**TITLE**

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ABSTRACT

KEYWORDS: amphitropic protein, molecular dynamics simulations, surface plasmon resonance, proteinase 3, neutrophil elastase

ABBREVIATIONS

PR3: proteinase 3, HNE: human neutrophil elastase, LUV: large unilamellar vesicle, POPC: 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine, SPR: surface plasmon resonance, MD: molecular dynamics

1. Introduction

Unlike integral membrane proteins, amphitropic proteins do not span the cell membranes. Theybind reversibly to biological membranes and weakly to lipid vesicles with equilibrium association constants typically between 103 M to 107 M[1](#_ENREF_1). Electrostatic interactions drive their positioning and orientation at the membrane surface thus facilitating the intercalation of a few hydrophobic groups. The latter can be hydrophobic amino acids or covalent lipid anchors such as palmitoyl, myristoyl or even glycophosphatidylinositol (GPI) anchors. It is generally acknowledged that the association of amphitropic proteins with lipid bilayers is fast while the dissociation is slow; the dissociation rate constant is thus the main determinant of the binding strength. As a consequence, in simple systems where the protein does not undergo conformational changes and does not interact with other proteins, the affinity for the membrane is mostly accounted for by interactions between the protein interfacial binding site and lipids. The energy and specificity of these interactions are generally estimated by including contributions from electrostatics, ~-1.4 kcal/mol for each positively charged amino acid interacting with the lipid headgroups[2](#_ENREF_2), and hydrophobic interactions with the bilayer core, ~-0.8 kcal/mol per acyl chain CH2 group interacting with the protein.

Neutrophil serine proteases.

Drug targets in chronic inflammatory diseases.

Proteinase 3 (PR3) is found at the surface of the plasma membrane (Baggiolini, Bretz, Dewald, & Feigenson, 1978). It is know that its expression at the membrane is a risk factor for vasculitis, rheumatoid arthritis and Wegener Granulomatosis (Witko-sarsat et al., 1999; Woude et al., 1985). PR3 has been identified as a diagnosis marker in Wegener granulomatosis because it is recognized by anti-neutrophil cytoplasmic antibody (ANCAs) and is now acknowledged to be the preferred target of ANCAs (Lüdemann, Utecht, & Gross, 1990).

**Prediction of PR3 IBS**

Computational studies on PR3 and implicit membrane model, mimicking the surface of the lipidic surface, have predicted a membrane binding site of PR3 to the plasma membrane. This simplistic model shows that the interfacial binding site (IBS) is made of basic and hydrophobic amino acids act jointly to respectively orient and anchor PR3 at the surface of the plasma membrane (Hajjar, Mihajlovic, Witko-Sarsat, Lazaridis, & Reuter, 2008). The involvement of these amino acids have been confirmed by ?*in silico*? mutagenesis experiments, where mutations of the four hydrophobic (F180, F181, L228, F229) or four basic (R193, R194, K195, R227) amino acids abrogated the membrane anchorage of PR3 (Kantari et al., 2011). All-atom molecular dynamics simulations pointed out the detailed mechanisms of interaction between PR3 and lipid bilayers and showed that the identified basic residues interacts via hydrogen bonds with the lipid headgroups to stabilize PR3, hydrophobic residues insert into the hydrophobic core below the carbonyl groups of the lipid bilayer, and aromatic residues contribute to electrostatic interactions via cation π interaction with the choline group of phosphocholine (Broemstrup & Reuter, 2010). Computational studies are so far supporting the hypothesis of a direct binding.

2. Material and Methods

2.1 Molecular dynamics simulations

We performed MD simulations of PR3 and HNE inserted in POPC lipid bilayers. The main steps of the procedure are the following: (1) equilibration of the lipid bilayer, (2) insertion of PR3 in the lipid bilayer and (3) simulation of the PR3-POPC complex and subsequent analysis of the resulting trajectories.

In this manuscript we consequently use the chymotrypsin numbering for both PR3 and HNE. It presents the advantage providing a consistent numbering for all enzymes of the family but introduces letters in addition to the numbering (e.g the two consecutive arginines labelled 186A and 186B).

**POPC bilayer.** A lipid bilayer made of 256 POPC was built using the CHARMM-GUI (Jo, Lim, Klauda, & Im, 2009). The lipid bilayer was subjected to energy minimization using NAMD (Phillips et al., 2005) and the CHARMM36 force field (Klauda et al., 2010). The system was then equilibrated for 300 ps at 310 K using a time step of 2 ps, and subsequently run into production for 60 ns. The SHAKE algorithm was applied to constraint bonds between a heavy atom and a hydrogen (Andersen, 1983). Non-bonded interactions were truncated using a cutoff of 12 Å, using a switch function for van der Waals and a shift function for electrostatics. For estimating long-range electrostatic forces, the Particle-Mesh-Ewald (PME) algorithm was used (Darden, York, & Pedersen, 1993; Essmann et al., 1995). The Langevin algorithm was used to control temperature (310K, damping coefficient: 10/ps) and pressure (target pressure: 1 atm, oscillation period: 75 fs, oscillation decay time: 25 fs) (Feller, Zhang, & Pastor, 1995). The area per lipid and the order parameter were monitored along the simulation to assess the properties of the bilayer. The order parameter SCD was calculated with VMD from the average value of the angles between each C-H bond of the lipid tails and the normal to the membrane. The profiles are consistent with those in (Klauda et al., 2010) The surface area was calculated to be 65.5 ± 0.8 Å on average during the simulation (cf. Fig. XX in Supporting Information). Klauda et al. (Klauda et al., 2010) report a surface area of 64.7 ± 0.2 Å for a simulated POPC bilayer using the CHARMM 36 force field, while Kucerka et al. report an estimate of 68.3 ± 1.5 Å using hybrid electron density models WHICH METHOD? (Kucerka, Tristram-Nagle, & Nagle, 2005).

**Insertion of Proteinase 3 and HNE at the interface of the lipid bilayer.** The cartesian coordinates of PR3 were taken from chain A of the crystal structure referenced 1FUJ in the Protein Data Bank (Fujinaga, Chernaia, Halenbeck, Koths, & James, 1996), and those of HNE from the 1PPF structure (Bode, Wei, Huber, & Meyer, 1986). PR3 was then oriented with respect to and inserted at the interface of the equilibrated POPC lipid bilayer as described previously (Broemstrup & Reuter, 2010). Briefly, each of the enzymes was positioned at the surface of a POPC lipid bilayer in the orientations predicted by implicit bilayer simulations (Hajjar et al., Proteins) and all-atoms simulations with a DMPC bilayer (PR3: Broemstrup et al., PCCP; HNE: unpublished data). They were then translated 2 Å above their initial position to account for the difference in width between POPC and DMPC bilayers. Six lipids overlapping with the protein were removed, in the case of PR3 and HNE, respectively.

**Simulations PR3-POPC and HNE-POPC.** The system was then solvated in a cubic box of TIP3 water and neutralized with VMD (version 1.8.7) (Humphrey, Dalke, & Schulten, 1996) using chloride anions. The system was then minimized with CHARMM (v33b1) (Brooks et al., 2009) using harmonic restraints on the protein backbone with first the steepest descent and then the conjugate gradient algorithms. The system was subsequently equilibrated for 600 ps with NAMD (Phillips et al., 2005). Pressure and temperature control, as well as the cutoff scheme and treatment of long-range electrostatics interactions are the same as for the equilibration of the bilayer. The integration of the equations of motion was done using a Multiple Time Step algorithm (Izaguirre, Reich, & Skeel, 1999); bonded interactions and short-range nonbonded forces were evaluated in every step and long range electrostatics every second step. The system was then run into production for 500 ns in the NPT ensemble.

**Analysis.** We calculated the root mean square deviation (RMSD) along the simulation (Cf. Figure xx in the Supporting Information). The RMSD for PR3 first stabilizes at xx ns, and at yy ns for HNE. For the sake of consistency between the two simulations we thus used the window between xx ns and yy ns as sampling window. All analyses described in the results section are performed on the sampling window.

Hydrogen bonds were calculated with Charmm (Brooks et al., 2009) using a 2.4 Å cutoff distance between hydrogen and acceptor and a 130° donor-hydrogen-acceptor angle criterion. The donor and acceptor definition are taken from the Charmm force field (MacKerell, et al., 1998).Hydrophobic contacts were calculated using a 3 Å cutoff distance between the aliphatic group of the lipids (Charmm atom types: ) and the enzymes (Charmm atom types ca; cb; cg1; cg2; cg2; ha\*; hb\*; hg; hg2\*; type cg except for hsd, hse, asn, asp; type hg1 except for cys, thr, ser; type cd except for arg, gln, glu; type cd1; type cd2 except for hsd, hse; type ce1, ce2, cz and associated hydrogens of phe, tyr, type cd1, cd2, ce2, ce3, cz2, cz3 and associated hydrogen of trp, type cay and type hy\*). Cation-π interactions between aromatic rings (phenylalanine, tyrosine and tryptophan) are considered to exist when all distances between the atoms of the aromatic ring and choline nitrogen are below 7 Å and when these distances do not differ by more than 1.5 Å (Chipot & Minoux, 1999; Petersen, Jensen, & Nielsen, 2005).

We evaluated the depth of anchorage of the proteins as described in Grauffel et al. (Grauffel, J Am Chem Soc). Briefly we used the average z coordinate of the phosphorus atoms as a reference plane. The center of mass of each residue was calculated and its difference to the reference plane was calculated. The corman module of Charmm program was used for coordinates statistics. Values reported are averages of the distances of the last 300 ns of simulations.

2.2 Sample preparation.

**Proteins.** PR3 and HNE were purchased from Athens Research & Technology and fatty acid free bovine serum albumine (BSA) was obtained from Sigma.

**Liposomes.** The lipids (POPC) were purchased from Avanti Polar Lipids. Liposomes were prepared as reported in (Jr, Ying, Baumann, & Kleppe, 2009). Lipids solvated in chloroform were added in glass tubes in the prerequisite amount. Lipids were handled and kept out of light and reactive atmosphere as much as possible by operation in hoods, flushing reagent bottles with dry N2, and using glass containers wrapped in aluminum foil. The chloroform solutions were dried under dry N2 pressure. Traces of chloroform were removed by subjecting the samples to vacuum for at least two hours. Lipid cakes were rehydrated with HBS-N buffer and vortexed vigorously until all films were suspended as slurry. For liposomes preparation, solutions were subjected to seven freeze-thaw cycles using liquid N2 and a warm water bath. The hydrated multilamellar structures were then extruded at room temperature and well above the lipid Tm using a Mini-Extruder (Avanti Polar Lipids) assembled using two Millipore filters of 100 nm pore size. Samples were forced thought the filters 10 times using Hamilton syringes and the resulting solution were transferred to clean, foil wrapped glass tubes and stored at 4°C. Final liposome composition was 100 % POPC and the total lipid concentration was 2.5 mM.

2.3 Surface Plasmon Resonance

The SPR analyses were carried out on a BIAcore T200 (BIAcore, GE Healthcare) and Biacore T200 Control Software. All experiments were carried at 25 °C. Protein and lipid interactions were monitored using a L1 sensor chip. A preparation procedure was performed before each experiment. The surface of the L1 sensor chip was first cleaned with a 1 min injection of octylglucosyl 40 mM at a flow rate of 10 μl/min. Liposome solutions were diluted to 1 mM concentration with running buffer (HBS-N: 0.1 M HEPES, 1.5 M NaCl, pH 7.4) and injected at a flow rate of 1 μl/min for 10 minutes until the maximum of binding was reached. Liposomes maximum deposition was 8500 RU for POPC. The surface of the L1 chip was then washed with a solution of NaOH 10 mM for 1min at a flow rate of 10 μl/min. The the completeness of the chip ocverage was assessed by injection of BSA 0.1 mg/ml at 10 μl/min for 60 s. Generally, this injection did not perturb the lipid-covered chip by more than 12.6 RU, and rapidly fell back to its original value when injection of BSA stopped. Binding assays were then performed on the validated chips. The two proteins (PR3 and HNE) were diluted to sets of at least 5 different concentrations ranging from 0 to 3 μM (0, 0.125, 0.5, 1, 2, 3) and were injected over the immobilized liposomes at a flow rate of 5 l/min during 120 s to 180 s until the equilibrium was reached. The dissociation phase were measured for at least 480 s after the addition of the sample. At the end of the binding assay, the surface of the sensor chip was regenerated with a solution of octylglucosyl 40 mM for 30s at a flow rate of 30 μl/min. No reference channel was used due to non specific binding and maximal coverage of the chip with liposome was achieved to fully cover the chip. The SPR data were analyzed with the Biacore T200 Evaluation Software. Binding affinities were calculated using the steady state affinity model (Langmuir model) and maximal resonance unit (RU) was plotted against concentration.

3. RESULTS

3.1 Molecular dynamics simulations

The simulations of PR3 and HNE positioned in the interfacial region of POPC lipid bilayers were analysed in order to characterize the interactions between their interfacial binding sites and the lipids. We report in Tables 2 and 3 the occupancy of significant hydrogen bonds along the sampling window (occupancy above 20%), as well as the average number of hydrophobic contacts for the amino acids that achieve on average more than one contact per frame of the trajectory.

**3.1.1. PR3**

Interactions between PR3 and the POPC bilayer are mediated almost exclusively by amino acids located on three different loops: 8-9 (163-180), 9-10 (184-197), 11-12 (215-225).

Most of the hydrogen bonds we observed involve the phosphate groups of POPC lipids. A low number of hydrogen bonds with occupancies below 20% involve glycerol groups. The only one with occupancy above our threshold involves Arg186A (20.1 %) and indicates that Arg186A is buried somewhat deeper in the interface than the other basic amino acids. The strongest hydrogen bonds involve basic amino acids (R177, R186A, R186B and K187) and have occupancies above 80%. Remarkably Lys187 is involved in hydrogen bonds through its side chain (74.5%) and backbone (90.4%). We have earlier predicted, using simulations with an implicit membrane model and mutagenesis experiments (Kantari et al., J Leukoc Biol), that R186A, R186B, K187 and R222 play a major role in PR3 interaction with cell membranes. In particular mutating these four amino acids into four alanines would abrogate PR3 membrane expression in Rat Basophil (RBL) cells. Further a 100 nanoseconds-long MD simulation of PR3 anchored on a DMPC bilayer, with an all-atom description of the system, show that R177, R186A, K187, R222 mediate the strongest hydrogen bonds with the phosphate groups of the lipids, while R186B contributes to a lower extent (Broemstrup et al, PCCP). Our results seen in light of previous work confirms the importance of this cluster of basic amino acids constituted of four arginines (177, 186A, 186B, 222) and one lysine (187) even though we only observe a low occupancy for Arg222 (occupancy of 8.6% with glycerol groups and 8.8% with phosphate groups) in the current simulation. Besides these, lysine 99 (K99) reported to be important for ligand binding (Hajjar et al., FEBS J) mediates hydrogen bonds with the lipids. It is also the case of F166 and W218 although they mediate interactions via their backbone atoms while their sidechains are heavily involved in hydrophobic contacts with the lipid tails.

We calculated the average number of hydrophobic contacts per frame along the sampling window (Cf. Table 3). Several amino acids of the predicted interfacial binding site display hydrophobic contacts with the POPC lipid bilayer. As expected these are aromatic (F165, F166, W218, F224) and hydrophobic amino acids (V163, T221, L223 and P225). Among these V163, F166, L223 have particularly high average number of contacts (2.3, 2.5 and 5.8, respectively). Simultaneous mutations of the four amino acids F165, F166, L223 and F224 did impair membrane expression of PR3 on RBL cells (Kantari et al., J Leukoc Biol). Interestingly the basic cluster involved in strong hydrogen bonding (R177, R186A, R186B, K187) is also involved in hydrophobic contacts with the lipid tails. In agreement with its involvement in hydrogen bonds with POPC glycerol groups, Arg186A is the basic amino acid with the higher number of hydrophobic contacts. The three aromatic residues F165, F166 and W218 have the highest bond lifetime percentage (above 75 %). These are the most embedded within the lipid bilayer. (I AM NOT SURE I UNDERSTAND WHAT YOU MEAN HERE).

While we observed strong cation-p interactions between W218 and DMPC lipids in our previous work, the occupancy of this interaction is of only 5.7 % in the present simulation, which we do not consider as being significant.

The position of the amino acids with respect to the average plan of the phosphorus atoms gives an indication of their depth of anchorage in the lipid bilayer. Two loops are anchored significantly beyond the phosphorus atoms; loop 11-12 appears to be the one that has the deepest anchorage, while the 8-9 loop with F165 and F166 also is anchored deeper than the plan of the phosphorus atoms. The 9-10 loop carries the basic cluster and its positioned slightly above the two other ones.

**3.1.2. HNE**

Using an implicit membrane model, we previously predicted that HNE, if it binds to membranes, would do so using the same interfacial binding site as PR3. We therefore inserted HNE in the POPC membrane similarly to Proteinase 3; using the same orientation and the same depth of anchoring. (HOW DOES THE DEPTH VARY ALONG THE MD FOR PR3 AND HNE?). With the implicit membrane model, we also observed a higher electrostatic contribution than in the case of PR3 and fewer contributions from hydrophobic amino acids.

HNE is stable on the POPC bilayer (RMSD?) and most of the interactions with POPC lipids are achieved by amino acids carried by the same three loops as PR3 (8-9, 9-10, 11-12). Unlike for PR3 two other loops interact with the lipids: 7-8 with R146 and the loop that links both barrels, 6-7, with R129 and N132. The valine numbered 97 (V97, 5-6), very close in sequence to K99 from PR3, is also involved in interaction with the lipids. Interestingly the amino acid L99, equivalent of PR3 K99 is not involved in any interaction.

Hydrogen bonds between HNE and the lipids are mediated by more amino acids than in the case of PR3 but only two of these have occupancies above 70%. (WE NEED TO CHECK HOW THESE EVOLVE DURING THE SIMULATION)

Only a few amino acids are involved in hydrophobic contacts (V97, S165, L166, R178 and L223), and only Leu 223 is anchored below the average level of the phosphate plane.

All together these results indicate that HNE interacts with the bilayer using mostly hydrogen bonds and very few hydrophobic anchors, suggesting a looser binding to lipid membranes than PR3.

## 3.2 Surface Plasmon Resonance

To experimentally verify the hypotheses resulting from the MD simulations, we conducted SPR assays to compare the affinity of PR3 and HNE for large unilamellar vesicles (LUVs) constituted of POPC lipids.

**Liposome immobilization.**

Liposomes were immobilized on the surface of the L1 sensor chip at a low flow rate (1 μL·min-1) until the maximal amount of deposition was reached. Liposome immobilization levels were monitored over time and the immobilization levels for POPC LUVs were 8563 ± 243 RU (Cf. Table 1 KEEP?) ADD HOW MAY EXPERIMENTS HAVE BEEN CONDUCTED. To avoid non-specific binding of proteins to the surface of the L1 chips, special care was taken on covering the chip surface at the highest possible levels of liposomes. The level of the coverage sensor chip was assessed with BSA injections (0.1 mg·ml-1). Resulting signals from BSA around 100 RU or less indicates a sufficient coverage (REF). In our case, BSA binding amounts to around 12 RUs (PROVIDE INSTEAD AN AVERAGE VALUE AND DEVIATIONS) and allowed us to pursue experiments further with POPC.

**Binding of PR3 to POPC LUVs**

We investigated the interaction of PR3 with neutral liposomes made of POPC using SPR. Liposomes were immobilized on the surface of the L1 sensor chip as described above. Binding assays were performed by injecting protein samples at increasing concentration and affinity calculations were carried out by steady state analysis. We monitored the association phase for 180 seconds and the dissociation phase for 420 seconds. Sensorgrams show that the protein response is concentration dependent and is reaching equilibrium towards the end of each injection (Figure 2). The calculated KD between PR3 and POPC is 9.24 10-7 M. During the dissociation phase, we also observed that the response signal of PR3 does not return to zero and thus demonstrates a persistent binding of PR3 to the liposomes. These results support the hypothesis of a direct binding of PR3 towards neutral liposomes (POPC).

**Binding of HNE to POPC LUVs**

The binding of HNE towards POPC was monitored in the same way as PR3. The association of the protein to the LUVs was monitored for 120 s (shorter than for PR3) and the dissociation for 420 s. The sensorgrams are presented on Figure XX and show that HNE can bind to liposomes made of POPC in a concentration-dependent manner which indicates a direct binding of the protein to the liposomes. During the dissociation phase, the signal drops immediately and returns to zero. This is in contrast to the behavior of PR3, and indicates that HNE lacks membrane-anchoring residues that are involved in slowing dissociation in PR3 (KEEP FOR DISCUSSION?). The kinetics of the protein-membrane interaction seems to be different for the two proteins. For the KD calculation, the data collected for HNE clearly shows that the equilibrium was not reached even at XXX (WRITE CONCENTRATION HERE). It was therefore not possible to calculate the affinity accurately. Yet, from these experiments, we can calculate a lower limit for the KD value of approximately 3.4 μM.

DISCUSSION (TODO)

**The direct binding of PR3 to lipid vesicles indicates that it might bind directly to the surface of neutrophils**

How PR3 interacts with the plasma membrane of the neutrophils remains in facts a controversial subject. Several studies identified potential protein partners of PR3 at the membrane, and among these are CD177 (NB1) (Hu et al., 2009; von Vietinghoff et al., 2007), Fcgamma receptor FcgRIIIb and p22phox subunit of cytochrome b558 (Alina David, Fridlich, & Aviram, 2005), β2 integrin adhesion molecule CD11b/CD18 (A David, Kacher, Specks, & Aviram, 2003), Protease Activated Receptor 2 (PAR2) (Jiang et al., n.d.; Kuckleburg & Newman, 2013) and Phospholipid Scramblase 1 (Kantari et al., 2007). The modalities of the membrane expression of PR3 are also depending on how neutrophils themselves are stimulated. A study made on cells has shown by quantitative fluorescence microscopy that PR3 is expressed at the surface of primed and activated neutrophils and that the level of PR3 membrane expression varies depending on which agonist is used to stimulate the neutrophils (Campbell, Campbell, & Owen, 2000). In addition to these experiments conducted on cellular models, a direct interaction between PR3 and liposomes has been reported by Goldmann et al. (Goldmann, Niles, & Arnaout, 1999). They studied the interactions between purified human PR3 with mixtures of zwitterionic and anionic reconstituted lipid bilayers using differential scanning calorimetry and lipid photolabeling and showed a direct interaction of PR3 with liposomes with dissociation constant (KD) in the micromolar range.

CONCLUSION

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FIGURE CAPTIONS

**Figure 1:** structural alignment of PR3 and HNE

**Figure 2:** Binding assay of PR3 and HNE to LUVs from Surface Plasmon Resonance. PR3 and HNE binding responses (left) and affinity data (right) over immobilized POPC. All data are blank substracted. No double referencing has been done due to high non specific binding to the reference channel (L1 chip with no liposomes – data not shown).

**Figure 3:**

TABLES

**Table 1:** LUVs immobilization levels and chip coverage accession by BSA binding (BSA is used at 0.1 mg/ml and is injected 60 s at 60 l.min-1). Values are the mean of four experiments.

|  |  |  |
| --- | --- | --- |
| Liposome type | Immobilization level (RU) | BSA binding level (RU) |
| POPC | 8669 ± 95 | 43 ± 2.6 |

**Table 2:** KD determination of PR3 and HNE with POPC LUVs. Values are the mean of six experiments for PR3 and four for HNE

|  |  |  |
| --- | --- | --- |
|  | KD (x10-7 M) | |
|  | PR3 | HNE |
| POPC | 9.22 ± 0.4 | 33.4 ± 4 |

**Table 3.** Anchorage of PR3 in a POPC lipid bilayer: inventory of interactions and depth of anchorage.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Amino acid | Depth *a* (Å) | Hydrophobic *b* | Hydrogen bond *c* (%) | Cation π *e*(%) |
| K99 | - 11.2 ± 2.1 |  | 20.1 |  |
| V163 | - 2.5 ± 1.6 | 2.3 |  |  |
| T164 | - 1.7 ± 1.6 |  | 45.6 |  |
| F165 | + 1.8 ± 1.5 | 1.5 |  |  |
| F166 | + 1.7 ± 1.6 | 2.5 | **28.1** |  |
| R177 | - 4.0 ± 1.6 | 1.1 | 87.9 |  |
| R186A | + 0.7 ± 2.1 | 4.2 | 85.9 |  |
| R186B | - 1.3 ± 2.2 | 1.5 | *20.1d/*82.9 |  |
| K187 | - 0.2 ± 2.4 | 1.6 | **90.4/**74.5 |  |
| F215 | - 8.6 ± 2.0 |  |  | 5.9 |
| W218 | + 0.8 ± 2.3 | 1.9 | **26.6** | 5.7 |
| T221 | + 0.1 ± 2.1 | 1.6 |  |  |
| L223 | + 3.5 ± 1.9 | 5.8 |  |  |
| F224 | + 1.2 ± 1.8 | 1.2 |  |  |
| P225 | - 1.6 ± 1.7 | 1.9 |  |  |

*a* Positive values indicate that the center of mass of the amino acid is buried in the bilayer beyond the plane defined by the phosphate groups. *b* Average number of hydrophobic contacts per frame (listed if above 1). *c* Occupancies of hydrogen bonds with POPC phosphate groups in % (if > 20; bold numbers are for hydrogen bonds involving the protein backbone) . *d* Hydrogen bond between Arg186B and POPC glycerols.  *e* Occupancy of cation-π adducts (if > 5%).

**Table 4.** Anchorage of HNE in a POPC lipid bilayer: inventory of interactions and depth of anchorage.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Amino acid | Depth *a* (Å) | Hydrophobic *b* | Hydrogen bond *c* (%) | Cation π *d*(%) |
| V97 | ? | 1.8 |  |  |
| R129 | -12.6 ± 4.9 |  | 34.8 |  |
| N132 | - 7.0 ± 3.2 |  | 25.0 |  |
| R146 | - 8.7 ± 4.9 |  | 59.1 |  |
| T164 | - 4.8 ± 2.4 |  | 25.7 |  |
| S165 | -2.12 ± 1.9 | 1.3 | 24.0 |  |
| L166 | -0.67 ± 1.7 | 6.7 |  |  |
| R177 | - 6.7 ± 2.3 |  | 59.8 |  |
| R178 | -4.91 ± 2.4 | 1.9 | **37.4/**85.6 |  |
| G186A | -4.4 ± 2.4 |  | **54.8** |  |
| R186B | -4.1 ± 1.9 |  | 73.4 |  |
| F192 | 14.9 ± 3.9 |  |  | 13.2 |
| R217 | -11.6 ± 4.3 |  | 55.9 |  |
| S221 | -4.0 ± 3.0 |  | 32.8 |  |
| G222 | -1.9 ± 2.2 |  | **28.4** |  |
| L223 | +0.3 ± 2.1 | 6.8 |  |  |

*a* Positive values indicate that the center of mass of the amino acid is buried in the bilayer beyond the plane defined by the phosphate groups. *b* Average number of hydrophobic contacts per frame (listed if above 1). *c* Occupancies of hydrogen bonds in % (if > 20; bold numbers for backbone hydrogen bonds). *d* Occupancy of cation-π adducts (if > 5%).

FIGURE 1

FIGURE 2

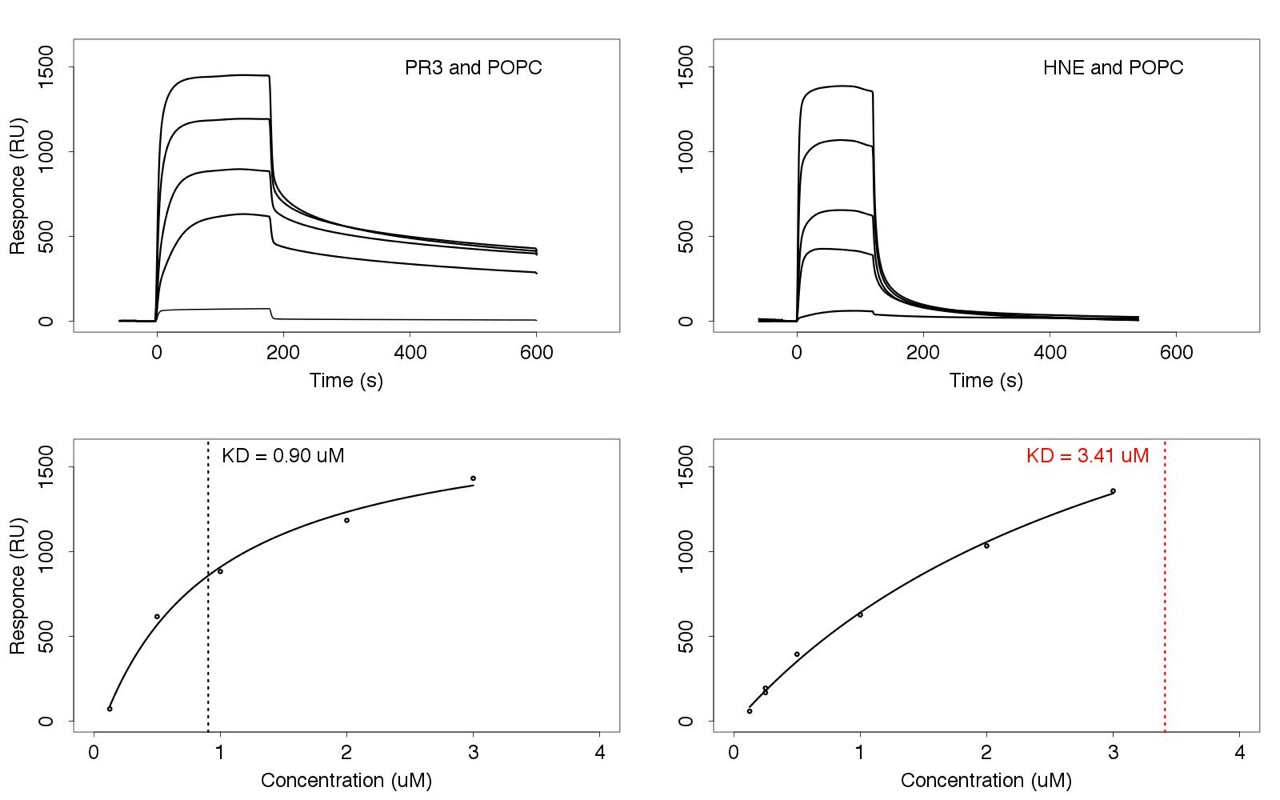


FIGURE 3

Images of the systems simulated, with a zoom on the interface showing the most important amino acids highlighted (the ones making the strongest interactions as obtained from the MD)

HIGHLIGHTS

(3 to 5 bullet points that convey the core findings of the article)

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GRAPHICAL ABSTRACT