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Structural insights on the effects of mutation of a charged binding pocket residue on phosphopeptide binding to 14-3-3 ζ Protein

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Mutation of an invariant aspartate residue in the binding pocket of 14-3-3 ζ isoform to alanine dramatically reduced phosphopeptide binding and induced opening of the binding pocket. Here we use extensive molecular dynamics simulations to understand the role of D124 residue in ligand binding. The simulations show that in the absence of phosphopeptide, the D124A mutation leads to binding pocket reorganization including widening up of the binding pocket at the major groove and repositioning of N173, a key residue that interacts with the main chain of phosphopeptide. These structural changes would interfere with the efficient binding of the peptide, corroborating the experimental observations. Both gain and loss of electrostatic interactions in the form of salt bridges strongly indicate a rearrangement of the network of interactions within the binding pocket. Limited proteolysis coupled mass spectrometry (lip-MS) of the apo and holo forms of WT and mutant protein shows a peptide binding helix otherwise buried in the WT protein was particularly accessible to trypsin in the apo form of the mutant protein and the region was mapped to 158-186 amino acid residues of 14-3-3 ζ). These results further confirm the dynamic nature of D124A mutant. Unlike other basic residues, the invariant D124 facilitates peptide bind-

ing by maintaining the geometry of interacting residues and by enforcing the structural integrity of amphipathic pocket.

KEYWORDS

14-3-3, signaling proteins, phosphopeptides, MD

1 | INTRODUCTION

14-3-3 proteins regulate multiple cellular process such as cell division, apoptosis, transcription, intracellular trafficking and cytoskeletal structure by interacting with many different client proteins [1, 2, 3, 4, 5, 6, 7, 8]. 14-3-3 protein family consists of highly conserved 30 kDa dimeric proteins that are present in all eukaryotic cells [9, 10]. There are seven known 14-3-3 isoforms (β , γ , ϵ , η , σ , τ and ζ) in mammals [11, 12]. Their sequence and structures are largely identical and yet their functions are often non redundant [13, 14]. The minor differences in sequence and structure of various isoforms may have a role to play in distinct functions even within the same pathway [15].

The heterogeneity and a sheer number of interacting partners of 14-3-3 proteins led to the speculation that there must be a common determinant for binding. The hypothesis was confirmed upon the identification of phosphoserine/phosphothreonine containing motifs present in many of the interacting partners [16, 17, 18, 19]. Peptide library screening studies led to the identification of two main 14-3-3 consensus motifs R-(S/Ar)-[+]-pS-[LEAM]-P (mode 1) and R-X-[Y/F]-[+]-pS-[LEAM]-P (mode 2). 14-3-3 can bind to certain non-phosphorylated substrates such as Exoenzyme S, R18 peptides. The overall three dimensional structure of the isoforms are near identical [16, 10, 20]. Each monomer is composed of nine antiparallel α -helices (α A- α H). The helices labelled α A, α B, α D form a dimer interface, whereas helices α C, α E, α G, α H form a cup-shaped amphipathic client protein pocket. The positively charged residues such as K49, R56 and R127 interact with the phosphorylated serine of the ligand (residue numbering according to ζ -isoform). Y128 stabilizes the incoming serine phosphate, maintaining the peptide in the requisite extended conformation. E180, V176, Y179 and W228 are involved in peptide side-chain interactions. The side-chains of N224, N173 and K120 form hydrogen bonds with the peptide's backbone. D124 lies at 3 Å from N173 and R127 residues and mutating D124 to alanine results in loss of peptide binding, primarily due to structural alteration as deduced by biophysical parameters [21].

To fully comprehend the impact of D124A mutation, in this study, we have used extensive molecular dynamics simulations to understand the effect of D124A mutation on the structure of 14-3-3 and phosphopeptide binding. We modelled the structure of D124A apo and holo form (bound to mode-2 1QJA peptide) and performed simulations to capture the dynamic changes induced by mutation. While large scale global changes do not occur on D124A mutations, subtle and specific alteration in the position of a network of residues in the binding pocket provides an explanation for reduction in peptide binding. This is an important result as it distinguishes mutation induced structural changes from events that purely affect binding. Moreover, using LiP-MS we mapped the conformational changes in different regions of the WT and mutant apo proteins. To our surprise, we recover a unique fragment present only in the apo form of the mutant protein, indicating that this region is labile in the mutant substantiating our previous claim that the mutation leads to opening of the binding pocket. Sequence and structural alignment indicates that the segment belongs to the α -helix located at the floor of the binding pocket. This observation also provides credence the hypothesis that, the small peptide will dissociate in the absence of stable interaction, while the large phosphopeptide/phosphorylated protein can potentially remodel the flexible binding pocket necessary for stable binding. Thus by a series of orthologous techniques we provide important insights into the structure, protein interaction and conserved

FIGURE 1 Cartoon representation of 14-3-3 WT ζ dimer system (PDB id: 1QJA) and the two monomers are referred to as M1 and M2. The relevant pocket residues whose mutations are considered in this study are labelled in orange (M1:D124, M2:D354) and the two bound phosphopeptides are shown in yellow (M1) and cyan (M2).

mechanism of binding and dissociation of this very important class of proteins.

2 | METHODS

2.1 Molecular Dynamics Simulation:

To understand the effect of D124A mutation on the binding of the phosphopeptide we simulate both the wild type (WT ζ , PDB id 1QJA) and mutated D124A ζ dimer system with (holo form : 1QJA mutated) and without the phosphopeptide (apo form : phosphopeptide removed from 1QJA). The details of all the systems studied are given in Fig. 2(a).The missing residues in the initial wild type PDB structure were modelled using Modeller software [22] and the correct protonation states of the ionizable residues were determined at the experimental pH of 6.2 (from the crystal structure) using DEPTH server [23] and appropriately changed before solvating the system. The dimeric form of 14-3-3 ζ with the phosphopeptide and the binding pocket residues mutated in this study are shown in Fig. 1.

Each system was solvated using TIP3P water model [24] and 150 mM NaCl salt was added to each system for overall charge neutralization and mimicking the physiological salt concentration. All the simulations were performed using Charmm36 forcefield [25, 26] in NAMD 2.12 software [27]. VMD software [28] and The PyMOL Molecular Graphics System, Version 2.4.2 Schrödinger, LLC [29] were used for visualisation and analyses were done using in-house analysis codes using TCL scripting language. Energy minimization was done for 5000 steps using conjugate gradient method [30] and harmonic restraints were applied with force constant of 1Kcal/mol/Å² on the complex for 1ns with a time step of 1fs towards the equilibration of the system. The constraints were slowly released over 10ns and all the systems were run for atleast 500 ns each with the total simulation of over 2 micro seconds. Langevin thermostat [31] was used to maintain constant temperature at 300K. Nose-Hoover Langevin piston [32, 33] with a

decay period of 100fs and a damping time of 50fs was used to maintain pressure at 1atm. A cut-off distance of 12Å was used to calculate short-range van der Waals (VDW) interactions and the long-range electrostatics interactions was calculated by the Particle Mesh Ewald (PME) method [34, 35].

2.2 Cloning, expression and purification of 14-3-3 ζ WT and mutants:

14-3-3 ζ WT and mutants D124A, N173V purified as described in Modi et al., [21].

2.3 Fluorescence anisotropy:

Fluorescence anisotropy (FA) was used to monitor the binding of FAM-RLYH(pS)LPA mode-2 1QJA (GL Biochem, Minhang, China) peptide to 14-3-3 WT and the mutant proteins. Various concentrations of 14-3-3 proteins in 50 mM Na-HEPES pH 8.0, 150 mM NaCl, 10% DMSO were incubated with 62.5 nM -labelled peptide, and the reaction was allowed to reach equilibrium for 30 min at room temperature in dark. The change in anisotropy was measured on Cytation 5, Biotek (Winooski, VT, USA). The G factor of the instrument was determined to be 1.2. Equilibrium KD was determined by fitting the data to a nonlinear regression model for single site-specific binding using GRAPHPAD PRISM 6 (GraphPad Software Inc, San Diego, CA, USA).

2.4 Determination of T_m :

The thermal and chemical denaturation of the WT and the mutant 14-3-3 proteins were performed using nano-DSF (Prometheus NT.48) from Nanotemper Technologies (Munich, Germany). The proteins at concentration of 15 μ M were used in 50 mM Na- HEPES buffer pH 8.0 containing 150 mM NaCl and 10 % glycerol.

2.5 Trypsin digestion:

14-3-3 WT ζ and D124A ζ were reduced with 12.5mM DTT for 30 min at 37°C, followed by alkylation with 40mM Iodoacetamide for 45 min at 25°C in the dark. Further trypsin was added in 1:75 ratio (trypsin: protein) and incubated overnight at 37°C. The trypsin digestion was stopped using formic acid. The peptide mixture was dried using speed vac. The dried pellet was resuspended into 0.1% formic acid. The peptide mixture was loaded onto desalting column and eluted with 80% acetonitrile. All the peptide samples were evaporated by using speed vac and redissolved in 0.1% formic acid followed by mass spectrometry [36].

2.6 Mass spectrometry:

The peptides were analyzed using Eksigent ekspertTM nano-LC 400 with cHiPLC® system, with trap column (200 μ m X 0.5 mm) and analytical column (75 μ m X 15 cm), both packed with 3 μ l ChromXp C18 (120 Å). For reverse phase HPLC, with a gradient elution was performed for 60 min. 0.1% FA in water and 0.1% FA in acetonitrile (ACN) served as solvent A and B respectively. Peptides were eluted with increasing percentage of Solvent B at flow rate of 300 nl/min. Eluted peptides were ionized using nanospray tip and analyzed in a positive ion mode on Triple TOF 5600+ mass spectrometer. Peptide analysis was done using software protein pilot TM software 4.5 and proteomics data Human + E. Coli downloaded on 2nd Jan 2019. Further analysis performed using Skyline software.

FIGURE 2 (a)Details of 14-3-3 ζ dimer systems simulated with (holo) and without (apo) the peptide. (b)RMSF of apo-WT ζ and apo-D124A ζ averaged over the last 100ns.

3 | RESULTS

To understand the effect of D124A mutation on the 14-3-3 ζ protein, analysis was first done on the apo-14-3-3 ζ trajectories. For convenience, chain A is referred as M1 and chain B is referred as M2 in the following results and discussion.

3.1 Global conformational and dynamics changes in apo-14-3-3 ζ upon mutating D124 to alanine:

To study the effect of D124A mutation on the binding of the phosphopeptide, the wild type (WT ζ , PDB ID : 1QJA) and mutant D124A ζ dimer systems with (holo form: 1QJA mutated) and without the phosphopeptide (apo- form: phosphopeptide removed from 1QJA) were simulated. In the crystal structure (PDB ID: 1QJA) [16] of 14-3-3 protein there are missing residues and these include: Q67-Q76, A134-D136 in M1 and A134-D137 in M2 of the dimer, which are modelled in the present study (see Methods). We compared the RMS deviations over the length of the simulation, to probe for global structural changes. Residue level changes are shown in Fig. 2(b), illustrating the C_{α} Root Mean Squared Fluctuations (RMSF) as a function of residue number, averaged over the last 100ns. D124A mutation caused a change in fluctuations in the residue patches 62-80 in M1 and 358-372 (corresponds to 128-142 in M1) in M2 (Fig. 2(b)). The results suggest that D124A mutation does not induce any significant changes in the overall structure of the protein.

3.2 D214A mutation alters conformation of binding pocket:

Modi et al. [21], using various biochemical and biophysical approaches, have shown that D124A ζ protein has a more open structure than WT ζ , resulting in loss of peptide binding. The opening up of structure was presumed to be due to local changes in the amphipathic groove upon mutating D124 to alanine. There are two openings in the binding pocket located at either end referred as major groove (M1=65 and 227, M2= 295 and 457) and minor groove (M1=40 and 209, M2= 270 and 439) at the either end of 14-3-3 ζ (Fig. 4(b), (d)). Simulations reveal that major groove width significantly increases after D124A mutation, though minor groove width does not change much in M1 monomer (Fig. 4(a,c)). However in M2 monomer, an increase in both minor and major groove width was observed (Figure S2(a,b)). The number of water molecules in the binding pocket before and after mutation were calculated in

FIGURE 3 Cartoon representation of the binding pocket of 14-3-3 WTζ M1 (PDB id: 1QJA). The bound phosphopeptide is shown in orange, charged residues in the phosphopeptide, R and phosphorylated serine, p(S), are shown as sticks. The interacting charged residues in the binding pocket which are within 4Å are shown in blue (basic) and red (acidic).

FIGURE 4 (a) cartoon representation showing minor groove defined as distance between C α atoms of residues 40 and 209 in M1 (b) cartoon representation showing major groove defined as distance between C α atoms of residues 65 and 227 in M1 (c) Binding pocket width distribution in M1 over the length of the simulation in minor groove (d) Binding pocket width distribution in M1 over the length of the simulation in major groove (e) Time evolution of number of waters in the binding pocket of M1- ζ

FIGURE 5 (a) T_m of WT ζ and D124A ζ (b) All the peptide intensities (whole protein) obtained by using skyline were summed up and considered 100%, followed by calculating % intensity of each peptide and plotted using the GraphPad prism.

WT ζ and D124A ζ . The data suggests that with the D124A mutation, there is an increase in the number of resident water molecules in the binding pocket (Fig. 4(e), Figure S2(c)). Taken together these results indicate that the D124A mutation causes structural rearrangement of the binding pocket making it more open and accessible, corroborating experimental observations.

3.3 Effect of mutation on structural stability of 14-3-3:

Although D124A mutation didn't show much change in global RMSF values, the effect on binding groove was quite evident. To validate the effect of mutation on the 14-3-3 structure observed by simulation data, thermal denaturation was monitored using intrinsic Tryptophan fluorescence as a probe and stability of the proteins was compared. T_m of the WT ζ protein was 61.8°C while that of D124A ζ was 50°C (Fig. 5 (a)) consistent with previous data published by [21]. The decrease in T_m value indicates the loss of structural stability due to mutation.

It is possible to predict specific conformational changes in the protein using LiP-MS. In LiP-MS, the WT and D124A digested with trypsin in limited amount and products are evaluated by mass spectrometry. Obtained peptide intensities are directly proportional to the trypsin-accessible surface. When the WT and D124A peptide profiles were analysed, most of the peptide intensities were equivalent except very specific peptide spanning the region 158-186 which showed drastic increase in peptide intensities in mutant and almost undetectable in the WT (Fig. 5 (b)). This segment belongs to α G helix, the part of amphipathic groove which harbours one of the key residues involved in the peptide binding i.e., N173. This result indicates very specific local changes in the structure that is confined to the binding pocket due to the mutation.

3.4 Effect of D124A peptide binding amino acid residues:

In both the monomers of WT ζ isoform, D124 interacts with other charged residues K120, R127 and N173 (Fig. 6 (a), Figure S3(a)). MD simulations of the apo-forms of both WT ζ and D124A ζ proteins reveal that these interactions are disrupted after the D124A mutation (Fig. 6(b-d), Figure S3(b-d)). D124 mutation brings about two changes a) conversion of charged residue to neutral residue b) replacement of bulky residue with smaller residue resulting into electrostatic and volume changes respectively. The loss of stabilising interactions between D124A mutated residue and other charged residues results in significant changes in the orientation of these residues inside the empty binding pocket (apo-form), which can have profound effect on the phosphopeptide binding.

The orientation of charged residues in the binding pockets of WT and D124A were compared in both apo and holo forms (Fig. 7(a)). In WT, R127 is closer to D124 and interacts with incoming phosphorylated serine [37, 38, 39] (Figure S1). After D124A mutation, due to the loss of interaction with A124, this residue moves away from its WT ζ position (Fig. 7(a), Figure S4(a), shown in purple). This new conformation allows R127 to interact with another binding pocket charged residue E180 (Fig. 7(b-d), Figure S4(b-d)). To further quantify this change in conformation of R127, time evolution of dihedral angle of the residue, CG-CD-NE-CZ in M1 (Fig. 7(e)) and CB-CG-CD-NE in M2 (Figure S4(e)) was calculated, which shows a prominent change after D124A mutation in apo-WT ζ .

Such conformation of R127 was observed only in D124 apo form (Fig. 7(a), Figure S4(a)), while its conformation in apo-WT ζ , holo-WT ζ and holo-D124A ζ are very similar. This prompted us to ask, how this gain of interaction of R127 and E180 would affect indirect peptide binding. This difference in conformation between the apo and holo D124A ζ systems is most likely due the presence of phosphopeptide which restricts conformational freedom of R127 favoring peptide interaction. The side chain of N173 forms a hydrogen bond with the peptide backbone NH and CO groups of the phosphopeptide residue Leu in the mode 2 crystal structure [40, 38] (Figure S1). In the simulated apo D124A ζ

FIGURE 6 (a) D124 residue and its interacting partners K120, R127 and N173 are shown in M1 (structure at end of the simulation) along with the distances between them. (b-d) Time evolution of distance plots showing the loss of interaction between D124 and K120, R127 and N173 after D124A mutation.

FIGURE 7 (a) change in the conformation of R127 in apo-D124Aζ-M1 (purple) as compared to apo-WTζ-M1 (blue), holo-WTζ-M1 (orange) and holo-D124Aζ-M1 (magenta). (b) New interaction of R127 with E180 after D124A mutation; (b) WTζ and (c) D124Aζ in M1 (d) Distance plot showing a consistent new interaction between E180 and R127. (e) Dihedral angle CG-CD-NE-CZ changes upon D124A mutation

FIGURE 8 (a) change in the orientation of sidechain in N173 in apo-D124Aζ-M1 (b) The difference in the dihedral angle CA-CB-CG-ND2 in apo-WTζ-M1 and apo-D124Aζ-M1. (c) FP assay for WT, D124A(D) and N173V(N)

structure, the orientation of the N173 side chain also flips, resulting in loss of N173 peptide backbone contact (Fig. 8(a), Figure S5(a)). This change in conformation of N173 can have profound implications on phosphopeptide binding to the groove in apo-D124Aζ system. To further quantify the change in conformation of N173, dihedral angle CA-CB-CG-ND2 was calculated (Fig. 8(b), Figure S5(b)) and a distinct change in dihedral angle CA-CB-CG-ND2 was measured and confirmed the local changes in conformation caused by D124A mutation. This is notably present in only D124A-apo form and not in holo form, where the bound peptide may potential stabilize these interactions.

3.5 14-3-3-peptide interaction and effect of mutations in vitro:

One of the major mechanistic reason for reduction in peptide binding in D124A mutation, from MD simulations here, is the loss of peptide backbone interaction with N173. We tested peptide binding ability of N173V mutant using D124A as a positive control for reduction in peptide binding. Various protein concentrations of WT and mutant proteins were titrated against the fixed concentration of 1QJA phosphopeptide by Fluorescence polarization. N173V like D124A showed very little binding to the peptide, confirming the role of D124 in stabilizing the network of interaction at amphipathic pocket (Fig. 8(c)).

4 | DISCUSSION AND CONCLUSION

Understanding per residue contribution to protein-ligand and protein-protein interaction from structure alone is a formidable task. Nevertheless, structure is an essential guide to probe the role of dominant interactions at protein interface, which can contribute via local conformational changes, allosteric interactions in the cycle of association, dissociation to the structure and the resulting function. The 14-3-3 family of proteins are integral to the regulation mediated by post translational modifications as they sequester several 100s of phosphoproteins and release them in a precisely regulated manner to regulate a myriad of processes in the cells [16, 17, 18, 19]. Crystal structures of

many peptide bound forms and few protein bound forms are available [41, 42, 43, 44, 45] and yet answers to many of the key questions remain elusive. Some of these outstanding questions include: a) how binding is initiated b) what is the lifetime of the complex c) what are the conformational changes in the 14-3-3 or the client protein required for association and dissociation d) how does peptide and protein binding differ e) what are the secondary binding sites that stabilize the protein interaction and f) what are the different mechanisms by which of protein selectivity versus affinity is determined between the isoforms. By taking advantage of the available structures one can do specific mutations and perform extensive molecular dynamics simulations to translate the structural information into an energetic and functional map that is no longer static but captures the nuances of local geometry, dynamics and long-range conformational changes. Besides interrogating mutations and their effect on binding, an experimentalist can complement the MD studies by performing what is termed as the LIP MS. This technique helps to precisely identify the regions that undergo conformational changes over a variety of conditions including the bound versus unbound form or the WT and mutant structure.

In this study we use these two approaches to investigate what happens when an invariant negatively charged residue in the binding pocket of 14-3-3 is mutated to a neutral and small residue such as Alanine? We also asked if we could find an answer to the stark difference between phosphopeptide binding *in vitro* and phosphoprotein binding *ex vivo* when such a mutation is created. The key observations are: a) D124A mutation affects peptide binding directly by altering the geometry of the N173 which is normally engaged in a hydrogen bond interaction with the peptide back bone. Mutation of N173 results in loss of peptide binding, confirming these observations; b) D124A mutation results in widening of the entry and exit port of the amphipathic pocket which will increase the rate of dissociation. C) A bound ligand locks the geometry of the network residues in the WT like conformation explaining why we could still measure peptide binding by a more sensitive assay such as SPR. D) The accessibility of a helix 7 which packs the floor of the binding pocket in the mutant may enhance the on-rate of association of a phospho-protein. It is likely that the disordered helix in the apoform of the mutant regains its structure in the phosphoprotein bound configuration. Increase in the number of water molecules in the binding pocket, via MD simulations, further confirms the dynamic nature and increased accessibility of the binding pocket in the mutant.

With these observations in place we propose a common mechanism of mutation induced structural and conformational changes that can be extrapolated to describe the energetic map of ligand binding and common rules that distinguish peptide and protein binding across 14-3-3 isoforms. And these are: (a) mutation of D124 has profound effects on binding of the phosphopeptide; gain and loss of electrostatic interactions in the form of salt bridges strongly result in net rearrangement of the residues only in the apo form of the mutant cumulative effect of which is to weaken the interactions at the binding pocket. This observation comes exclusively from the MD simulations. (b) As predicted from our previous experimental studies on the γ isoform [21, 15] and reiterated by investigations here on the ζ isoforms, D124A mutation induces local conformational changes and opens up the minor and major grooves that likely guard the entry and exit of the ligand; such a precise mapping is made possible by the comparative simulation of the WT and mutant proteins. (c) complementing these studies, the LIP-MS identifies a segment spanning 158-186 residues that is exclusively dynamic in the apo D124A mutant. We argue that such an opening would knock out a shorter peptide with limited area of interaction enhancing its dissociation while a large protein ligand such as seen with the 14-3-3 γ -NPM1 protein interaction, would cause 'binding induced folding' that rearranges the network of residues leading to the retention of the ligand at the binding pocket. Aspartate is invariant at this position because it is required for precise geometric orientation of interacting residues and disallowing intra protein interaction that would cost key interaction with the ligand and it is required for stability and compactness of the amphipathic groove that precisely determines the on-rate of association and off-rate of dissociation acting as a timer of binding events. Finally, we conclude that application of multiple techniques that probe atomic level interactions and account for protein dynamics

are important for arriving at some of the basic rules of protein interactions and go beyond a static snapshot provided by crystal structure. Unduly longer association between two proteins can be as detrimental as weak protein interactions and can be potent enough to deregulate homeostasis. Evolution seems to guard cellular functions by defining amino acid conservation that appear counterintuitive such as negatively charged Asp residue in 14-3-3 very close to the strongly negatively charged phosphorylated ligand in the binding pocket. Thus by two different techniques, we map the structural and conformational changes in 14-3-3 ζ in response to the mutation of an invariant negatively charged amino acid and provide plausible explanation in atomic details for the observed experimental observations.

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