

Supporting Information

1 Phosphorylation

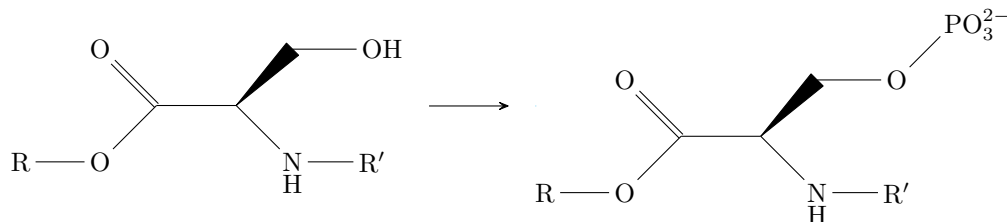


Figure 1: Phosphorylation of Serine

2 Enzymes phosphorylating AS

PLK2	GKMYACKR [...]	QIVSGLKYLHEQEILHRDLK
CK1	GEEVAIKL [...]	QMISRIEYIHSKNFIHRDVK
CK2	NEKVVVKI [...]	EILKALDYCHSMGIMHRDVK
GRK5	NKVYAAKI [...]	EILCGLEDLHHENTVYRDLK
LRRK2	GEEVAVKI [...]	HVADGLRYLHSAMIIYRDLK
	.: . *	.: : * :***:

Figure 2: Sequence comparison of the binding site and active site of PLK2, CK1, CK2, GRK5 and LRRK2: in the last row "*" indicates the same residue in all chains, ":" indicate strongly similar residues across all chains and "." indicates weakly similar residues.

The enzymes phosphorylating AS include: CK1 [1], CK2 [2], GRK5 [3] and LRRK2 [4] (see Fig. 3). The binding sites and/or the region around them in the first three are positively charged, as already observed [3][5]. The active site of LRRK2 is instead negatively charged. Thus, we may expect a similar electrostatic steering of AS for S129 phosphorylation in all of them but LRRK2.

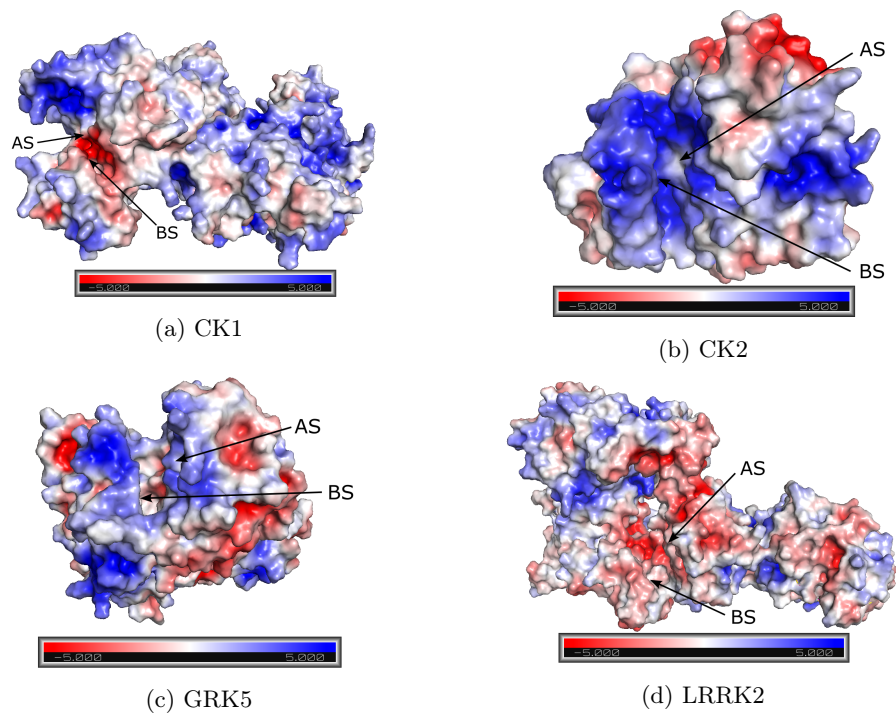


Figure 3: CK1 (a)[1], CK2 (b)[2], GRK5 (c)[3] and LRRK2 (d)[4] with indications for the binding site (BS) and active site (AS).

3 Prevalence of clusters

Table 1: Percentage of occurrence of the ten most representative structures of non-phosphorylated AS and pAS in the DES-Amber simulation.

Number	AS [%]	pAS [%]
1	7.6	6.9
2	5.5	5.0
3	3.4	3.8
4	3.2	3.5
5	2.9	3.3
6	2.8	2.5
7	2.7	2.4
8	2.3	2.3
9	2.3	2.2
10	2.3	1.8

4 AMBER a99SB-*disp* force field results

Here we present simulations using the AMBER a99SB-*disp* force field for comparison with those based the newly developed force field by Piana et al.[6]

Table 2: Number of atoms in the simulation box for the three systems simulated here. The TIP4P-D water model (used by Robustelli et al. [7] and us together with the a99SB-*disp* force field) uses a forth dummy atom to simulate water, thus the total number of atoms including dummy atoms is given in brackets.

	Protein	Water	Sodium	Chlorine
AS	2020	68370 (91160)	75	65
pASH	2024	79431 (105908)	86	75
pAS	2023	68361 (91148)	77	65

Table 3: Calculated properties of AS and pAS using the a99SB-*disp* force field. (i) Gyration (R_g) and hydrodynamic R_H radii of the entire proteins and of the NAC regions. The experimental value of R_H is 28.7 Å [8]. (ii) Average number of hydrogen bonds. (iii) Average number of salt bridges. Standard deviations are indicated in parenthesis.

	R_g [Å]	R_H [Å]	$R_g(\text{NAC})$ [Å]	$R_H(\text{NAC})$ [Å]	N_{HB}	N_{SB}
AS	27.6 (± 5.7)	30.8 (± 3.5)	16.7 (± 5.7)	22.8 (± 5.2)	21.51 (± 4.19)	2.86 (± 1.96)
pAS	29.3 (± 4.5)	31.8 (± 2.7)	18.7 (± 7.6)	24.4 (± 6.4)	22.13 (± 4.18)	1.85 (± 1.62)

Table 4: Average solvent accessible surface areas (SASA) in AS and pAS in the NAC-Region and N- and C-terminus using the a99SB-*disp* force field.

	SASA _N [nm ²]	SASA _{NAC} [nm ²]	SASA _C [nm ²]
AS	0.87 (± 0.38)	0.83 (± 0.40)	1.07 (± 0.34)
pAS	0.86 (± 0.46)	0.85 (± 0.39)	1.07 (± 0.39)

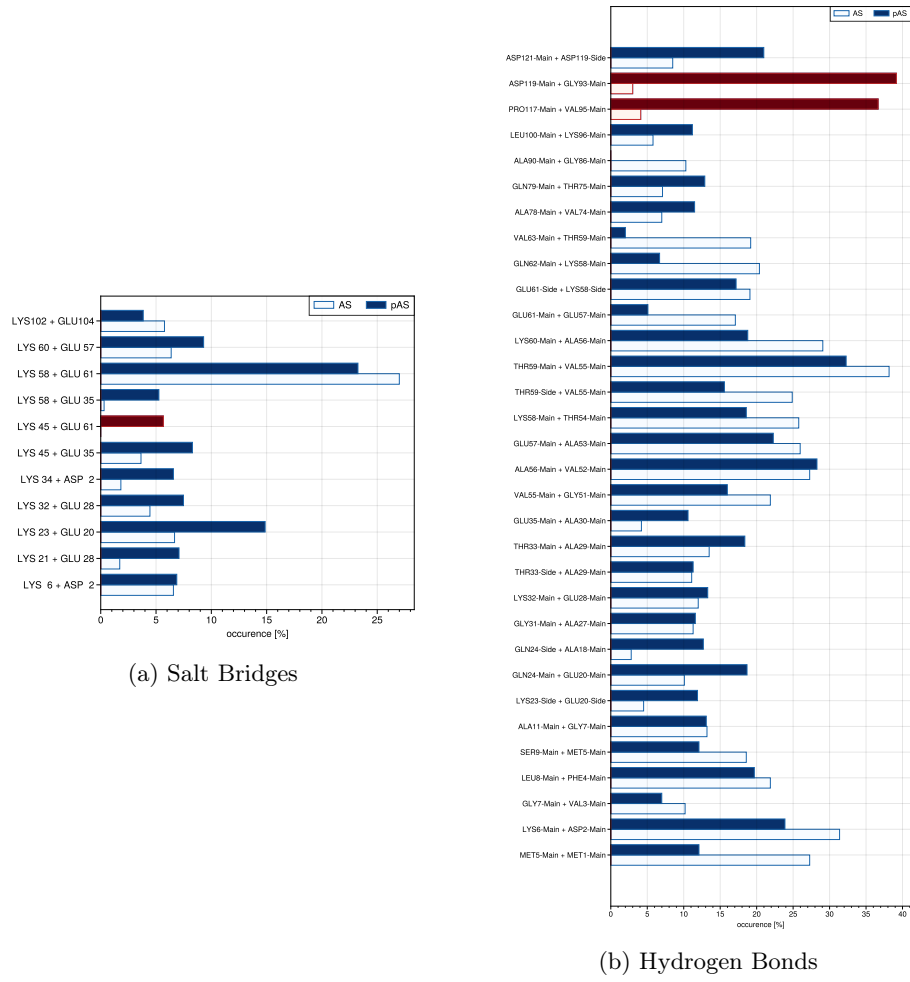


Figure 4: Occurrence of intradomain (blue) and interdomain (red) salt bridges (a) and H-bonds (b) in AS and pAS using the a99SB-*disp* force field.

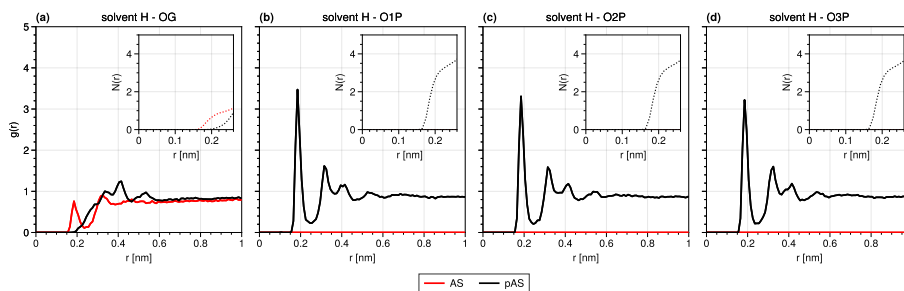


Figure 6: RDF ($g(r)$) of the oxygen atoms of residue 129 and water hydrogens (a-e) using the a99SB-*disp* force field. Compare the RDF for pAS (black) and AS (red) for the oxygen atom in the sidechain (a) and the carbonyl oxygen (e). (b)-(d) illustrate the RDF for the terminal phosphoryl oxygens.

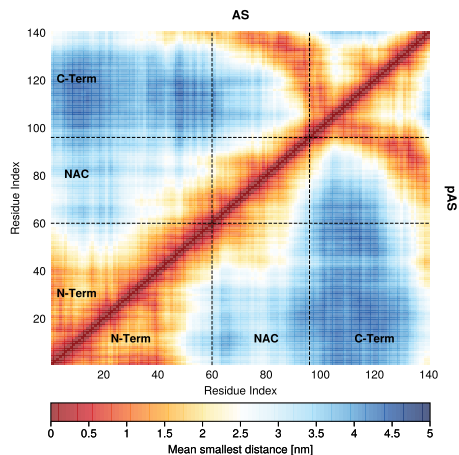


Figure 5: Contact maps in AS (triangle above) and pAS (triangle below) using the a99SB-*disp* force field.

5 Convergence tests

This section investigates the convergence of the simulations of the a99SB-*disp* simulations analogous to Rossetti et al.[9] The running time averaged values of chemical shifts and secondary structure percentages were analyzed for AS (Figure 7), pASH (Figure 8) and pAS (Figure 9).

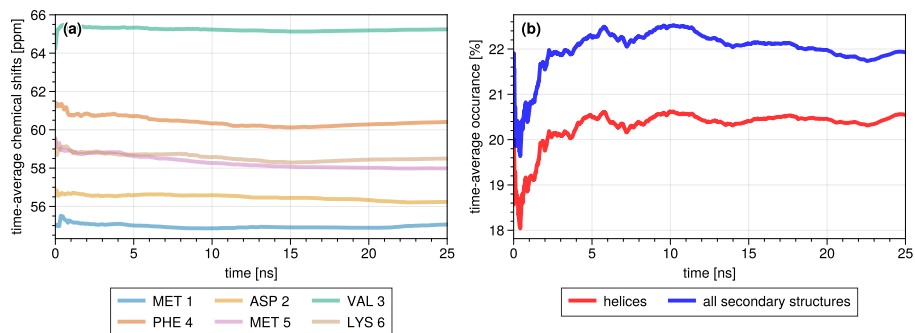


Figure 7: AS (a) running time averaged chemical shifts of the C α atoms of the first six residues; (b) running time averaged occurrence of secondary structures (red) and helices (blue) as percent of all residues.

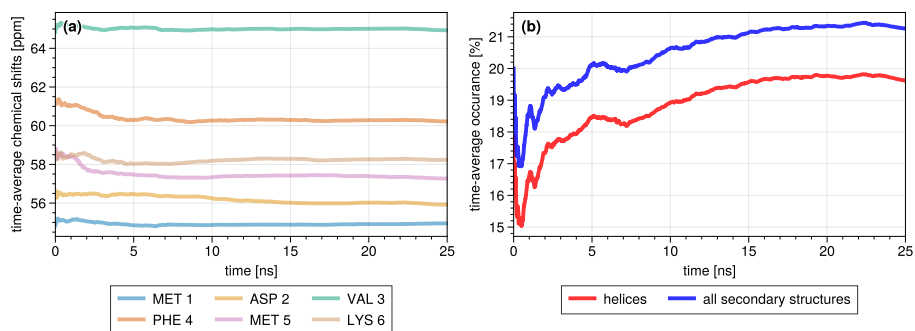


Figure 8: pASH (a) running time averaged chemical shifts of the C α atoms of the first six residues; (b) running time averaged occurrence of secondary structures (red) and helices (blue) as percent of all residues.

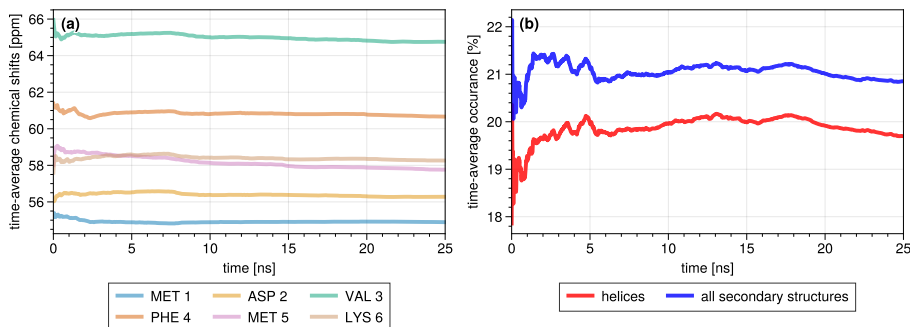


Figure 9: pAS (a) running time averaged chemical shifts of the C_{α} atoms of the first six residues; (b) running time averaged occurrence of secondary structures (red) and helices (blue) as percent of all residues.

6 Comparison with experimental data

The properties were calculated from the trajectories between 12 and 25 ns. This section covers both the DES-Amber and the a99SB-*disp* force field simulations.

NMR properties For each residue in the protein, the chemical shifts of C_{α} , C_{β} , N (backbone) and H (backbone) atoms were calculated using ShiftX2 [10]. Comparison was made with the experimental values in ref. [11] (Figures 10 and 11, tables 5 and 6).

The prediction of the ^{13}C -NMR chemical shifts of C_{α} , C_{β} is excellent, even better than that reported in Ref. [9] (from a correlation of 0.86 and 0.94 when only using the first 12 residues) for the all residues. The ^{14}N -NMR shows also good a correlation, 0.931 or 0.951, with the experiment. The prediction of ^1H -NMR atoms is less accurate but still acceptable (correlation of 0.295 for a99SB-*disp* and 0.487 for DES-AMBER), as seen in [9].

CD spectra The calculated CD spectrum of AS (Fig. 12a) is in fair accord as that experimentally measured [8]. The experimental minimum is at a 10 nm lower values than the theoretical value (198 nm and 208 nm, respectively), similarly to what found in ref. [9].

The minima of the calculated spectra range up to $-15 \cdot 10^{-3} \text{ deg cm}^2 \text{ dmol}^{-1}$ for specific conformations, similarly to what found in Rossetti et al, as to be expected given the improvement of the forcefields used.[9], and they average to $-7 \cdot 10^{-3} \text{ deg cm}^2 \text{ dmol}^{-1}$ for a99SB-*disp* and $-8 \cdot 10^{-3} \text{ deg cm}^2 \text{ dmol}^{-1}$ for DES-Amber, which is in much better agreement with the experimental results compared to Rossetti et al. The minima in the experimental data of Maltsev et al are at $-19 \cdot 10^{-3} \text{ deg cm}^2 \text{ dmol}^{-1}$.

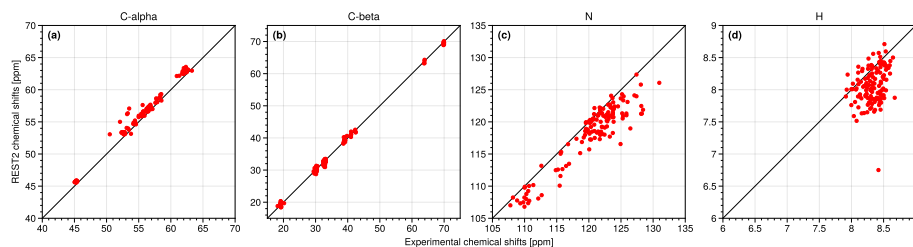


Figure 10: Calculated chemical shifts of C_α , C_β , N and H in AS from the a99SB-*disp* simulation plotted against the experimental data from Roche et al.[11]

Table 5: Correlation of calculated and measured chemical shifts of AS in the a99SB-*disp* simulation.

C_α	C_β	N	H
0.988	0.999	0.931	0.295

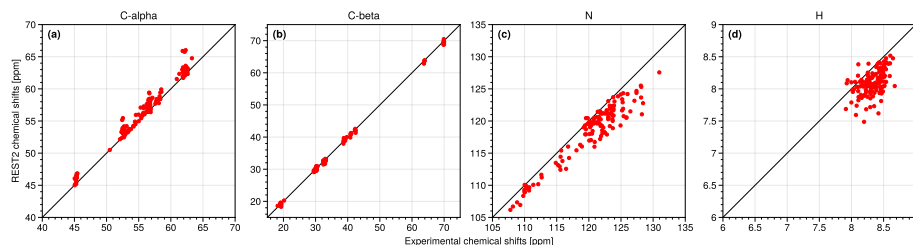


Figure 11: Calculated chemical shifts of C_α , C_β , N and H in AS from the DES-Amber simulation plotted against the experimental data from Roche et al.[11]

Table 6: Correlation of calculated and measured chemical shifts of AP from the DES-Amber simulation.

C_α	C_β	N	H
0.987	0.999	0.954	0.487

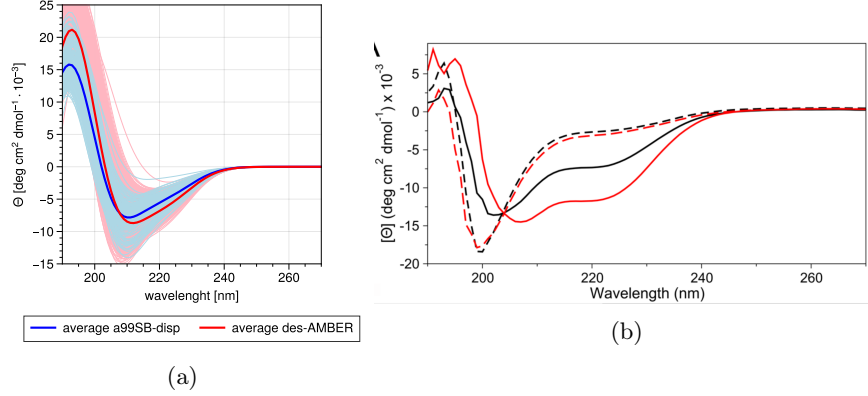


Figure 12: (a) Circular Dichroism spectra of pAS simulations from 12 ns to 25 ns simulation time calculated with DichroCalc [12]; average CD spectrum (red) and CD spectra of all clusters (lightred). (b) Experimental CD spectra: spectra of wild type alpha-synuclein (dashed, black) and N-terminally acetylated (dashed, red) from the works of Maltsev et al [8].

7 Calculated properties

The additional properties were calculated from the trajectories between 12 and 25 ns of the a99SB-*disp*.

Root-mean-square deviations (RMSDs) of atom positions . The RMSDs of AS, pAS and pASH relative to the initial structures (Figure 13) fluctuates along a average value between 15 Å and 40 Å after 5 ns (Table 7. The values of pAS and of pASH are slightly larger than those of AS.

N- to C-terminus distance. In neither the plot (see Figure 14) nor the averages (see Table 8) the distance from the N- to C-terminus shows a clear trend.

Ramachandran plot .The Ramachandran plots (Figure 15) show no discernible differences and exhibit allowed ϕ and Ψ values.

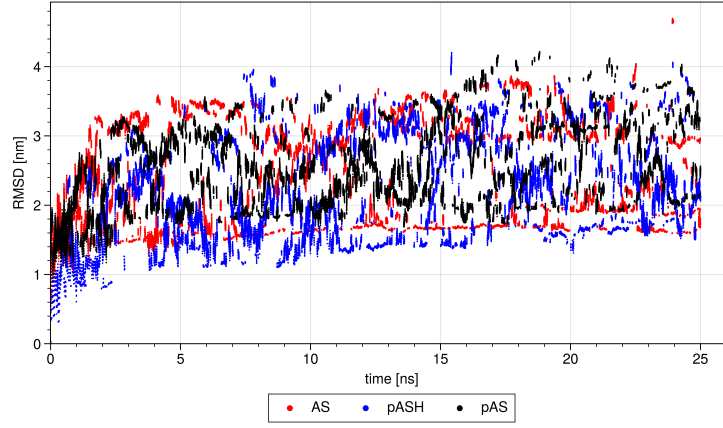


Figure 13: RMSDs of AS, pAS and pASH plotted as function of time from the a99SB-*disp* simulation.

Table 7: RMSDs of atom positions of AS, pAS and pASH averaged over the the 12 ns to 25 ns simulation period from the a99SB-*disp* simulation.

Protein	RMSD [nm]	σ_{RMSD} [nm]
AS	2.41	0.75
pASH	2.46	0.74
pAS	2.77	0.60

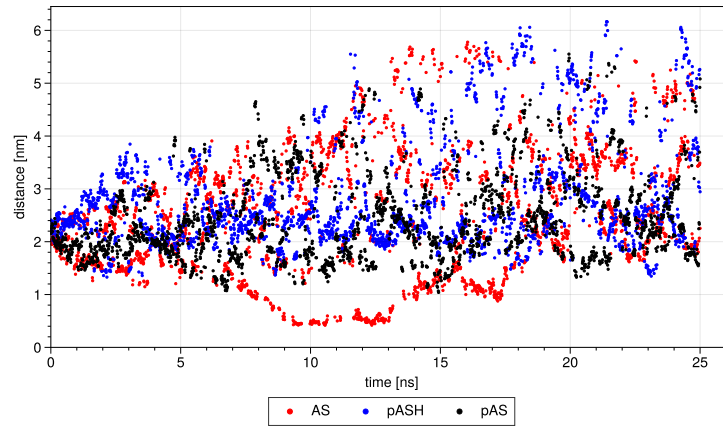


Figure 14: Distance from the N- to C-terminus for all three proteins against simulation time in the a99SB-*disp* simulation.

Table 8: N-terminus/ C-terminus distances of AP, pAP, pAPh averaged over the 12 ns to 25 ns simulation period in the a99SB-*disp* simulation.

Protein	Distance d [nm]	σ_d [nm]
AS	2.78	1.29
pASH	3.08	1.19
pAS	2.59	0.86

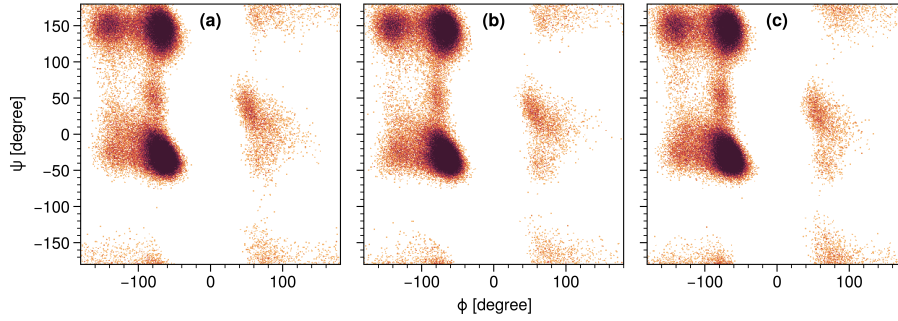


Figure 15: Ramachandran plots for all (a) AS, (b) pASH and (c) pAS from the a99SB-*disp* simulation.

8 AS with protonated phosphate (pASH)

All results in this section used the a99SB-*disp* force field simulations.

The phosphate group is expected to be deprotonated at physiological pH, as its pKa is 5.6[13]. However, in the presence of the negative potential of the C-Term, a fraction of the protein population might exhibit a protonated phosphate (pASH system). Here we have carried out the simulations of such form, following exactly the same protocol as that of pAS.

The RMSD (see Fig. 13), the gyration and hydrodynamic radii (see Table 9 and 10), the SASA (see Table 13 and 14) and RDFs (see 20) turn out to increase on passing from AS to pASH to pAS. The hydrogen bonds and salt bridges of pASH (see Table 11 and 12 respectively) are lower than the those in pAS. The contact maps of pAS and pASH (Fig. 19) are similar. The N-terminus/C-terminus distance (Table 8) and the Ramachandran plot (Fig. 15) are similar to those of AS and pAS.

Table 9: Radii of gyration R_g and hydrodynamic radii R_H of AS, pAS, pASH, as well as the experimental hydrodynamic radius of the non-phosphorylated protein.[8] Results for the simulation only after the first 12 ns of the a99SB-*disp* simulation.

System	R_g [Å]	σ_{R_g} [Å]	R_H [Å]	σ_{R_H} [Å]
AS	27.6	5.7	30.8	3.5
pASH	27.8	5.5	30.9	3.
pAS	29.3	4.5	31.8	2.7
AS (exp)	–	–	28.7	–

Table 10: Radii of gyration R_g and hydrodynamic radii R_H of the NAC region of AS, pAS, pASH , averaged over the 12 ns -25 ns period from the a99SB-*disp* simulation.

System	R_g [Å]	σ_{R_g} [Å]	R_H [Å]	σ_{R_H} [Å]
AS	16.7	5.7	22.8	5.2
pASH	17.4	7.7	23.3	6.7
pAS	18.7	7.6	24.4	6.4

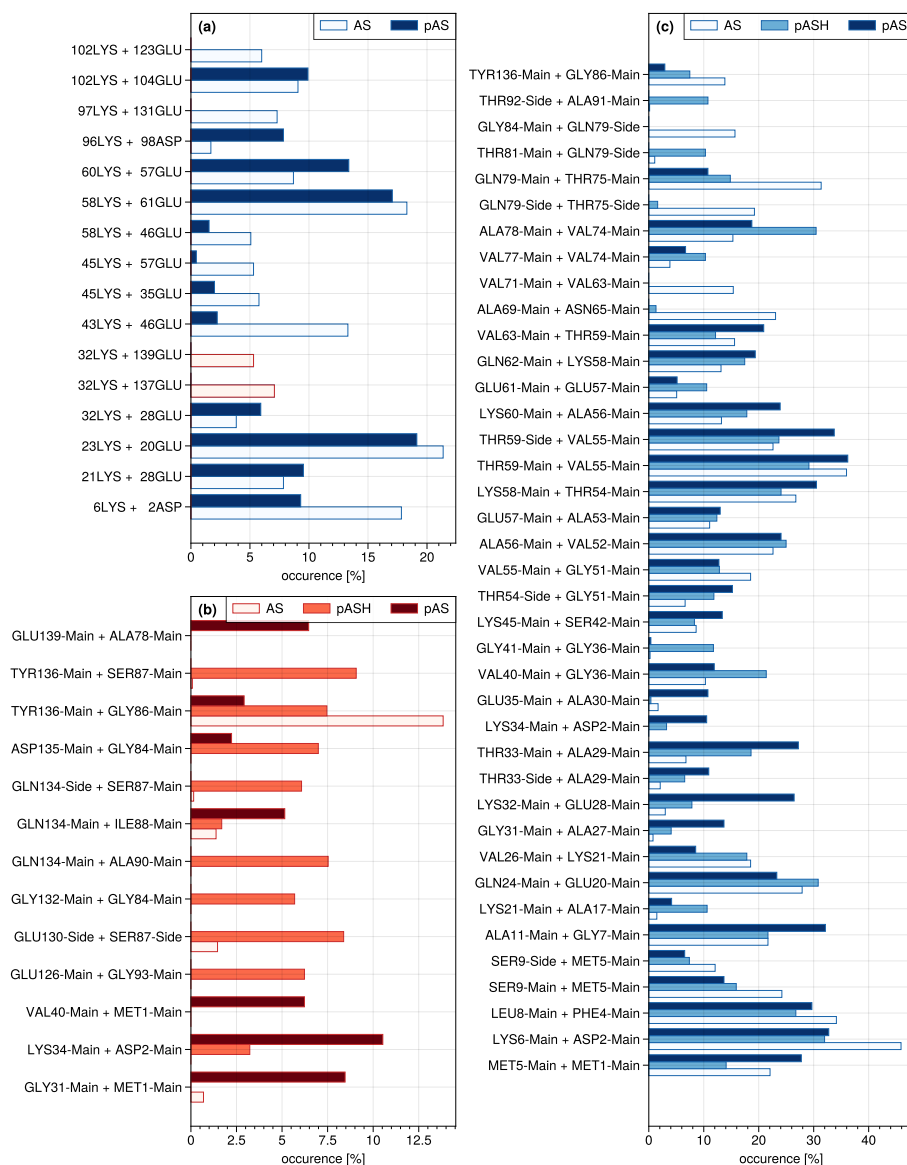


Figure 16: Comparison of properties in AS, pAS and pASH in the a99SB-*disp* simulation: (a) Persistency of salt bridges in across the 12 ns to 25 ns simulation period. The inter-domain salt bridges are highlighted in red; (b) inter-domain hydrogen bonds in all three proteins; (c) intra-domain hydrogen bonds in all three proteins.

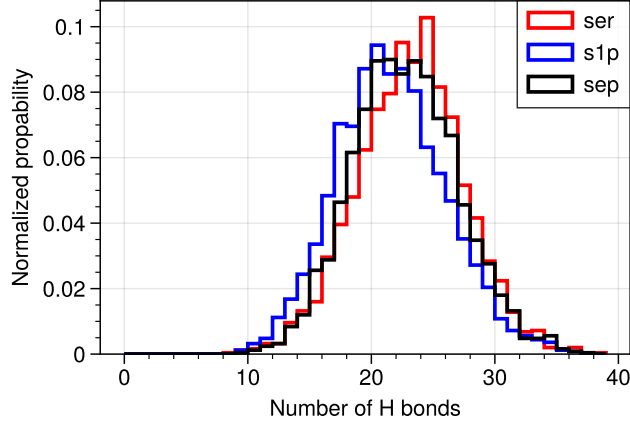


Figure 17: Normalized probability to find a number of hydrogen bonds over the 12 ns to 25 ns simulation period for AS (red), pASH (blue) or pAS (black) in the a99SB-*disp* simulation.

Table 11: Number of intermolecular hydrogen bonds of AS, pAS, pASH averaged over the 12 ns to 25 ns simulation period of the a99SB-*disp* simulation.

System	N_{HB}	σ_{N-HB}
AS	21.51	4.19
pASH	21.02	4.61
pAS	22.13	4.18

Table 12: Number of salt bridges of AS, pAS, pASH over the 12 ns to 25 ns simulation period of the a99SB-*disp* simulation.

System	N_{SB}	σ_{N-SB}
AS	2.86	1.96
pASH	1.77	1.53
pAS	1.85	1.62

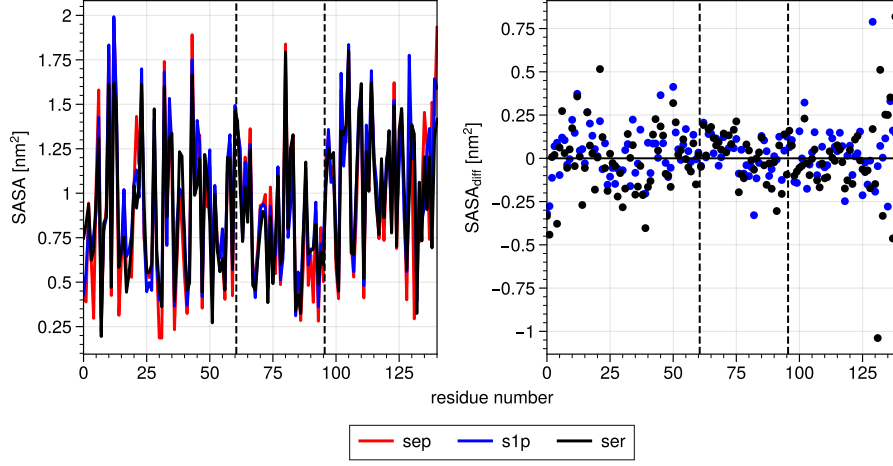


Figure 18: (a) Solvent accessible surface area per residue in AS, pAS, pASH averaged over the 12 ns to 25 ns simulation period. The NAC-region is indicated by dotted lines; (b) difference in solvent accessible surface area between AS and pASH or pAS, averaged in the same time frame from the a99SB-*disp* simulation.

Table 13: Solvent accessible surface area for the NAC region in AS, pAS, pASH, averaged over the 12 ns to 25 ns simulation period from the a99SB-*disp* simulation; for the other regions of the proteins, see Figure 14 in the SI.

System	$SASA_{NAC}$ [nm ²]	σ_{SASA} [nm ²]
AS	0.83	0.40
pASH	0.84	0.36
pAS	0.85	0.39

Table 14: Solvent accessible surface area for N/C-term region of AS, pAS, pASH averaged over the 12 ns to 25 ns simulation period from the a99SB-*disp* simulation.

System	$SASA_{N-Term}$ [nm ²]	σ_{SASA} [nm ²]	$SASA_{C-Term}$ [nm ²]	σ_{SASA}
AS	0.87	0.38	1.07	0.34
pASH	0.90	0.42	1.12	0.36
pAS	0.86	0.46	1.07	0.39

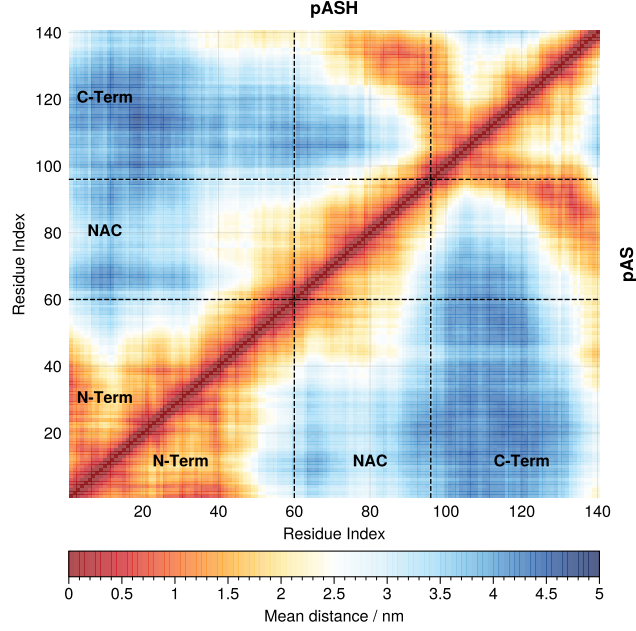


Figure 19: Contact maps in pASH (triangle above) and pAS (triangle below) of the a99SB-*disp* simulation.

9 Radial distribution functions

This section covers additional RDFs from the a99SB-*disp* simulations.

Some RDFs (Figure 20) did not converge to 1, because the system was not isotropic. The coordination number reads:

$$N(r) = \rho \cdot 4\pi \int_0^r g(r) r^2 dr \quad (1)$$

with ρ being the density of atoms in the simulation box. The integrals are shown up to 0.26 nm (Figure 21) as this covers the peaks visible in Figure 20 which can be attributed to hydrogen bonds.

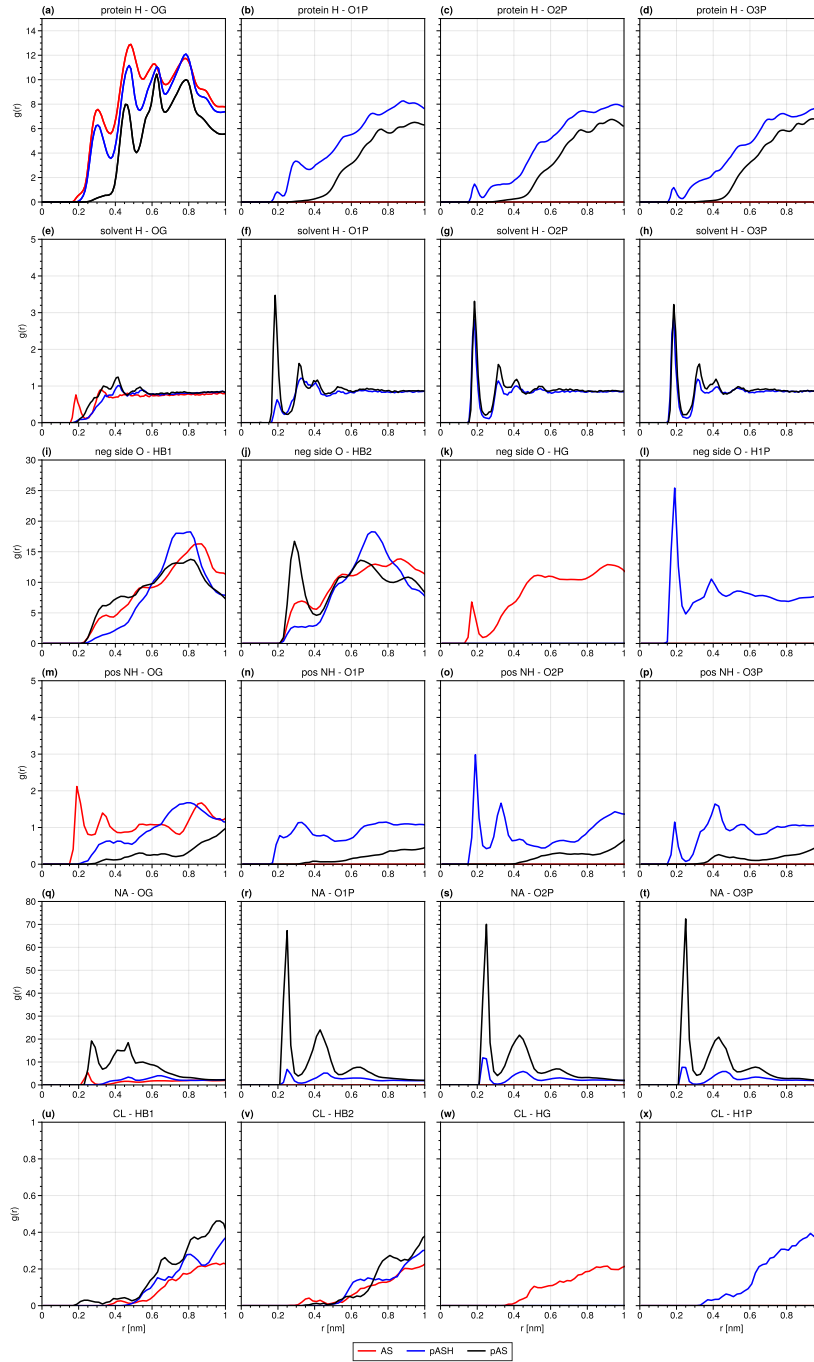


Figure 20: a99SB-*disp* simulation: RDF $g(r)$ of the oxygen atoms of residue 129 side chain and hydrogen atoms bonded to polar atoms of all other residues (a-d), water hydrogens (e-h), hydrogens atoms bonded to positively charged nitrogen (m-p), and sodium ions (q-t) and RDF of hydrogen atoms of residue 129 side chain and negatively charged oxygens of all other residues (i-l) and chlorine ions (u-x). (a), (e), (m), (q) compare the RDF for pAS (black) and AS (red) for the oxygen atom in the sidechain. (b)-(d), (f)-(h), (n-p), (r-t) illustrate the rdf for the terminal phosphate oxygens. (i)-(j), (u)-(v) compare the rdf of pAS (black) and AS (red) for the sidechain hydrogens bonded to carbon. (k), (w) show the rdf of the hydroxy group. (l), (x) show the rdf of the monoprotonated phosphoryl group.

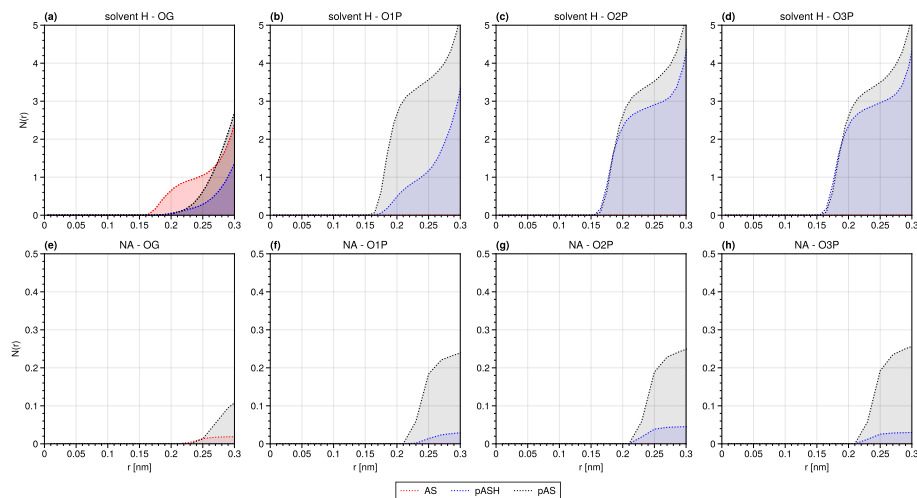


Figure 21: Spherical integrals of the RDFs presented in Fig. 20. (a-d) show the integrals of (e-h) in Fig. 20 and (e-h) who the integrals of (q-t) in Fig. 20. The other integrals are not shown as they are almost zero.

To eliminate the possibility of double counting solvent molecules, the RDF and integral in figure 22 show that there are on average 12 solvent molecules surrounding the phosphoryl group in pAS and a slightly reduced 9 around pASH. This is more than the expected maximum of 3 solvent molecules that can be bound via hydrogen bonding per oxygen atom. This was confirmed through visual inspection along the converged part of the trajectories. This over-hydration is attributable to either the high charge density at the site, or an insufficient description of the hydration of phosphoryl moieties by the TIP4P-D water model[7] employed in the current study.

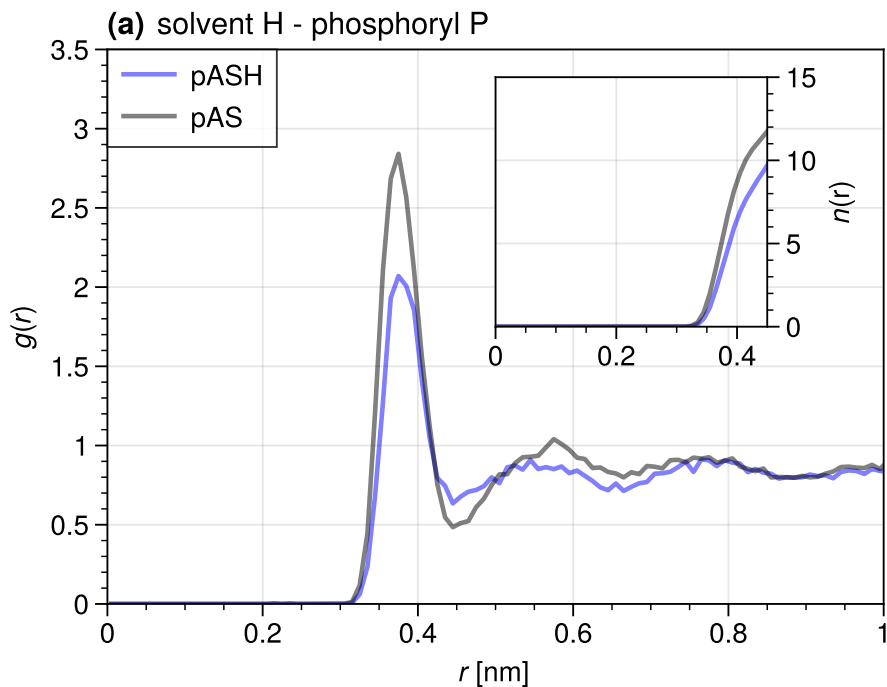


Figure 22: a99SB-*disp* simulation: RDF $g(r)$ of the phosphoryl phosphor atom and solvent hydrogen atoms. The inset shows the cumulative integral $n(r)$ of $g(r)$ up to 4.5 nm (the end of the first solvation shell/peak around the three phosphoryl terminal oxygens).

10 Simulation Parameters

10.1 Force field parameters

The force field parameters for phosphorylated amino acids in Amber were taken from [14] .

10.2 Simulation parameters

Tables 15 to 20 show the parameters used to initiate the different runs in GRO-MACS. Tables 15 to 19 describe both the preparation for the simulations using REST2 and the unbiased MD simulations. Table 20 was used for the REST2 run.

Table 15: Parameters used in GROMACS for the energy minimisation.

parameter	value
integrator	steep
nsteps	100000
emtol [kJ mol ⁻¹ nm ⁻¹]	10
emstep [nm]	0.01
nstlog	1000
nstenergy	100
energygrps	System
cutoff-scheme	Verlet
nstlist	1
rlist	1
pbc	xyz
pme_order	4
vdwtype	Cut-off
vdw-modifier	Potential-shift-Verlet
rcoulomb [nm]	1.2
rvdw [nm]	1.2
epsilon_r [nm]	1
fourierspacing [nm]	0.12

Table 16: Parameters used in GROMACS for the NVT equilibration.

parameter	value
integrator	md
nsteps	50000
dt [ps]	0.002
nstxout	500
nstvout	500
nstenergy	500
nstlog	500
continuation	no
constraint_algorithm	lincs
constraints	h-bonds
lincs_iter	1
lincs_order	4
cutoff-scheme	Verlet
nstlist	10
rcoulomb [nm]	1.2
rvdw [nm]	1.2
DispCorr	EnerPres
coulombtype	PME
pme_order	4
fourierspacing [nm]	0.12
tcoupl	Nose-Hoover
tc-grps	Protein, Water, Ion
tau_t [ps]	0.5
ref_t [K]	300
pcoupl	no
pbcs	xyz
gen_vel	yes
gen_temp [K]	300
gen_seed	-1
Define	-DPOSRES

Table 17: Parameters used in GROMACS for the simulated annealing.

parameter		value
integrator		md
nsteps		50000
dt [ps]		0.001
nstxout		2000
nstvout		2000
nstenergy		100
nstlog		100
nstxout-compressed		100
compressed-x-grps		System
energygrps	Protein, Water, Ion	
continuation		no
constraint_algorithm		lincs
constraints		none
lincs_iter		1
lincs_order		4
lincs-warnangle		30
morse		no
cutoff-scheme		Verlet
nstlist		1
coulombtype		PME
pme_order		4
vdwtype		Cut-off
vdw-modifier	Potential-shift-Verlet	
rcoulomb [nm]		1.2
rvdw [nm]		1.2
epsilon_r [nm]		1
fourierspacing [nm]		0.12
tcoupl	Nose-Hoover	
tc-grps	System	
tau_t [ps]		0.1
ref_t [K]		300
pcoupl	Parrinello-Rahman	
pcoupltype	isotropic	
tau_p [ps]		0.5
ref_p [bar]		1.0
compressibility [bar ⁻¹]		4.5e-5
nstcomm		1
comm-mode		linear
comm-grps	Protein, Water, Ion	
pbc		xyz
DispCorr	EnerPres	
gen_vel		yes
gen-temp		2
gen-seed	23	173529
annealing		single
annealing_npoints		40
Define		-DPOSRES

Table 18: Temperature and time steps in the simulated annealing run.

annealing_time	annealing_temp
0	2
50	5
75	10
100	15
125	20
150	25
175	30
200	35
225	40
250	45
275	50
300	55
325	60
350	65
375	70
400	75
425	80
450	85
475	90
500	100
525	110
550	120
575	130
600	140
625	150
650	160
675	170
700	180
725	190
750	200
775	210
800	220
825	230
850	240
875	250
900	260
925	270
950	280
975	290
1000	300

Table 19: Parameters used in GROMACS for the NPT equilibration.

parameter	value
integrator	md
nsteps	50000
dt [ps]	0.002
nstxout	500
nstvout	500
nstenergy	500
nstlog	500
continuation	yes
constraint_algorithm	lincs
constraints	h-bonds
lincs_iter	1
lincs_order	4
cutoff-scheme	Verlet
nstlist	10
rcoulomb [nm]	1.2
rvdw [nm]	1.2
DispCorr	EnerPres
coulombtype	PME
pme_order	4
fourierspacing [nm]	0.12
tcoupl	Nose-Hoover
tc-grps	Protein, Water, Ion
tau_t [ps]	0.5
ref_t [K]	300
pcoupl	Parrinello-Rahman
pcoupltype	semiisotropic
tau_p [ps]	2.0
ref_p [bar]	1.0
compressibility [bar ⁻¹]	4.5e-5
refcoord_scaling	com
pbc	xyz
gen_vel	no
Define	-DPOSRES

Table 20: Parameters used in GROMACS for the production run using REST2. Unbiased production runs used the same parameters apart of **nsteps**; 50000000 steps were taken instead.

parameter	value
integrator	md
nsteps	12500000
dt [ps]	0.002
nstxout	5000
nstvout	5000
nstenergy	1000
nstlog	5000
nstxout-compressed	10
nstcalcenergy	100
compressed-x-grps	Protein, Ion
energygrps	Protein, Water, Ion
continuation	yes
constraint_algorithm	lincs
constraints	h-bonds
lincs_iter	1
lincs_order	4
cutoff-scheme	Verlet
nstlist	10
coulombtype	PME
pme_order	4
rlist [nm]	1.2
rcoulomb [nm]	1.2
rvdw [nm]	1.2
fourierspacing [nm]	0.12
tcoupl	Nose-Hoover
tc-grps	Protein, Water, Ion
tau_t [ps]	0.5
ref_t [K]	300
pcoupl	Parrinello-Rahman
pcoupltype	isotropic
tau_p [ps]	2.0
ref_p [bar]	1.0
compressibility [bar ⁻¹]	4.5e-5
DispCorr	EnerPres
pbcs	xyz
gen_vel	no
nstcomm	1
comm-grps	Protein, Water, Ion

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