence of seventeen unique water molecules (IW1 to IW17) that are only available either in hGRK2 with $G_{\beta}G_{\gamma}$ or without $G_{\beta}G_{\gamma}$ conformation. Moreover, the present computational investigation also indicates four additional water molecules (W18 to W21) located at the kinase domain of the hGRK2 protein with a putative functional role. MD simulation results suggest that above mentioned four crystal water sites are accessible by crystal water molecules in MD simulation and allow entering of these crystal water molecules at their concerning sites. Apparently, the kinase domain is also been stabilized by three salt-bridges via Lys210---Asp212, Lys220---Glu239, and Lys220---Asp335, and it also gets interconnected with RH and AGC domain via the interaction Lys215---Glu523, and Lys228---Asn502 respectively.

ABBREVIATIONS

hGRK2: Human G protein-coupled receptor kinase2; KD: Kinase Domain; SASA: Solvent Accessible Surface Area; RMSF: Root mean square fluctuations; MD: Molecular Dynamics

INTRODUCTION

The human GRKs are G protein-coupled receptor kinase 2 (GRK2), which is emerging as a key hub in cell signalling cascades, and they also act as a pharmaceutical target for the treatment of cardiovascular diseases. Notably, GRK2 expression and activity are controlled by several mechanisms, and changes in those mechanisms in humans result in hypertension [1], heart failure [2], metabolic syndrome, inflammation, and certain tumors [3], suggesting that changes in GRK2 function may be involved in the triggering or development of relevant pathological situations and that this protein could be a useful biomarker or therapeutic target. Based on sequence similarity, human GRKs are classified into three subfamilies: GRK1,(comprising GRK1 (Rhodopsin Kinase) and GRK7 (Cone Opsin Kinase)); GRK2, (comprising GRK2 and GRK3); and GRK4 (comprising GRK4, GRK5, and GRK6) [4]. GRK2 is responsible for the desensitization in HEK293 cells that are mostly observed in the cytoplasmic region of the cell [5]. All GRKs are ~530-700 amino acid residues long containing multi-domains. The proteins consist of an N-terminal region, followed by the N-terminal RH domain, a Ser-Thr protein kinase domain (KD), an AGC C-Tail domain and the C-terminal RH domain [4]. In GRK2, the C-terminal region contains one additional structural domain, the Pleckstrin Homology (PH) domain that binds $G_{\beta}\gamma$ subunits and is responsible for membrane targeting [6]. The GRKs have an unusual structure, suggesting that they evolved only once. A phylogenetic analysis revealed that most kinase groups and families are of ancient origin among the eukaryotes, whereas subfamilies generally emerged later. The majority of the kinase active site specificities present today in eukaryotic species have occurred early on during the evolution of eukaryotes.

Proteins having multi-domains often have a beaded necklace-like structure since the domains align one after the other. In contrast, in the case of GRKs, the Kinase domain is inserted into a loop within the RH domain. Analysis of the KD sequence reveals that the Kinase domain of GRKs is closely related to the kinases of the AGC kinase family which are ubiquitous in unicellular eukaryotes. This provides an insight that the KD region of GRKs has been initiated by duplication of an AGC kinase.

The Ser-Thr protein kinase domain (KD) is a central catalytic

Kinase Domain (res id: 185-455 aa) (Figure 1), which has been observed as mostly conserved among all the GRKs whereas the RH and other domains show the highest degree of divergence [7]. This catalytic region is responsible for the phosphorylation of the protein substrates by transferring the phosphate group from nucleoside triphosphates (ATP) to the side chain of an amino acid residue [8,9], resulting in various cellular processes such as transcription, cell cycle progression, cytoskeletal rearrangement, and cell movement, apoptosis, and differentiation. The kinase domain is comprised of a small lobe (residues 181–268) and a large lobe (residues 269–454), with the active site situated in a cleft between them. Basic residues located on surface of the large lobe are required for binding peptide substrates. Interestingly, a well-ordered ligand in the active site has not yet been reported because all of the GRK2 crystal structures of the kinase domain adopt an "open" conformation, in which the nucleotide-binding site is not fully predicted. The X-ray structures of balance and structural analogs of balance reveal that the compounds bind to a conformation of PKA intermediate to the both "open" (inactive) and "closed" (active) states of the kinase domain [10]. The recent study suggests that paroxetine binds in the active site of GRK2 and stabilizes the kinase domain in a novel conformation in which a unique regulatory loop forms part of the ligand binding site.

The GRKs are mainly responsible for homologous desensitization of GPCRs by phosphorylating the cytoplasmic loop which allows the cells to adapt extracellular signals. Based on the type of GRKs (GRK1, GRK2, GRK4) different sets of serine-threonine residues are phosphorylated which is further read by a β-Arrestin GRKs phosphorylates different sets of Serine-Threonine residues which leads to different fates like, if GRK2-mediated phosphorylation occurs, β-Arrestin could read the pattern and as a result, it brings the Adaptor protein (AP2) and the Cavoline-mediated endocytosis occurs. GRK activation by binding to an active GPCR highlight that GRKs phosphorylate nearby proteins with accessible loops, i.e., with the highest probability the intracellular loops and tails of the receptor molecules they bind to.

As we know, water molecules play a vital role in protein structure, enzyme catalysis, protein architecture, conformational stability, protein plasticity, stabilization of salt bridges, ligand binding, and selectivity for specific interactions [11,12]. Crystallographic data suggest the conservation of at least one water molecule in the active site of the pocket which is known to be essential for the enzymatic reactions [13]. However, these water sites (W) of human GRK2 (hGRK2) protein are defined as confined space regions close to the residues of the protein surface, exhibiting a high probability of hosting water molecules. The positions of water sites, (W), are chosen by the coordinates of the maximum probability point using a surface residue of the protein as a reference that can make H-bonds favourably with the water molecules [14]. It is well known that, due to the impacts of various environmental factors, it is difficult to identify the conserved water molecules from free water molecules directly as conserved water molecules are normally deeply embedded in proteins and form strong hydrogen bonds with surrounding polar groups [15]. Mainly due to the limitations in biophysical techniques, the geometrical or position information of these water molecules is often inaccurate or inaccessible. Therefore,

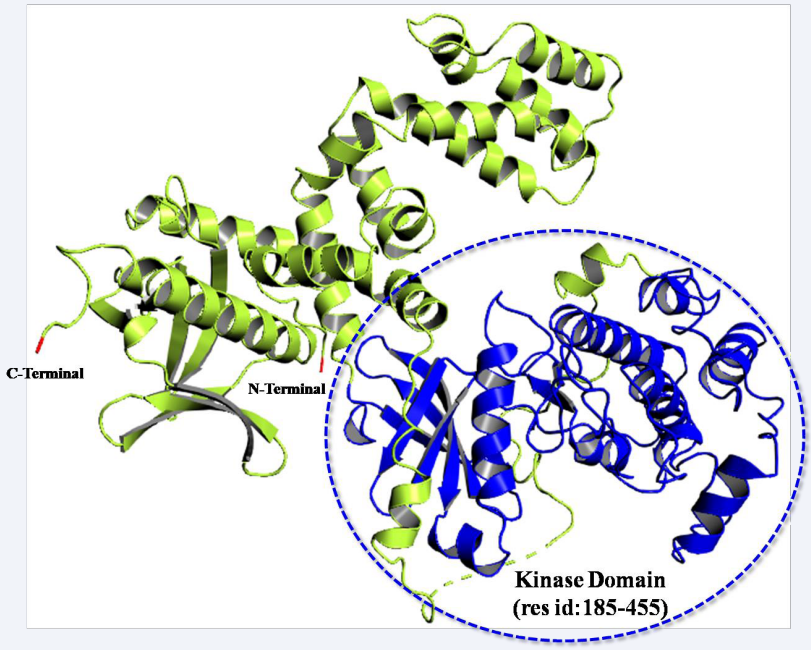

Figure 1 The representation of Central Catalytic Kinase Domain (res id:185-455) marked as blue in the crystal structure of human GRK2 (hGRK2) protein (PDB Id. 3V5W).

it is difficult to identify the conserved water molecules in the binding sites directly. Thus, we expect that our study on conserved or semi-conserved water molecules may extract relevant information about the presence of water molecules. Different researchers have considered hGRK2 as a significant drug target; hence they adopt various computational strategies to investigate some promising selective inhibitors for hGRK2 to treat heart failure [16]. Moreover, no detailed computational investigation has yet been reported for evaluating the following information about kinase domain:

1. Identifications of conserved and semi-conserved water molecules in the structures of the kinase domain of hGRK2.

2. Comparative analysis of conserved water molecules in the kinase domain between crystal and MD structures.

3. Identification and characterization of salt bridges in the kinase domain of hGRK2.

To the best of our knowledge, this is the first computational investigation that is incorporating the results of water dynamics analysis and focused on identifications of four (W18 to W21) conserved water molecules.

MATERIALS AND METHODS

Structure Collection

To identify the conserved water molecules at the kinase domain of human GRK2 (hGRK2) protein, nine hGRK2 crystal structures (PDB Id. 5UKL, 5UKK, 5HE1, 4PNK, 4MK0, 3V5W, 3KRX, 3KRW, and 3CIK) were obtained from the Protein Data Bank (PDB) [17]. Due to the availability of a reasonable number of water molecules in the protein structure, the PDB Id: 3V5W, 5UKL, 3KRW, and 3CIK were included in the present study whereas 5UKK, 5HE1, 4PNK, 4MK0, and 3KRX structures were excluded. The crystal structures with low solvent content diffract better than those with high resolution. Hence, a crystal structure 3V5W has low resolutions and it is considered a template for present structural and dynamic analysis [18].

Identification of Conserved Water Molecules in hGRK2

Two conserved water molecules (W18 to W19) and two semi-conserved water molecules (W20 and W21) were identified at the kinase domain of hGRK2 using the 3-Dimensional Structural Superposition (3dSS) program [19]. However, the above-mentioned four water molecular positions were also verified by the Swiss PDB Viewer program [20]. Two water molecules (oxygen atoms) or two atoms (oxygen atoms between two water molecules or oxygen atom of the water molecule and specific atom of ligand) whose centre-to-centre distance was within 1.80Å, [21,22], in between reference (PDB Id: 35W), and movable structures (5UKL, 3KRW, and 3CIK); that were assigned as conserved [23-25]. Each conserved water molecule also makes at least one H-bond with protein molecules. In addition, the water molecules W18 and W19 are available in 3V5W, 5UKL, 3KRW, and 3CIK and they are considered conserved whereas W20 and W21 are either present two or three structures are also considered semi-conserved.

Calculation of SASA of Water Molecules

The solvent accessible surface area (SASA) of four conserved water molecules were calculated using an in-house TCL script with a probe radius of 1.40 Å. Water molecules that had the SASA value of less than or equal to 2.50 $Å^2$ were assigned to be buried. The side-chain accessibility surface area (ASA) of reference residues of each of the twenty-one conserved water molecules was calculated using the Get Area web server [26,27].

Calculation of Conformational Stability Free Energy and RMSF of Each Domain

The conformational stability-free energy ofthe 3V5W crystal structure was calculated by the FoldX program [28]. The temperature, ionic strength, pH, and VDW were assigned as 300 K, 0.05 (M), 7.0 and 2 Å. The conformational stability free energy of each domain was calculated with the presence or absence of crystal water molecules. Moreover, the experimental B-factors of the 3V5W crystal structure of each domain were converted to RMSF value by using the Debye-Waller formula: $(RMSF=3B/8\pi2)^{½}$ [29].

Calculation of Pka Values of the Ionizable Residues

The pKa values and protonation states of all titratable residues in 3V5W, 5UKL, 3KRW, and 3CIK crystal structures were determined using the Protein Prepare application module of the PlayMolecule program [30]. The Protein Prepare program calculates the pK_a values of the side chains of those titratable residues using an empirical method PROPKA (version 3.1) at pH 6.00. To investigate the validation of the method described above, we also measured the pK_a values of those corresponding titratable residues in 3V5W, 5UKL, 3KRW, and 3CIK crystal structures compared the pK_a values with 5UKL and 5UVCstructures, respectively (**Table S1**).

Molecular Dynamics Simulations and Trajectory Analysis

The 3V5W, 5UKL, 3KRW, and 3CIK structures at pH 6.0 were prepared for further water dynamic studies in the presence of crystal water molecules to identify the conserved water molecules at hGRK protein to evaluate their structural properties. Using the AutoPSF module of the Visual Molecular Dynamics (VMD v.1.9.3.) program [31], missing hydrogen atoms were added to each structure. Each structure was then solvated with the presence of crystal water molecules in a cubic box of around 16,646 TIP3P water molecules extending at least 5 Å from the protein surface. Sodium and chloride ions were employed to neutralize the overall charge of the system; the resulting system consisted of around 150000 atoms. MD simulation was performed for the 5UKL and 5UVC structure using the *Nano-scale Molecular Dynamics* (NAMD v.2.11) program by assigning the CHARMM-36 all-atom force field (with map correction) for protein [32]. The energy minimizations were adopted to stabilize the system using the steepest-descent method by 5000 steps. Then each system was equilibrated by fixing protein atoms for *10ns* under *NPT* [33,34]. To mimic physiological conditions, the temperature was kept at

310 K using Langevin dynamics with a damping coefficient of 5 ps-1. The pressure was maintained at 1 atm using the Langevin piston Nose–Hoover method, with a piston period of 100 fs and a decay time of 50 fs.

During the water dynamics, Nos´e-Hoover Langevin piston barostat and Langevin thermostats [35], were used to enforce constant pressure and temperature. The SHAKE algorithm [36], was used to keep bonds involving H atoms at their equilibrium length, allowing a 2 fs time step. The van der Waals interactions were truncated at 12.0 Å with switching from 10.0 Å. Electrostatic interactions were modelled accordingly with a dielectric Constant of 1.0 throughout the equilibration and production runs. The standard Particle–Mesh Ewald method was used with periodic boundary conditions to compute the long-range electrostatic interaction of the system by specifying an appropriate Particle–Mesh Ewald grid size. Finally, each structure's production runs of water dynamics were performed in the NPT ensemble for 10 ns. The atomic coordinates were recorded at every 2 ps for further data analysis (5,000 frames). Each MD trajectory, especially 5000 recorded snapshots, was considered for further data analysis using the VMD program.

RESULTS AND DISCUSSION

The nine crystal structures of the human GRK2 (hGRK2) protein were obtained from the Protein Data Bank but only four PDB structures (3V5W, 5UKL, 3KRW, and 3CIK) were considered for the present computational study. Several research groups have independently solved these structures using REFMAC refinement programs at different pH (4.6 to 7.0) levels with different resolutions (2.07 to 3.15) (Table S2).The average B-factor and residual flexibility (RMSF) of the kinase domain are 65.56 $Å^2$ and 1.58 Å respectively, and its conformational stability free energy is 5.00 kcal/mol (Table S3). The computational data suggest that the kinase domain (KD) is comparatively more stable than the AGC domain.

Previous computational investigations on multiple analyses of crystal structures (bound and unbound conformation of $G_\beta G_\gamma$) suggest the presence of seventeen unique water molecules (IW1 to IW17) those play a decisive role in maintaining the structural architecture of human GRK2 protein. They are only available either in hGRK2 with $G_\beta G_\gamma$ or without $G_\beta G_\gamma$ [11]. The previous result also reveals the importance of biochemical information about the association and dissociation mechanism of GRK2 protein with GβGγ and provides a testable hypothesis for experimental validation. In the present computational study, the PDB Id: 3V5W, 5UKL, 3KRW, and 3CIK revealed the existence of four water molecules (W18, W19, W20, and W21) at the different domains of the hGRK2 protein, which may have pivotal roles in the structure, function, and stabilization of the kinase domain in hGRK2 protein.

The corresponding water molecule for W18 is W890 in 3V5W, W937 in 5UKL, W693 in 3KRW, and W696 in 3CIK, W19 for W905 in 3V5W, W948 in 5UKL, W692 in 3KRW, and W695 in 3CIK. The semi-conserved water molecules W21 is W836 in 3V5W, W905 in 5UKL, no water molecules in 3KRW, and W701 in 3CIK are observed in the kinase domain whereas W20 for W816 in 3V5W, W841 in 5UKL, no water molecules in 3KRW, and W700 in 3CIK is in the RH domain. The W18 and W19 water sites are available in four crystal structures with residential frequency 1 and they are considered as conserved whereas W20 and W21 are available only three crystal structures with residential frequency 0.75 and are considered as semi-conserved (Table 1). Furthermore, the conserved water molecule W18 makes H-bond with Asn275 and W19 with Leu273 in all the crystal structures. However, the semi-conserved water molecules W20 and W21 stabilize the Lys62 and Asp212 respectively (Table 2 and Figure 2). The conserved water molecules W18 and W19 are highly flexible in crystal structures as their experimental B-factors and calculated RMSF values are very high. The water molecules with a restricted solvent-accessible surface area (SASA) of less than 20% were considered to be buried water molecules [37], because they are shielded due to other neighbouring atoms preventing water from accessing the atom of interest. Therefore, the solvent-accessible surface area (SASA) of W18 and W19 water sites are buried semi-buried respectively and their corresponding values are 11.36 and 34.07 ($Å^2$), the semi-conserved water molecules W20 and W21 are also highly flexible because their B-factors and computed RMSF values are high. Moreover, the solvent-accessible surface area (SASA) of these water sites are 13.29 and 0.00 ($Å^2$) respectively and W20 seems to be buried whereas W21 is semi-buried (Table 3). Furthermore, the water dynamics study is employed in all four X-ray structures with the presence of crystal water molecules to investigate whether the conserved and semi-conserved water molecular positions are also available in the MD structures. The present computational analysis presumably highlights that water molecular positions in crystal and MD structures are similar and the four water sites are available in both static and dynamic structures (Table 4 and Figure 3).

Salt bridge contributes an essential role in the stabilization of protein structure, and inter or intra-domain recognition, and is also involved in chemical signaling or electron transfer processes [38,39]. Salt bridge-mediated stabilization in hGRK2 plays a significant role in the regulation of their differential activity in biological system. The Lys210 and Asp212 make a salt-bridge mediated H-bonding interaction in the kinase domain; furthermore, the NZ atom of Lys220 also forms a salt-bridge mediated interaction with Glu239 and Asp 335. Therefore, three salt-bridges provide stability to the kinase domain of human GRK2 protein (Table 5 and Figure 4). Moreover, the salt-bridge mediated inter-domain recognitions are also observed in hGRK2 protein, in detail, the Lys215 and Lys238 of the kinase domain produce the salt bridges and recognized Glu523 of RH and Asn502 of AGC domain respectively. In addition, His185 of the RH domain also gets associated with Asp637 of the PH domain (Table 6 and Figure 5). Possibly, these salt bridges may involve a chemical signaling process between kinase and RH or AGC domain. Interestingly, the water-mediated salt bridge interaction is also observed in the kinase domain between Lys210 and Asp212. The W21 is also associated with a water-based salt bridge interaction study.

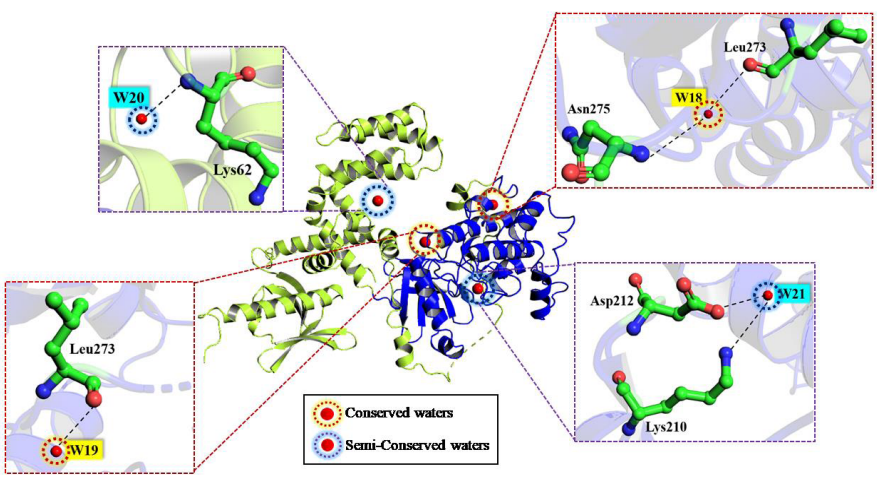

Figure 2 Presence of conserved and semi-conserved water sites in crystal structure of kinase domain (PDB Id. 3V5W) and their H-Bond interactions (Å) with the neighboring residues.

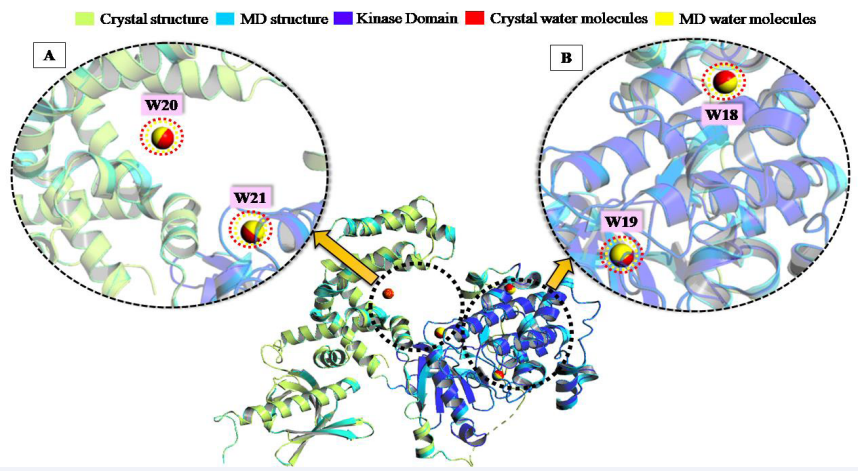

Figure 3 The Superimposed complex between X-ray and MD structures (PDB Id. 3V5W). (A) Superimposition of Conserved water sites W18 and W19 (B) Superimposition of Semi-conserved water sites W20 and W21.

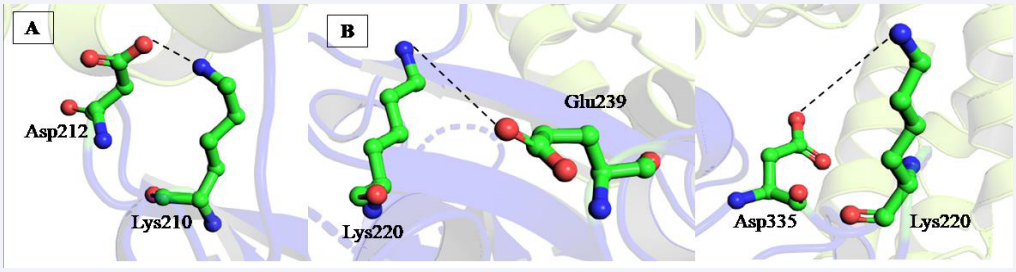

Figure 4 The Superimposed complex between X-ray and MD structures (PDB Id. 3V5W). (A) Superimposition of Conserved water sites W18 and W19 (B) Superimposition of Semi-conserved water sites W20 and W21.

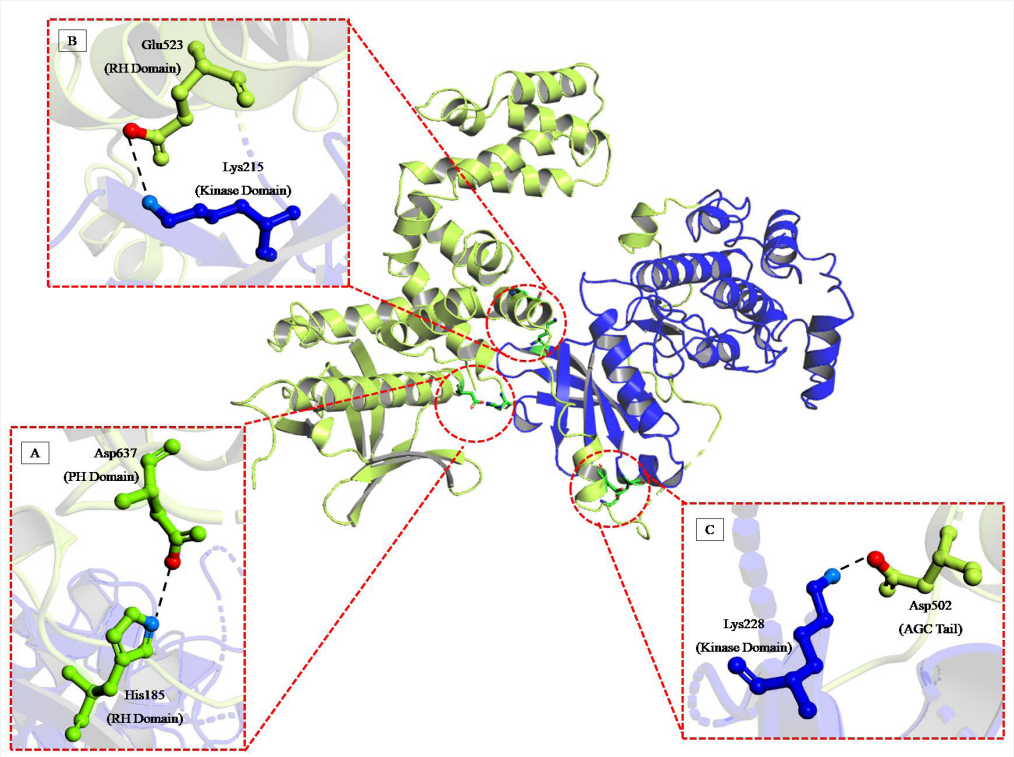

Figure 5 The Salt Bridge mediated H-binding interactions (Å) of Kinase Domain with other domains in crystal structure of PDB Id. 3V5W. (A) His185--- Asp637, (B) Lys215---Glu523, (C) Lys228---Asp502.

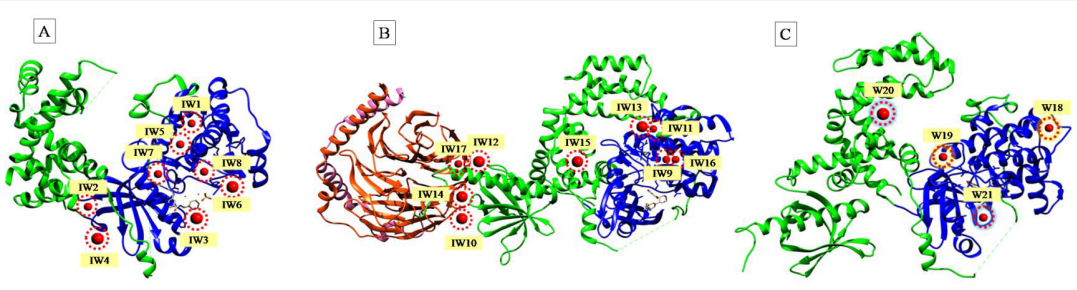

Figure 6 (A) Presence of unique water molecules in hGRK2 Protein with unbound $G_\beta G_\gamma$ (PDB Id: 5UVC) (B) Presence of unique water molecules in hGRK2 Protein with human $G_\beta G_\gamma$ complex (PDB Id: 5UKL) (C) Presence of conserved and semi-conserved water molecules in kinase domain of hGRK2 (PDB Id: 3V5W).

Table 1: Conserved and Semi-conserved water molecules in Kinase Domain (KD) of crystal structures of human GRK2 (hGRK2) protein

Water Id.	PDB Id: 3V5W	PDB Id: 5UKL	PDB Id: 3KRW	PDB Id: 3CIK	Residential Frequency	Domain Position
Conserved						
W18	W890	W937	W693	W696	1	KD
W19	W905	W948	W692	W695	1	KD
Semi-Conserved						
W20	W816	W905	NA	W701	0.75	*RH (N)
W21	W 836	W841	NA	W700	0.75	KD

*RH (N): N-Terminal RH domain

Table 2: Interactions of Conserved and Semi-conserved water molecules with neighbouring residues in crystal structures of hGRK2 protein

Water Id.	PDB Id: 3V5W	PDB Id: 5UKL	PDB Id: 3KRW	PDB Id: 3CIK
W18	Leu273/**Asn275**	**Asn275**/Glu327	**Asn275**	Leu273/**Asn275**
W19	**Leu273**	**Leu273**	**Leu273**	**Leu273**
W20	**Lys62**	**Lys62**	NA	**Lys62**
W21	**Asp212**	Lys210/**Asp212**	NA	Lys210/**Asp212**

Table 3: The B-factors, SASA values, and H-Bonding interactions of conserved and semi-conserved water molecules in crystal structures of hGRK2 protein

Water Id.	Water Id	B-Factor (Å²)	RMSF (Å)	SASA (Å²)		Position of Water	H-Bonds
				SASA Total	SASA Exposed		
W18	W890	42.49	1.27	107.14	11.36	Buried	2
W19	W905	37.04	1.19	107.14	34.07	Semi-Buried	1
W20	W816	31.62	1.10	107.14	13.29	Buried	1
W21	W836	52.52	1.41	107.14	0.00	Semi-Buried	2

Table 4: Comparative analysis of conserved and semi-conserved water molecules in kinase Domain (KD) between crystal and MD structures

Water No.	PDB Id: 3V5W		PDB Id: 5UKL		PDB Id: 3KRW		PDB Id: 3CIK	
	Crystal Structure	MD Structure	Crystal Structure	MD Structure	Crystal Structure	MD Structure	Crystal Structure	MD Structure
W18	W890	W890	W937	W937	W693	W693	W696	W696
W19	W905	W905	W948	W948	W692	W692	W695	W695
W20	W816	W816	W905	W905	NA	NA	W701	W701
W21	W836	W836	W841	W841	NA	NA	W700	W700

Table 5: Salt Bridge mediated H-bonding interactions within Kinase Domain (KD) in X-Ray structure of hGRK2 protein

Res. Id	Res. Id	Distance (Å)	Domains
Lys 210 (NZ)	Asp 212 (OD2)	2.70	KD
Lys 220 (NZ)	Glu239 (OD)	2.93	KD
Lys 220 (NZ)	Asp 335 (OD)	2.99	KD

Table 6: Salt Bridge mediated H-bonding interactions of Kinase Domain (KD) with other domains in X-Ray structure of hGRK2 protein

Res Id	Res. Id.	Distance (Å)	Other Domains
His 185 (NE2)	Asp 637 (OD2)	3.06	*PH
Lys 215 (NZ)	Glu 523 (OE1)	3.06	**RH (C)
Lys 228 (NZ)	Asp 502 (OD1)	2.58	***AGC

*PH: Pleckstrin Homology *RH (C): C-Terminal RH domain ***AGC: AGC C-Tail domain.

CONCLUSION

The water molecules play a decisive role in maintaining the kinase domain architecture of human GRK2 protein. Our previous computational investigations on multiple analyses of crystal structures (bound and unbound conformation of $G_{\beta}G_{\gamma}$) reveal the presence of seventeen unique water molecules (IW1 to IW17) that are only available either in hGRK2 with $G_{\beta}G_{\gamma}$ or without $G_{\beta}G_{\gamma}$ complex. Furthermore, the present study also indicates an additional four water molecules (W18 to W21) that are located at the kinase domain of the hGRK2 protein with a putative functional role (Figure 6). The W18, W19, and W20 provide the structural stability to the kinase and RH domain of the GRK2 protein respectively. The W21 makes salt-bridge mediated H-bonding interaction and gives the stability of the kinase domain. This water-mediated salt-bridge is associated with the chemical signaling process of inter-domain recognition of the kinase domain. The MD simulation results suggest that four crystal water sites are accessible by crystal water in MD simulation and allow entering these crystal water molecules at their concerningsites. Furthermore, the kinase domain is also been stabilized by three salt-bridge mediated interaction by Lys210---Asp212, Lys220---Glu239, and Lys220---Asp335. In addition, the kinase domain gets interconnected with the RH and AGC domain through Lys215---Glu523, and Lys228---Asp502 respectively. The result of the present study suggests the importance of biochemical information about the association of conserved, semi conserved, and salt-bridges of kinase domain of hGRK2 protein that provides a testable hypothesis for experimental validation.

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