| **Reliability and reproducibility checklist for molecular dynamics simulations**<br>***All boxes must be marked YES by acceptance unless an N/A option is available** | **Yes** | **N/A** | **Response**<br>**(Please state where this information can be found in the text)** |
|---|---|---|---|
| **1. Convergence of simulations and analysis** | | | |
| 1a. Is an evaluation presented in the text to show that the property being measured has equilibrated in the simulations (*e.g.* time-course analysis)? | ☒ | | Supplementary Figures |
| 1b. Then, is it described in the text how simulations are split into equilibration and production runs and how much data were analyzed from production runs? | ☒ | | Methods |
| 1c. Are there at least 3 simulations per simulation condition with statistical analysis? | ☒ | | Supplementary Figures 18, 24 and 33 |
| 1d. Is evidence provided in the text that the simulation results presented are independent of initial configuration? | ☒ | | Supplementary Figures 18, 24 and 33 |
| **2. Connection to experiments** | | | |
| 2a. Are calculations provided that can connect to experiments (*e.g.* loss or gain in function from mutagenesis, binding assays, NMR chemical shifts, J-couplings, SAXS curves, interaction distances or FRET distances, structure factors, diffusion coefficients, bulk modulus and other mechanical properties, *etc*.)? | ☒ | | Results |
| **3. Method choice** | | | |
| 3a. Is it described in the text what force field and water model are used and why? | ☒ | | Methods |
| 3b. Do simulations contain membranes, membrane proteins, intrinsically disordered proteins, glycans, nucleic acids, polymers, or cryptic ligand binding? | ☐ | ☒ | Response not needed if **N/A** |
| If 3b is **YES**, are enhanced sampling methods used? | ☒ | ☐ | Enhanced sampling is used to obtain potential energy surfaces for the R142 side chain in the presence and absence of the aspartyl-AMP ligand. |
| If enhanced sampling methods are used, are the convergence criteria clearly stated? | ☒ | | Convergence data are shown in Supplementary Figures 35 and 36. A script for analyzing the enhanced sampling data (uploaded into the Zenodo repository) is provided as Supplementary Data 2 |
| If 3b is **YES**, is it explained in the text why or why not enhanced sampling methods are used? | ☒ | | Results and Supplementary Fig. 19 |
| **4. Code and reproducibility** | | | |
| 4a. Is a table provided describing the system setup, such as simulation box dimensions, total number of atoms, total number of water molecules, salt concentration, lipid composition (number of molecules and type)? | ☒ | | Source data |
| 4b. Is it described in the text what simulation and analysis software and which versions are used? | ☒ | | A list of the software packages used to perform |

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| | | | | the MD simulations, and to make Supplementary Movies 11-14, is provided in the Reporting Summary |
| 4c. Are initial coordinate and simulation input files and a coordinate file of the final output provided as supplementary files or in a public repository? | | | ☒ | | All MD coordinate and trajectory files are available the Zenodo repository, as shown in Supplementry Table 8. |
| 4d. Is there custom code or custom force field parameters? | | | ☒ | ☐ | Custom parameters were used for the β-aspartyl-AMP ligand |
| | If **YES**, are they provided as supplementary profiles or in a public repository? | | ☒ | | These custom parameters are provided as Supplementary Data 1 |