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Binding site multiplicity with fatty acid ligands: Implications for the regulation of PKR kinase autophosphorylation with palmitate

Liang Fang^{+,‡}, Hyun Ju Cho^{+,§}, Christina Chan[§], and Michael Feig^{*,‡}

[‡]Department of Biochemistry and Molecular Biology; Department of Chemistry, Michigan State University, East Lansing, Michigan 48824, United States

[§]Department of Chemical Engineering and Materials Science, Michigan State University, East Lansing, Michigan 48824, United States

Abstract

Saturated long chain-free fatty acids (FFAs), especially palmitate, have been implicated in apoptosis by inhibiting the activity of PKR (double-stranded RNA-dependent protein kinase). We recently found evidence that palmitate interacts directly with the kinase domain of PKR, subsequently inhibiting the autophosphorylation of PKR. To investigate the interactions of palmitate with PKR and its effects on PKR autophosphorylation, we performed extensive unbiased MD simulations combined with biochemical and biophysical experiments. The simulations predict multiple putative binding sites of palmitate on both the phosphorylated and unphosphorylated PKR with similar binding affinities. Ligand-protein interactions involving a large variety of different binding modes challenge the conventional view of highly specific, single binding sites. Key interactions of palmitate involve the α C-helix of PKR, especially near residue R307. Experimental mutation of R307 was found to affect palmitate binding and reduce its inhibitory effect. Based on this study a new allosteric mechanism is proposed where palmitate binding to the α C-helix prevents the inactive-to-active transition of PKR and subsequently reduces its ability to autophosphorylate.

Keywords

binding site; phosphorylation; MD simulation; mutation; competition assay; inhibitory mechanism

Introduction

Long chain FFAs are major sources of metabolic energy¹ and essential components of cell membranes.² On the other hand, FFAs, especially saturated FFAs, can cause apoptosis in many types of cells, including pancreatic β -cells,³ cardiomyocytes,⁴ and hepatocytes.⁵ They

*Corresponding author: 603 Wilson Road, East Lansing, MI, 48824, USA; feig@msu.edu.

[†]Co-first authors

Supporting Information Available: Figures for the starting positions of palmitate; the density distributions of palmitate around pPKR between the 110–160 ns and 110–250 intervals, respectively; the C α -RMSDs of the 25 individual MD simulations in both PKR and pPKR; the methods for binding free energy calculation. This material is available free of charge via the Internet at <http://onlinelibrary.wiley.com>.

are implicated in a variety of diseases, including diabetes, heart disease, and non-alcoholic fatty liver disease.^{6,7}

FFAs have low aqueous solubility in the hydrophilic, intracellular environment, which can be overcome by interacting with proteins, such as human serum albumin (HSA),⁸ or the family of fatty acid-binding proteins (FABPs).⁹ The binding modes of FFAs to those proteins appear to vary greatly. For example, crystallographic studies identified seven FFA-binding sites distributed across all three domains of HAS⁸ and two binding sites inside the inner cavity of human liver FABP¹⁰ with different binding-affinities. Several lipid-binding sites have also been identified on kinase proteins during these years.¹¹⁻¹³ Diskin et al. found a novel lipid-binding site that is able to accommodate both, a lipid (n-octyl β -glucopyranoside) and fatty acids (15(s)-hydroxyeicosatetraenoic acid and arachidonic acid) on the kinase C-terminal domain of p38 α MAPK.¹⁴ The authors suggest that the lipid/fatty acid binding modulates the conformation of the activation loop, thereby regulating p38 α MAP kinase activity.

In previous work from our group, we found evidence that palmitate binds to the kinase domain of double-stranded (ds) RNA-dependent protein kinase (PKR), subsequently inhibiting the autophosphorylation of PKR at the two residues T446 and T451.¹⁵ PKR is an interferon-induced serine/threonine kinase¹⁶ that plays a variety of central roles in cellular functions such as cell growth, differentiation, signal transduction, tumorigenesis, and apoptosis.^{17,18} The modulation of PKR activity by fatty acids is, therefore, of great interest for connecting metabolic disorders that result in high FFA concentrations to diseases that result from disruptions in kinase signaling pathways. PKR consists of an N-terminal dsRNA-binding domain (dsRBD) and a C-terminal kinase domain. PKR is thought to exist in cells in a latent monomeric state.¹⁹ dsRNA binding releases the RBD from the kinase domain and promotes PKR dimerization and autophosphorylation that lead to the activation of kinase.^{20,21} Activated PKR is able to phosphorylate the α -subunit of the translational initiation factor eIF2 α , thus causing the reduction of protein synthesis and the induction of cellular apoptosis.¹⁷ The X-ray crystal structure of the kinase domain of phosphorylated human PKR in complex with eIF2 α was resolved.²² A back-to-back dimeric configuration of the protein kinase domain is formed by the interaction of two N lobes, while the C lobe composes the binding site for eIF2 α . The phosphorylation site at T446 located on the activation loop is critical for the stabilization of active conformation of the kinase as well as the substrate recognition. It has been well established that phosphorylation on the activation loop induces significant conformation changes related to α C-helix and activation loop from inactive (unphosphorylated) to active (phosphorylated) state in the eukaryotic protein kinase.^{23,24}

In a first computational study, we complemented the experimental data with *in silico* docking results. Because of the nature of standard docking methods, we focused on finding specific binding sites in the vicinity of hypothesized regions where palmitate may interact and identified a possible site near the ATP-binding site.¹⁵ We consequently hypothesized that palmitate may modulate PKR activity simply through competition with ATP binding. However, traditional docking methods are ill-suited to capture broader ensembles that consist of a variety of different binding sites such as FFA interactions with other

proteins.^{8,10} Therefore, we are revisiting here the palmitate-PKR system from the perspective of extensive molecular dynamics (MD) simulations.

MD simulations involve physical energy functions that are expected to be more accurate and robust than scoring functions used in docking experiments.^{25,26} Furthermore, docking via MD simulations can identify both high- and low-affinity binding sites without any *a priori* bias, while, at the same time, allowing for full flexibility of both, the ligand and receptor. The downside of docking via MD simulations is that extensive sampling is required at a substantial computational cost. As a result, docking via MD so far been limited to only a few cases.²⁵⁻²⁹ In one recent example, Shaw et al. conducted long, unguided MD simulations in which a ligand (in this case the cancer drug dasatinib or the kinase inhibitor PP1) was initially placed arbitrarily with respect to the protein (Src kinase) within the simulation box.²⁶ In these simulations, the ligand found its target binding site, which was consistent with the crystallographically identified binding site's location. Here, we follow a similar protocol using extensive unbiased MD simulations to study palmitate-PKR interactions starting from random initial locations of palmitate around PKR. The main result from these simulations is an ensemble of multiple binding sites with similar affinities that include the previously hypothesized interaction site near the ATP binding site but more prominently feature interaction sites near the α C-helix. The newly found interaction with the α C-helix gave rise to an alternate hypothesis for explaining the experimentally observed inhibitory function of palmitate that was further tested with biochemical and biophysical experiments on PKR mutants.

Materials and Methods

Simulation Methods

Simulation models—The structure for phosphorylated human PKR was obtained from the crystal structure in the Protein Data Bank (PDB ID: 2A19).²² Missing loop residues (334-442) were generated by MODELLER (version: 9v7).³⁰ The structure for unphosphorylated human PKR was built by homology modeling based on GCN2 (PDB ID: 1ZY4),³¹ which was identified by using a PSI-BLAST search.³² The best model was selected using MODELLER's DOPE assessment method³³ and from visual inspection. The structures for the experimentally unresolved loop regions (residues 256-259, 344-358 and 438-452 in the unphosphorylated model and residues 334-442 in the phosphorylated structure) were subsequently refined further. Using MODELLER's loop modeling module, 1,000 different models were generated for each loop. The generated structures were then clustered via the K-means method based on RMSD using a radius of 2 Å and the best clusters were selected according to the DFIRE scoring function.³⁴ From the best-scoring clusters, the structure closest to the geometrical center was then selected as the respective representative structure for PKR and pPKR (Fig. 1). The resulting models were completed by adding hydrogen atoms. Histidine ionization states (protonation on N δ or N ϵ) were predicted using PROPKA3.1³⁵ and confirmed visually based on the local protein environment. The two resulting models for phosphorylated PKR (pPKR) and unphosphorylated PKR (PKR) are shown in Fig. 1.

We further performed a short simulation to optimize the conformation of the PKR homology model, using the CHARMM22/CMAP force field^{36,37} for the protein with a distance dependent dielectric function. The equilibration process involved initial minimization over 1000 steps with the conjugate gradient method, followed by heating from 0 to 300 K in six 2-ps steps. Subsequently, 100 ps were run to further relax the structure. During the simulation, C_α and C_β atoms of α-helix in the C lobe and β-strand in the N lobe were restrained to the corresponding crystal structure of pPKR with a force constant of 5 kcal/mol/Å² while the other part of the protein (residues 300-321 and 409-466), including the αC-helix and activation loop, were restrained to the initial position of the homology model of PKR with a force constant of 0.5 kcal/mol/Å². The choice of restraints was based on the assumption that, similarly to other eukaryotic protein kinases, the main conformation changes from inactive PKR to active pPKR occur on the activation loop and αC-helix while the remaining parts of PKR keep the same conformation with pPKR.

For pPKR, the resulting starting structure was relaxed by 200-step steepest descent energy minimization followed by 1000-step conjugate gradient minimization, using a force constant of 2 kcal/mol/Å² on C_α and C_β atoms of protein. The two minimized proteins were then solvated in a rectangular box using the TIP3P water model³⁸ with a minimum distance of 20 Å from the protein surface to the nearest edge of the box. The total dimension of each system was ~94 Å × 105 Å × 85 Å. Nine counter ions (Cl⁻) were added by randomly replacing nine water molecules to neutralize the system. No additional salt was added. Energy minimization with subsequent equilibration was performed on the initial solvated system with computational details described in more detail below. Palmitate was then embedded into the final box at a distance of ~10 Å from the surface of PKR at 25 different positions around the protein. The positions of palmitate were generated as follows: The first one was placed along the y-direction at coordinates (0, 40, 0). Then the positions 2, 3 and 4 were generated by rotation of the first position around z-direction by 45°, 90° and 135° in clockwise direction. The other 21 positions were obtained by rotation of positions 2, 3, and 4 with an increment of 45° around the y-direction. The 25 starting positions of palmitate are shown in supplementary Fig. S1. In each simulation only one palmitate was present so that palmitate oligomerization was not possible in our simulations. Once PKR was added, one Cl⁻ was removed from the system to maintain system neutrality. Each system contained ~91,600 atoms.

Molecular dynamics simulations—The equilibration process involved initial minimization over 1000 steps with the conjugate gradient method, followed by heating from 0 to 300 K in six 20-ps steps. Subsequent production runs were carried out over 250 ns for pPKR and over 160 ns for PKR, respectively. The systems were simulated in an NTP (constant number of particles, pressure, and temperature) ensemble with a temperature of 300 K and a pressure of 1 bar that were maintained using a Langevin thermostat and Langevin piston barostat. The CHARMM22/CMAP force field was used for the proteins.^{36,37} Palmitate parameters were taken from the CHARMM27 lipid force field.³⁹ The particle-mesh Ewald (PME) method⁴⁰ was used to calculate long-range electrostatic interactions. The direct electrostatic sum and the Lennard-Jones potential were truncated at 10 Å with a switching function effective at 8.5 Å. The SHAKE algorithm⁴¹ was applied to

constrain the lengths of all bonds involving hydrogen atoms. The integration time step of the simulations was set to 2 fs and the coordinates of the trajectories were saved every 2 ps. In the production runs, no restraints were applied to the protein. For each system, 25 independent MD simulations, one for each palmitate starting structure, were run using NAMD,⁴² version 2.7b2, in conjunction with the MMTSB Tool Set.⁴³

Analysis—Most of the analysis was carried out with the MMTSB Tool Set and CHARMM version c36a1,⁴⁴ based on the simulation interval of 110-160 ns in both systems for each of the 25 simulations. Hence, the first 110 ns were considered equilibration and discarded (see below). The density distribution of palmitate was calculated by using an in-house program that counts a 3D volumetric histogram on a grid after reorienting each snapshot with respect to the initial structure of PKR at the beginning of the production run. Visual Molecular Dynamics (VMD)⁴⁵ and PyMOL⁴⁶ were used for visualization.

Experimental Methods

Materials—Bodipy-labeled palmitic acid, Bodipy-PA (4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-hexadecanoic acid), was obtained from Invitrogen. Anti-PKR and anti-PKR-pT446 were purchased from Novus Biologicals. All other materials were of the highest reagent grade that is commercially available.

Plasmids and Mutagenesis: DNA recombinant plasmid (pET11a-PKR-WT/PPase) containing the full length of human PKR wild-type (WT) gene and the λ protein phosphatase (PPase) gene was kindly provided by Dr. James L. Cole's group.⁴⁷ As previously reported, coexpression of PKR with PPase produces predominantly PKR protein in *Escherichia coli* cells.^{47,48} Site-directed mutagenesis was performed using the QuikChange Lightning Site-Directed Mutagenesis Kit (Agilent Technologies, Inc.) and the mutation was confirmed by DNA sequencing services (Eurofins MWG Operon).

Protein Expression and Purification of PKR Proteins: The experiments were performed as described previously.¹⁵ Briefly, WT and mutant plasmids were transformed into *E. coli* BL21(DE3) pLysS (Novagen) and the proteins were expressed in the cells with addition of IPTG (isopropyl β -D-1-thiogalactopyranoside). The cells were collected and resuspended in the buffer (20 mM HEPES (pH 7.5), 50 mM NaCl, 0.1 mM EDTA, 1 mM TCEP (tris(2-carboxyethyl)phosphine), and 5% glycerol) containing a protease inhibitor cocktail (Roche). In the presence of lysozyme (0.5 mg/mL), sonication was performed to extract proteins. The lysate was clarified by centrifugation and the supernatant was loaded onto a HiTrap heparin HP column (GE Healthcare). The PKR proteins were eluted with a NaCl gradient using the AKTA FPLC system (GE Healthcare). All purified PKR proteins were confirmed by SDS-PAGE and Western blotting analysis. In addition, the proteins were quantified by a Bradford assay (Bio-Rad).

Competition assay and IC₅₀ calculations: In the absence and presence of Bodipy-PA, the PKR proteins were incubated with phosphorylation buffer (20 mM HEPES (pH 7.5), 20 mM KCl, 2 mM MgCl₂, 0.04 mM EDTA, and 0.4 mM β -mercaptoethanol) for 20 min. The kinase reaction was performed by adding 100 μ M ATP at room temperature for 30 min. The

autophosphorylation reaction was stopped by heating to 95 °C for 5 min. The samples were loaded onto 9% tris/glycine SDS-PAGE gel and the phosphorylation level of T446 was quantified by Western blotting analysis. The phosphorylation level was normalized to the sample without Bodipy-PA. The concentration of Bodipy-PA required for 50% inhibition (IC_{50}) was estimated using a sigmoidal dose-response model and the regression was performed by KaleidaGraph software:

$$Y = Y_{\min} + \frac{(Y_{\max} - Y_{\min})}{1 + 10^{\{(X - \log IC_{50}) / (HillCoefficient)\}}}$$

where X and Y denote log (Bodipy-PA concentration) and the phosphorylated T446 level is quantified from Western blotting analyses, respectively. $Y_{\min}$ and $Y_{\max}$ are the levels of phosphorylated T446 when the concentration of Bodipy-PA is zero and when the concentration of Bodipy-PA is infinite, respectively.

Results

Computational studies

We performed unbiased molecular dynamics simulations to investigate possible binding modes of palmitate around the kinase domain of PKR. Using the crystal structure of the phosphorylated human PKR and a homology model based on the unphosphorylated GCN2 structure as starting structures (Fig. 1), we observed possible interactions of palmitate with PKR in both the phosphorylated (pPKR) and unphosphorylated states (PKR). Our results are based on a total of 4 μ s MD simulation (comprised of 25 simulations over $\sim$ 160 ns) for each state.

To estimate the convergence of the MD simulations with respect to the palmitate-PKR interactions, we calculated the average volume sampled by palmitate (O1) vs. time (Fig. 2). The sampled volume was determined by counting the number of different grid voxels within the simulation box that palmitate visits in each interval (1 ns), averaged over the 25 simulations. For both simulations, the sampled volume decreases over time and becomes stable after 110 ns for PKR but continues to decrease beyond 110 ns for pPKR. To further investigate the convergence of the pPKR simulations, we extended the simulation time to 250 ns revealing convergence after about 150 ns (Fig. S2). We then compared the 3D density distributions of palmitate around pPKR using either the 110-160 ns or the 110-250 ns intervals. We found that there was not a significant difference in the resulting binding sites when the longer interval was used (Fig. S3). Finally, we also applied a subtractive analysis introduced by Guvench et al.²⁸, where the volume histogram bins during the last 50 ns are compared with results from the previous 50 ns (see Fig. S4). According to this analysis, we also find only a slight difference in sampling suggesting again that convergence has been reached. Therefore, we used the interval of 110-160 ns in the following analysis for both the PKR and pPKR systems in order to avoid using different simulation time intervals when comparing the two systems.

The C α -RMSDs of the 25 individual MD simulations with respect to the initial structure reach about 3 Å for pPKR but 4-5 Å for PKR (Fig. S5). The larger conformational fluctuations in PKR are mainly concentrated in the activation loop and the structural variability coincides with increased contacts between palmitate and the activation loop (see below).

Density distributions of palmitate around the PKR structures, averaged over all of the simulations, are shown in Fig. 3. Densities were calculated separately for palmitate O1 and C16 atoms to distinguish palmitate head and tail interactions. Both are shown together in Fig. 3. Overall, we found that palmitate interacts extensively with different parts of the kinase involving interaction sites that spread over most of the entire kinase surface. Interactions appeared to be slightly stronger around the α C-helix where the highest palmitate densities were found for both, phosphorylated and unphosphorylated PKR. However, the specific interaction modes differed between pPKR (Fig. 3A) and PKR (Fig. 3B). For PKR, high densities of the palmitate head (O1) were found near the N-terminal part of the α C-helix while the highest densities of the tail (C16) were distributed between the N and C lobes. For pPKR, on the other hand, the highest density distributions of the head (O1) were observed near the C-terminal part of the α C-helix and the density distributions of the tail (C16) were concentrated between the α C-helix and the β -sheets in the N lobe. In both systems, high-density distributions were also observed at the top of the N lobe. For pPKR, density distributions of palmitate were also observed in the ATP binding site although the density was relatively low, indicating that this is a secondary binding site. Other high palmitate densities were found between the α H- and α I-helices at the bottom of the C lobe of pPKR, and, for PKR, significant additional density distributions were located around the activation loop. Additionally, considerable density distributions are observed below the ATP binding site, located between the α D- and α E-helix in the C lobe.

To further investigate the specific interactions between palmitate and PKR, we calculated the distances of heavy atoms between the amino acids and palmitate divided into the carboxyl head (O1, O2 and C1) and the methylene tail (C2-C16) regions. Distances of less than 4 Å were defined as a contact. We used a 4 Å cutoff between heavy atoms to capture both head and tail contacts but most of the palmitate head interactions are in fact less than 3 Å (data not shown) since they involve salt bridges while the larger aliphatic groups largely prevent contacts below 3 Å. Fig. 4 shows a PKR-centric view by highlighting the residues of PKR that palmitate contacts with based on the percentage of time during the simulations. Contacts present for at least 4% of the simulation time are listed in Table I. For both, PKR and pPKR, one of the most frequent contacts with the head of palmitate was found for R287 (Fig. 4A and 4E), which lies in the connection loop between the β 2- and β 3-sheets and near the back-to-back dimer interface in the crystal structure of human PKR. These contacts were presented during 12.67% and 14.51% of the simulation time, respectively. Another frequent palmitate head interaction with PKR involved R307 on the α C-helix (shown in orange in Fig. 4G). The percentage of time that R307 was in contact with palmitate was 11.63%. Moreover, several residues on the activation loop, especially R445 and R453 in PKR (Fig. 4E and 4G), were also in contact with the head of palmitate for a high percentage of time (9.02% and 9.95%, respectively). In addition, significant contacts involving residue R381 were observed in PKR (see Fig. 4E). For pPKR, the most frequent contacts with the head of

palmitate were also located on the α C-helix but involved different residues than in PKR. Instead of extensive contacts with R307, palmitate contacts with K314 of pPKR were found during 13.14% of the simulation time (shown in orange in Fig. 4C). Contacts of the palmitate tail with residues in the ATP binding site occurred with relatively low probability (see Fig. 4B). Additionally, multiple residues at the bottom of the C lobe of pPKR were in contact with palmitate (see Fig. 4C and 4D).

Representative binding conformations based on clustering analysis are shown in Figs. 5 and 6, respectively. In general, the head carboxyl group of palmitate interacted with positively charged residues in PKR and appeared to be less flexible than the methylene tail. Multiple binding modes were found for palmitate interactions with R287 in PKR. Among these, the major binding modes observed with both PKR and pPKR (see Figs. 5C and 6B) include the head carboxyl moiety of palmitate interacting with R287 and K268 nearby. As can be seen in Fig. 5, panels A and D-H, the positions in the PKR structure that palmitate interacted with are located near the α C-helix and the activation loop. In each of these binding modes, the head carboxyl moiety of palmitate interacted with positive charged residues nearby, such as R307, K314, R445 and R453; the tail of palmitate displayed greater conformational flexibility and involved various nearby hydrophobic residues. The most favorable palmitate binding mode with the α C-helix is shown in Fig. 5E. In this structure, the head of palmitate was anchored through interactions with R307 in the N-terminal of the α C-helix and the tail was located between the N and C lobes. Fig. 5B shows another prominent binding mode where part of the tail of palmitate was accommodated in a hydrophobic pocket between the α D- and α E-helices. At the same time, the head was forming contacts with R381. For pPKR, the predominant binding conformations (Fig. 6) appeared to be significantly different from those around PKR (Fig. 5). The head of palmitate was anchored by interactions with K314 in the C terminal part of the α C-helix of pPKR and the tail extended to the hydrophobic site above the α C-helix (see Fig. 6A). Additionally, there were fewer contacts with the activation loop of pPKR than PKR. Only one significant interaction between the tail of palmitate and the activation loop was found (see Fig. 6G). Another binding mode of palmitate was observed in pPKR where the tail of palmitate was stabilized in a hydrophobic site above the β -sheets and the head interacted with the positive charged residues nearby (see Fig. 6C). The interaction of palmitate with the ATP binding site that was observed only in pPKR is shown in Fig. 6D. In this mode, the methylene tail was accommodated in the ATP-binding site through interactions with multiple hydrophobic residues. Finally, additional specific interactions were identified between palmitate and the C lobe around the α G-, α H-, and α I- helices in pPKR (see Figs. 6 E, 6F and 6H).

The relative sampling of different interactions between palmitate and PKR/pPKR allowed us to calculate relative binding affinities. The resulting values (given in Figs. 5 and 6) are quite small, with the least favorable and most favorable binding sites estimated to differ by only 1 kcal/mol. This further underlines the broad sampling of different interactions between palmitate and PKR with only slight preference for certain sites. This mode of interaction is in stark contrast to classic ligand-substrate interactions with a single, highly specific binding site.

Finally, we also estimated the overall binding affinity of palmitate to PKR and pPKR using MMPB/SA analysis (see supporting information). To obtain a total binding affinity in the presence of multiple binding modes, we calculated the weighted average of binding free energies associated with individual binding mode. The total binding affinities are estimated to be -22.58 and -21.36 kcal/mol for PKR and pPKR, respectively. Individual contributions to the total free energies are -208.59 and -196.81 kcal/mol for internal energies, 188.12 and 178.22 kcal/mol for solvation energies, and 2.11 and 2.78 kcal/mol for the $T \Delta S$ term for palmitate-PKR and palmitate-pPKR interactions, respectively. This suggests highly favorable binding to either PKR or pPKR as long as palmitate is present in the environment and similar binding affinities to either PKR or pPKR are in accordance with experimental data from our previous work.¹⁵

Experimental Studies

To experimentally identify the palmitate-binding sites, which could modulate the autophosphorylation of the PKR protein, we selected potential binding residues (R287, R307, R381, R445, R453) where the head of palmitate has the top 5 strongest interactions with the PKR protein according to the simulation results (See Table I). All five residues of the PKR protein were individually mutated to alanine using site-directed mutagenesis and the mutant PKR proteins were expressed in *E.coli* cells, followed by affinity chromatography. To test the impact of the alanine mutations on palmitate binding affinity, we used a fluorescence polarization (FP)-based assay. We previously developed a FP-based assay to measure the interaction between Bodipy-labeled palmitate and the PKR proteins, which was successfully applied to quantitatively measure the binding affinity of the palmitate binding proteins.¹⁵ At constant concentrations of Bodipy-PA and PKR proteins, the binding affinity was compared to that of the PKR-WT protein. As shown in Fig. 7, only the R307A mutant protein significantly decreased the palmitate binding affinity, suggesting that palmitate interacts with the R307 residue located on the α C-helix.

To further assess whether the R307A mutation alters the kinase activity of the PKR protein in the presence of Bodipy-PA, we performed an in-vitro competition assay. The binding of Bodipy-PA results in a dose-dependent decrease on the autophosphorylation of both the WT and R307 mutant proteins (Fig. 8A). However, as shown in Fig. 8B, a reduction in kinase inhibition by Bodipy-PA is observed with the R307A mutation as compared with the WT, with a 1.66-fold increase in the half-maximum inhibitory concentration (WT ($IC_{50} = 48.29 \pm 8.57 \mu M$, Hill coefficient = 2.47); R307A ($IC_{50} = 80.34 \pm 5.63 \mu M$, Hill coefficient = 2.70)). Thus, the experimental results suggest that palmitate binding at or near R307 is capable of inhibiting the autophosphorylation of the PKR protein.

Discussion and Conclusions

Experimental evidence has shown that palmitate interacts with proteins in a variety of binding modes, including multiple binding modes for the same protein.^{8,10} A common feature of the different modes is that the palmitate head carboxyl group is anchored by electrostatic interactions to positively charged residues while the methylene tail is accommodated within the hydrophobic cavities. Our simulation results also suggest multiple binding modes of palmitate with both the phosphorylated and unphosphorylated PKR that

involves a number of different sites with similar binding affinities. As expected, the most frequent interactions involve interactions between the head of palmitate with Arg and Lys residues and between the tail and hydrophobic residues (see Table I). However, in detail, the specific binding modes differ greatly between the different sites and between PKR and pPKR (see Figs. 5 and 6). In binding sites with the highest affinities, key interactions with the α C-helix appear to involve R307 in PKR and K314 in pPKR. A possible role of R307 as a key residue for PKR-palmitate interactions is further supported by the experimental binding and kinase assays. As shown in Fig. 7, the R307A mutant protein had a reduction of about 25 % in the palmitate binding affinity compared to WT. A modest reduction in binding affinity in the single R307A mutant is consistent with the presence of multiple binding sites involving more than just R307. Furthermore, the modest effect in the kinase assay together with the simulation results suggests that palmitate may exhibit its inhibitory effect in a number of ways such that any single mutant would only lead to a partial reduction of palmitate's inhibitory function. The possibility of multiple binding sites with similar affinities is further supported by the fact that experimental Hill coefficients are greater than 1 for both WT and the R307A mutant (see Fig. 8).

The α C-helix is a key structural element in the activation of protein kinases that switches between inactive (α C-helix “out”) and active (α C-helix “in”) conformations.^{23,24} In the active state, a conserved salt bridge is formed between the Glu residue in the α C-helix and the Lys residue in the β 3 strand, which coordinates the α - and β -phosphate groups of ATP in a geometry that promotes the phosphoryl-transfer reaction. The disruption of the catalytically crucial Lys–Glu pair is a strong indicator of protein kinase inactivity. Recently, two allosteric inhibitors (8-anilino-1-naphthalene sulfonate) have been found to bind to CDK kinase in the pocket located between the α C-helix, the β 4- and β 5-strands of the N lobe and the ATP-binding site while inducing a remarkable outward movement of the α C-helix.⁴⁹ This induced change in conformation of the kinase is presumably a major contribution to the inhibitory mechanism of CDK.

In PKR, the conserved E308 residue is located on the α C-helix and immediately adjacent to R307. As shown in Fig. 9A, palmitate binding to R307 in the site between the α C-helix and the C lobe (the same structure as shown in Fig. 5E) is presumed to stabilize the α C-helix in the “out” conformation. This would keep E308 pointed away from the catalytic site and prevent the salt bridge K296-E308 from forming, which may block the inactive-to-active transition of PKR and inhibit the phosphate transfer. The conformation shown in Fig. 5E involving R307 most directly supports this mechanism but alternate interactions with the α C-helix such as the conformation in Fig. 5F that does not involve R307 may function in a similar manner. The proposed inhibitory mechanism suggests that palmitate would act as a potential allosteric inhibitor.

In pPKR, the α C-helix interacts not just with K296 but is also connected to the activation loop through the interaction of K304 and R307 with the phosphate moiety of T446 (see Fig. 9B). This interaction is believed to play a key role in the stabilization of the active conformation state of the α C-helix and the activation loop. From our simulation results of pPKR, these intramolecular interactions involving pT446 are very stable, while, at the same time, palmitate is prevented effectively from forming interactions with those residues. On

the other hand, phosphorylation on its activation loop, as in other protein kinases, maintains the activation loop in an open conformation, which in turn enhances nucleotide or other ligand binding to its kinase catalytic site. Hence, palmitate would be more likely to interact with the ATP-binding site in the active state of PKR. This is consistent with our observation that the binding mode of palmitate in the ATP-binding site is only found in pPKR (see Fig. 6D).

Previously we suggested that palmitate may act as a competitive inhibitor that prevents ATP from binding to the active site.¹⁵ Our simulations are still consistent with this hypothesis since the interactions of palmitate with the ATP-binding site of pPKR would conflict with ATP binding. However, in detail, the binding mode of palmitate in the ATP-binding site differs from the docking result in the previous study. The docking results suggested that the methylene tail of palmitate is located near K296 or D432 in the ATP-binding site with a direction of the head carboxyl group pointing to the activation loop. On the other hand, the simulation results suggest a different pose of palmitate, namely that the methylene tail interacts with multiple hydrophobic residues in the ATP-binding site and the head carboxyl group interacts with K283, opposite to the activation loop (see Fig. 6D). Given the more accurate energy function and more extensive sampling in the MD simulations, we favor the MD results over our previous docking results, but, ultimately, additional experiments (i.e. crystallography) would be needed to unambiguously determine the exact interaction(s) of palmitate with PKR.

Our simulation results also show that there are significant contacts between palmitate and residue R453, which are located near the PKR/eIF2 α binding interface in the crystal structure of pPKR and are believed to be important in substrate recognition.²² It has been shown that eIF2 α contacts the activation loop of PKR directly through a hydrogen-bond interaction involving R453. Palmitate contacts with R453 (Fig. 5A) in the site between the activation loop and α G-helix may therefore block the binding of eIF2 α to PKR. Experimental results show that the R453A mutant protein does not significantly decrease palmitate binding affinities (Fig. 7). However, if binding to the α C-helix is indeed the predominant binding mode and binding to this site is only of secondary importance, it may be difficult to capture the contribution of abolishing binding at R453 while still allowing for binding to the major R307 site. Another possible complication is that due to steric effects, the Bodipy-PA used in the experiments might block the interaction between the methylene tail of palmitate and the hydrophobic cavity in this site. Although Bodipy-labeled palmitate has been used successfully to approximate the binding of FFAs,^{15,50,51} it could nevertheless impact the interactions with certain binding sites that are not able to accommodate the Bodipy moiety.

The prediction of strong interactions between palmitate and residues R287, R381 and R445 were also not supported by the experiments. Again, the effect may be small if the binding affinities are somewhat reduced compare to the R307 site. However, from the corresponding binding conformations shown in Figs. 5B, 5C and 5D, we found that besides those residues, there are other positively charged residues K426, K268 and R413 nearby which could provide alternate contacts for palmitate in the mutants.

Under *in vivo* conditions, FFAs in the plasma are transported by plasma fatty acid binding proteins, in particular albumin. As a result, the concentration of unbound FFAs is ~ 10 nM in normal human physiology.⁵² In *in vitro* experiments, FFAs, are typically introduced bound to bovine serum albumin (BSA) as a carrier protein. Similarly, the concentration of unbound palmitate was close to physiological level (10 nM) in our previous palmitate-PKR binding assay.¹⁵ In comparison, the concentration of palmitate in our simulation model is about 2 mM based on one palmitate in a simulation box with the size $94 \text{ \AA} \times 105 \text{ \AA} \times 85 \text{ \AA}$, which is much higher than the physiological level. Lower concentrations could be achieved in simulations by increasing the simulation volume (by adding more water). However, the computational cost would increase dramatically if the water box was increased enough to reach nM concentrations. At nM concentrations, palmitate binding kinetics are presumed to be diffusion limited and would depend on free palmitate being able to reach PKR without binding to carrier proteins, entering surrounding membranes, or forming aggregates to reduce unfavorable interactions with water. However, our calculations suggest that palmitate binding to PKR is highly favorable so that if palmitate reaches the vicinity of PKR it is likely going to bind. Another possibility is that carrier protein bound palmitate may be able to dissociate and rebind to kinase if the carrier and kinase proteins are found in sufficiently close vicinity. Our simulations are not able to fully consider some of these possibilities since we did not explicitly consider carrier proteins or membranes in our simulations, and oligomerization was not possible with only a single copy of palmitate in each simulation.

Finally, although the MD simulations are much more extensive than our previous docking simulations, there are still uncertainties that could possibly affect some of the results reported here. One issue is with the homology model of PKR. We built the PKR model in which the α C-helix and activation loop were obtained by homology modeling based on the inactive structure of GCN2 (64% and 63% sequence identities for the α C-helix and the activation loop, respectively) and the other parts of PKR were then constrained to the crystal structure of pPKR. However, we note that a similar protocol was used in a recent study of protein conformational transitions where the inactive structure of CDK5 (cyclin-dependent kinase 5) was built by homology modeling on the inactive structure of CDK2.⁵³ The fact that we were able to predict R307 as a key residue for palmitate interactions with PKR further validates our approach, but uncertainties remain in the absence of direct structural data for unphosphorylated PKR.

Another potential issue is related to the parameters of palmitate. We used parameters from the CHARMM lipid force field that are tuned to represent lipid bilayer properties for representing palmitate in aqueous solvent and in interactions with PKR. However, the design of CHARMM aims at transferability for these types of applications and there are a few previous MD simulations of fatty acid – protein interactions with other force fields that appear to have been successful.^{54,55} This suggests that our simulations should be at least qualitatively reliable.

Finally, convergence is always a concern in any simulation study. It appears that 25×160 ns simulations with different initial starting configuration are sufficient, but we note that the Shaw group has run much longer simulations for the ligand (dasatinib or PP1) and Src kinase.²⁶ A comparison between 160 ns and 250 ns simulations shows little difference but it

is possible that additional binding modes may be revealed with simulations over much longer time scales. Since the use MD simulations for direct docking is still an emerging area, it remains unclear how many simulations on what time scales are sufficient for reaching good equilibrium in MD-based protein-ligand modeling.

In summary, we employed extensive molecular dynamics simulation to investigate the interaction of palmitate with both unphosphorylated and phosphorylated PKR. A main finding is the multitude of different binding modes at different sites but with similar binding affinities suggesting an alternate paradigm for binding of FFAs to proteins that differs from the conventional single, high-affinity binding site view. Especially important interactions appear to be near the α C-helix, the activation loop, and the ATP-binding site. In particular, interactions with the α C-helix are suggested to play a key role in inhibiting PKR activation by blocking the inactive-to-active transition thus inhibiting autophosphorylation of T446. The predictions from the simulations were partially validated through experiments where mutation of a key residue, R307, resulted in reduced palmitate binding as well as reduced kinase activity inhibition by palmitate. Our simulations further continue to support an alternate mechanism where palmitate binds competitively near the ATP binding site thereby preventing kinase activity.¹⁵ Finally, yet another mechanism may involve inhibition of eIF2 α substrate binding although the experiments reported here could not confirm such a mechanism.

We believe that we have arrived at a much more detailed structural view of palmitate-PKR than in our previous study. Further biochemical experiments, for example involving double or triple mutants could further validate our predictions. However, ultimately, PKR-palmitate co-crystal structures would be desirable to complement and validate our simulation study and gain deeper insights into the effect of palmitate on disrupting kinase function.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

BSA	bovine serum albumin
CDK	cyclin-dependent kinase
DFIRE	Distance-scaled, Finite-Ideal gas Reference
DOPE	Discrete optimized Protein Energy
dsRNA	double-stranded RNA
dsRBD	dsRNA-binding domain

eIF	eukaryotic translation initiation factor
FABPs	fatty acid-binding proteins
FFA	free fatty acid
GCN2	general control nonrepressed 2
HSA	human serum albumin
IC50	half maximal inhibitory concentration
MD	molecular dynamics
MAPK	mitogen-activated protein kinase
PDB	Protein Data Bank
PKR	double-stranded RNA-dependent protein kinase
PME	particle-mesh Ewald
RMSD	root-mean-square deviation
WT	wild type

Figure 1. Structures of the kinase domain of human PKR. (A) Phosphorylated PKR (pPKR): crystal structure of human PKR (PDB ID: 2A19). (B) Unphosphorylated PKR: homology model based on inactive GCN2 (PDB ID: 1ZY4). The activation loop is colored green. The phosphorylation site at T446 located on the activation loop is shown in stick representation. Loops built by *ab initio* modeling are shown in blue. The α -helix in the C lobe and the β -strand in the N lobe residues that were restrained to the corresponding crystal structure of pPKR during the model building of PKR are shown in purple while residues kept near the initial position of the homology model of PKR are shown in orange.

Figure 2. Average volume sampled by palmitate (O1) vs time around PKR (A) and pPKR (B). The columns are averages over 25 simulations in each interval (1 ns) based on the volume grid points that palmitate samples during the simulation.

A phosphorylated PKR **B unphosphorylated PKR** **Figure 3.**

Density distribution of palmitate around PKR for pPKR crystal structure (A) and PKR homology model (B). Contours show number densities of palmitate O1 (red) and C16 (blue) atoms at a level of $0.0004/\text{\AA}^3$. For each structure, two views are shown. The second view was generated by rotation around the long axis of PKR by 180° .

Figure 4.

Surface representation of PKR colored by the percentage of time that different PKR residues are in contact with palmitate during the 110-160 ns simulation interval. Contacts are defined based on a distance cutoff of 4 Å for distances of heavy atoms between PKR and the head of palmitate (including atoms O1, O2 and C1) in (A), (C), (E) and (G) or the tail of palmitate (including atoms from C2 to C16) in (B), (D), (F) and (H). The color scale ranges from blue at the lowest percentage of time to red at the highest percentage of time as indicated by the color bar. The arrow indicates the position of the α C-helix.

Figure 5.

Representative structures of palmitate contacts with PKR based on clustering. Palmitate and PKR residues are shown in sphere and stick representations, respectively. The oxygen atoms at the head of palmitate are shown in red, the tail carbon atoms in black. The activation loop is shown in green. Relative binding free energies G_b were determined from relative populations as $G_b = -k_b T \ln[p_n/p_{ref}]$ where the structure in (H) is used as the reference. Based on a subtractive analysis (see Fig. S4) relative populations are assumed to be converged within 0.01 of relative probabilities, which translates into errors of less than 0.01 kcal/mol when comparing the most populated states.

Figure 6.
Representative structures of palmitate contacts with pPKR as in Figure 5.

Figure 7. Bodipy-PA binding to PKR-WT and mutant proteins. 10 nM of Bodipy-PA was mixed with 50 nM of the PKR proteins in PBS buffer (pH 7.4) at room temperature. After 10 min of incubation, fluorescence polarization measurements were performed at 488 nm/515 nm using a spectrofluorometer. Each error bar represents the mean of triplicates $\pm$ SD. The * indicates statistically different results compared to the WT protein ($p < 0.05$).

Figure 8. Inhibitory effects of Bodipy-PA on PKR-WT and PKR-R307A autophosphorylation. (A): *In-vitro* competition assay with Western blot analysis. In the presence of 100 μM ATP and 1 μM PKR proteins, Bodipy-PA at the indicated concentrations was added to the phosphorylation buffer. After the kinase reaction, the phosphorylated fractions were visualized by Western blot analysis using anti-pT446. (B): Quantification of the phosphorylated T446. From Figure 6A, the phosphorylation levels of PKR-WT and PKR-R307A were quantified and normalized against the levels in the negative control (without Bodipy-PA) for each protein. Data shown are the means and standard deviations from three independent experiments. * indicates statistically significant different values for the mutant vs. WT protein at the same condition ($p < 0.05$).

Figure 9.

Superimposition of the α C-helix and catalytic site between the palmitate-bound PKR (A: PKR; B: pPKR) and the crystal structure of pPKR (PDB ID: 2A19). The crystal structure of pPKR is shown in light grey and the α C-helix and residues are colored in green, whereas the palmitate-bound PKR is shown in light orange and the α C-helix and residues are colored in blue. Palmitate is shown in sphere representation. AMP-PNP and Mg^{2+} ions in the crystal structure of pPKR are shown in cyan and the interaction between K296-E308 is indicated as a black dotted line.

Palmitate-PKR contacts present for at least 4% of the simulation time (110-160 ns) based on heavy atom distances of <4 Å between PKR residues and palmitate

Table 1

	PKR		pPKR	
	residue number	head of palmitate	percentage of time (%)	percentage of time (%)
αC-helix	R287		12.67	14.51
	R307		11.63	13.14
activation loop	R453		9.95	5.77
activation loop	R445		9.02	4.90
	R381		8.38	4.67
	K268		6.79	
	K426		4.12	
	R413		4.06	
	tail of palmitate			
	R287		15.77	21.07
	M265		14.91	18.16
	W377		11.20	14.56
	L394		10.02	13.14
αC-helix	A311		9.82	10.51
	K261		8.97	10.41
αC-helix	R307		8.48	10.28
	L390		7.91	10.24
	L422		7.55	8.75
	L410		7.41	8.06
	I378		7.36	7.88
αC-helix	K314		6.78	7.35
	M455		6.35	6.90
	L452		6.27	6.12
	L374		6.19	6.03
	L315		5.73	5.53
	αC-helix			
	R287		15.77	21.07
	M265		14.91	18.16
	W377		11.20	14.56
	L394		10.02	13.14
αC-helix	A311		9.82	10.51
	K261		8.97	10.41
αC-helix	R307		8.48	10.28
	L390		7.91	10.24
	L422		7.55	8.75
	L410		7.41	8.06
	I378		7.36	7.88
αC-helix	K314		6.78	7.35
	M455		6.35	6.90
	L452		6.27	6.12
	L374		6.19	6.03
	L315		5.73	5.53

PKR			pPKR		
residue number	head of palmitate	percentage of time (%)	residue number	head of palmitate	percentage of time (%)
I460		5.48	F263		5.47
K426		5.47	K517		5.42
R499		5.20	R499		5.40
R262		5.14	F363		4.93
K408		5.09	R262		4.86
E308		4.48	E531		4.59
T258		4.46	V259		4.18
			F495		4.13
			Y472		4.11
			R447		4.04
			activation loop		