This copy of the ESI replaces the previous version published on 03/11/2021

ELECTRONIC SUPPLEMENTARY INFORMATION (ESI)

Hofmeister Effects on Protein Stability are dependent on the Nature of the Unfolded State

Sara S. Ribeiro[a‡], Tarsila G. Castro[b], Cláudio M. Gomes[c], João C. Marcos[a*]

[a] *Centre of Chemistry, University of Minho, Campus de Gualtar, Braga, Portugal*

[b] *Centre of Biological Engineering, University of Minho, Campus de Gualtar, Braga, Portugal*

[c] *Biosystems and Integrative Sciences Institute, Faculdade de Ciências and Departamento de Química e Bioquímica, Universidade de Lisboa, 1749-016 Lisboa, Portugal*

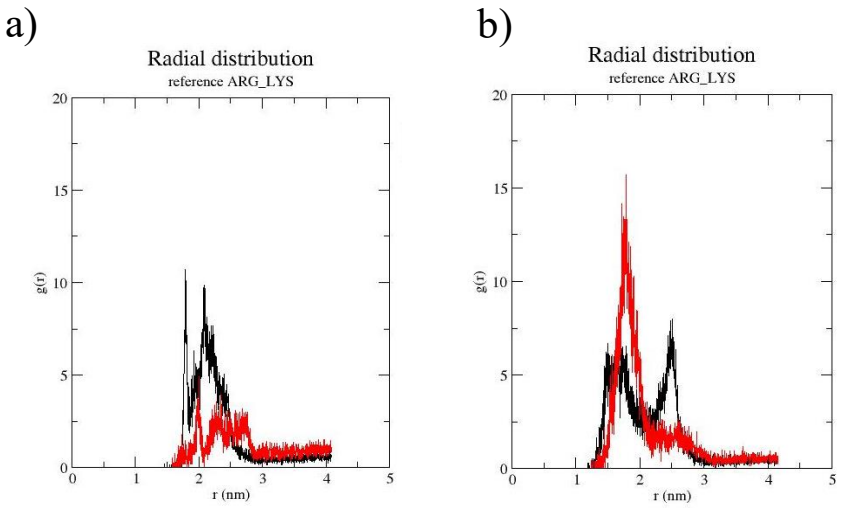

Figure S1. Radial distribution functions of dihydrogen phosphate $[H_2PO_4]^-$ (black line) and monohydrogen phosphate $[HPO_4]^{2-}$ (red line) in the vicinity of positive side (lysine (Lys) and arginine (Arg)) chain residues for the native **(a)** and thermally **(b)** unfolded states during 50 ns of MD simulation.

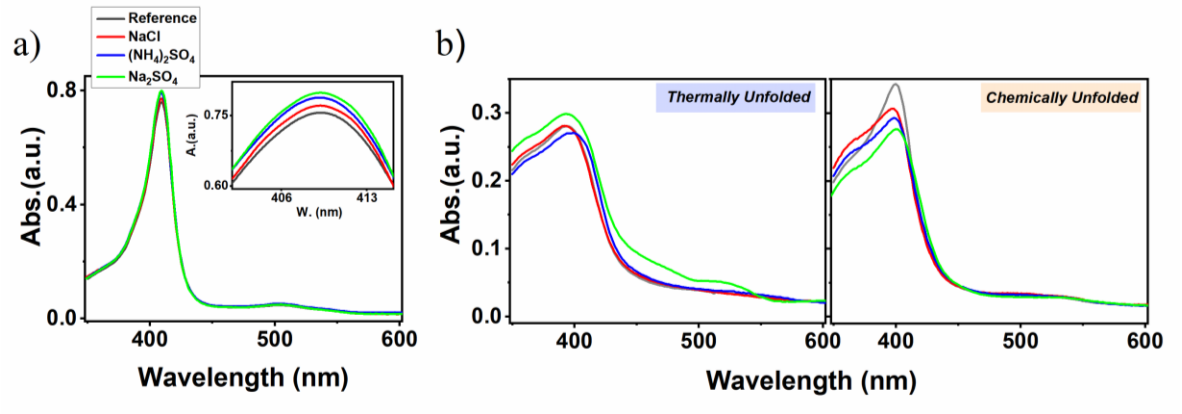

Figure S2. Spectra of heme group in 0.05 M phosphate buffer pH 7 (or reference) and in the presence of each studied salt at 0.3 M for the folded **(a)**, thermal and chemically **(b)** unfolded myoglobin.

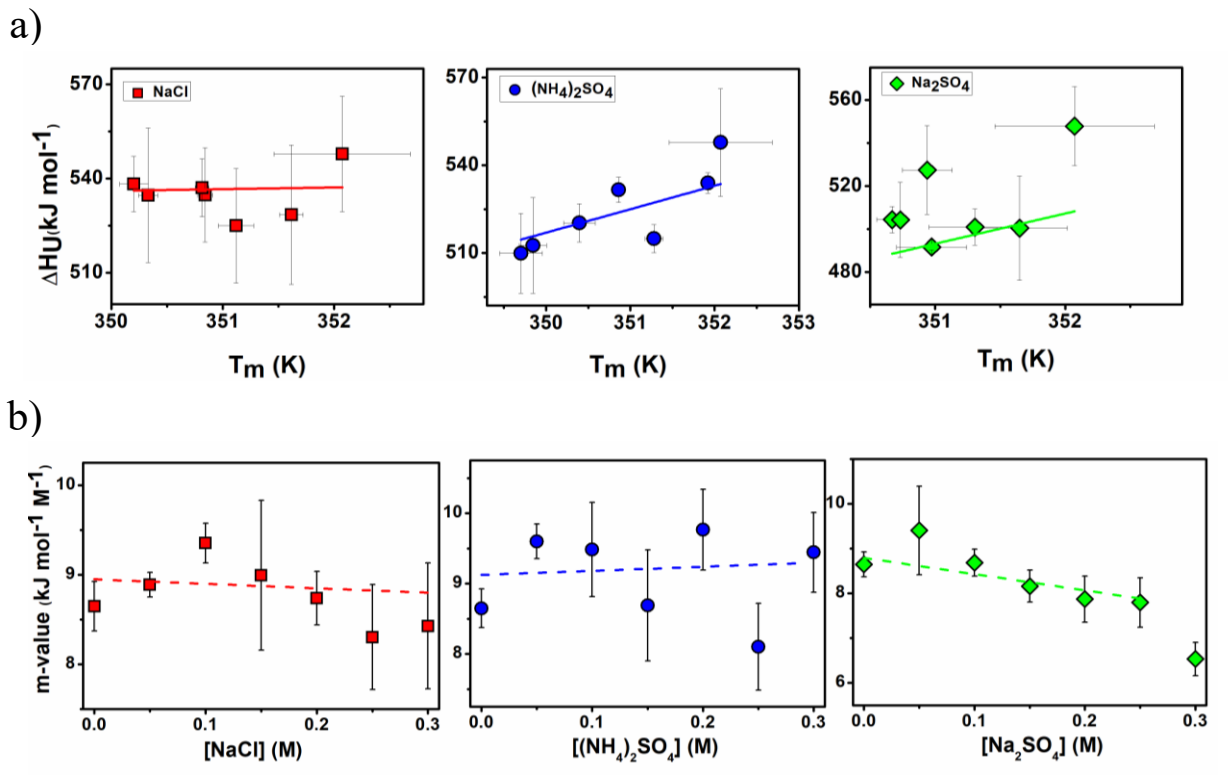

Figure S3. **(a)** ΔH_U as function of T_m plots for each salt obtained from the myoglobin thermal denaturation. The slope of these plots defines ΔC_p^*, the change in the heat capacity of unfolding induced by the salt at 0.3 M. No linear fit was successful (r^2 were all below 0.157). **(b)** m-values determined from chemical denaturation of myoglobin in the presence of each salt for the different concentrations. Similar to thermal unfolding, no linear relation was found, with all r^2 below 0.562.

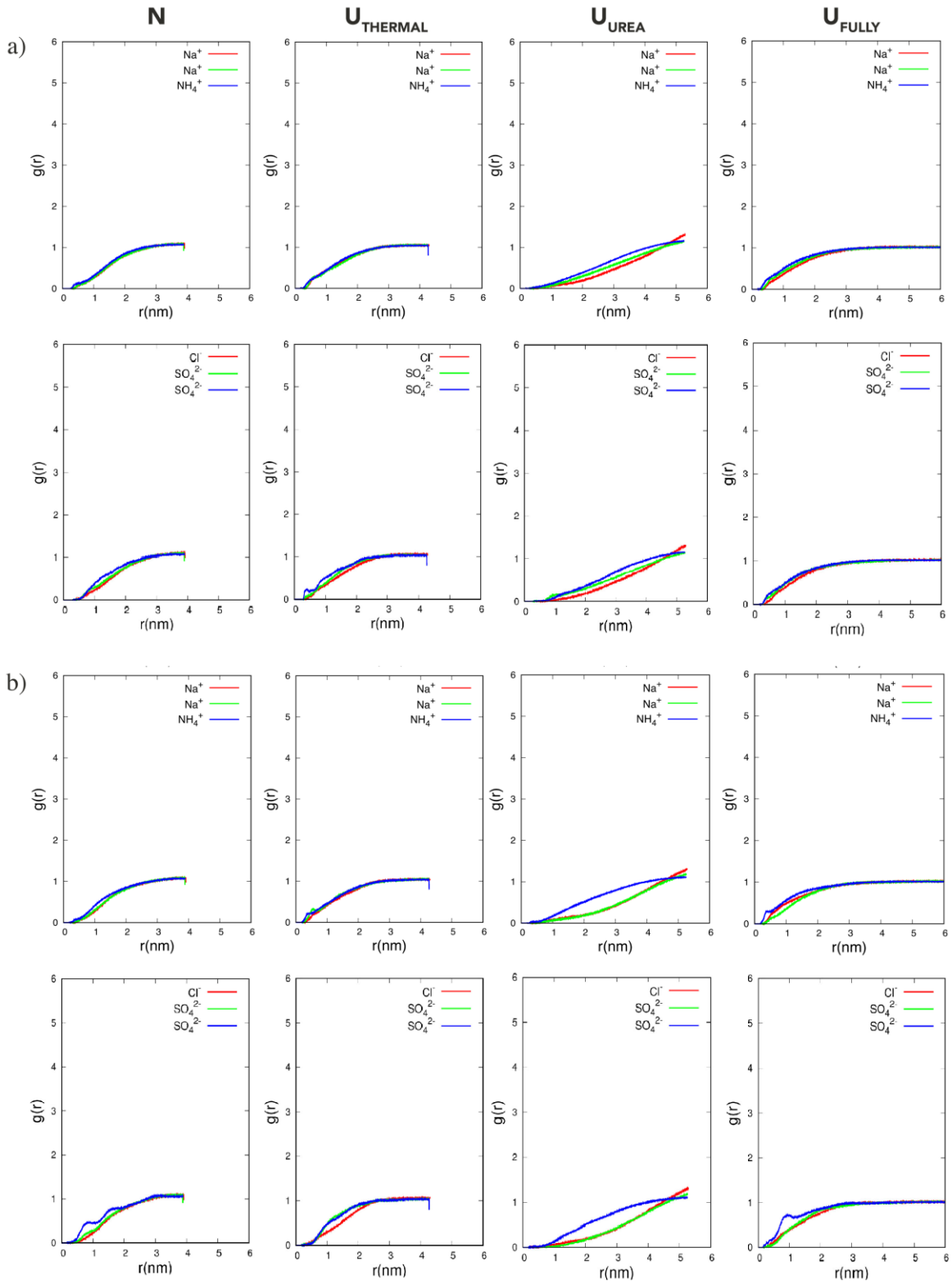

Figure S4. Radial distribution functions of cations (Na^+ and NH_4^+) and anions (Cl^- and SO_4^{2-}) in the vicinity of aliphatic **(a)** and aromatic **(b)** side-chains of myoglobin during the last 60ns of MD simulation in the four different conformations investigated. Red refers to NaCl, green to Na_2SO_4 and blue to $(NH_4)_2SO_4$.

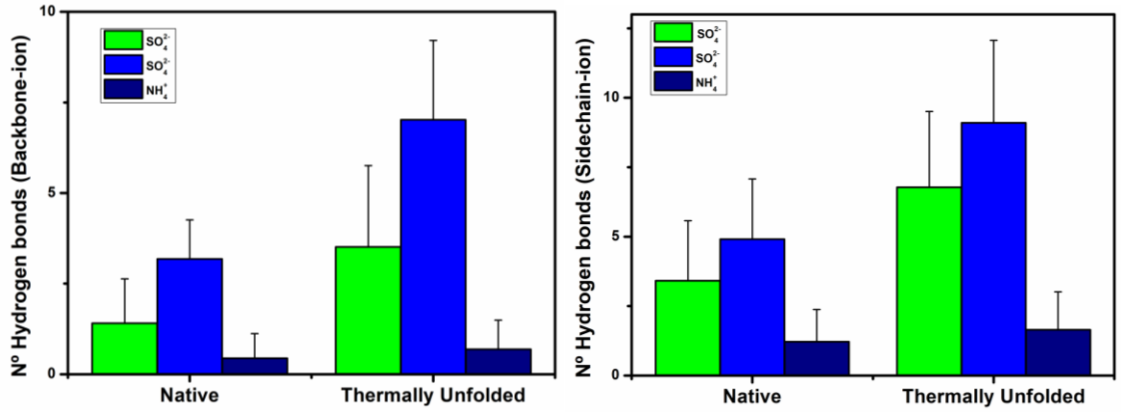

Figure S5. Average number of hydrogen bonds between myoglobin backbone (**left**) or sidechain (**right**) and SO_4^{2-} (green from Na_2SO_4) (blue from $(NH_4)_2SO_4$) or NH_4^+ during the last 60 ns of simulation.

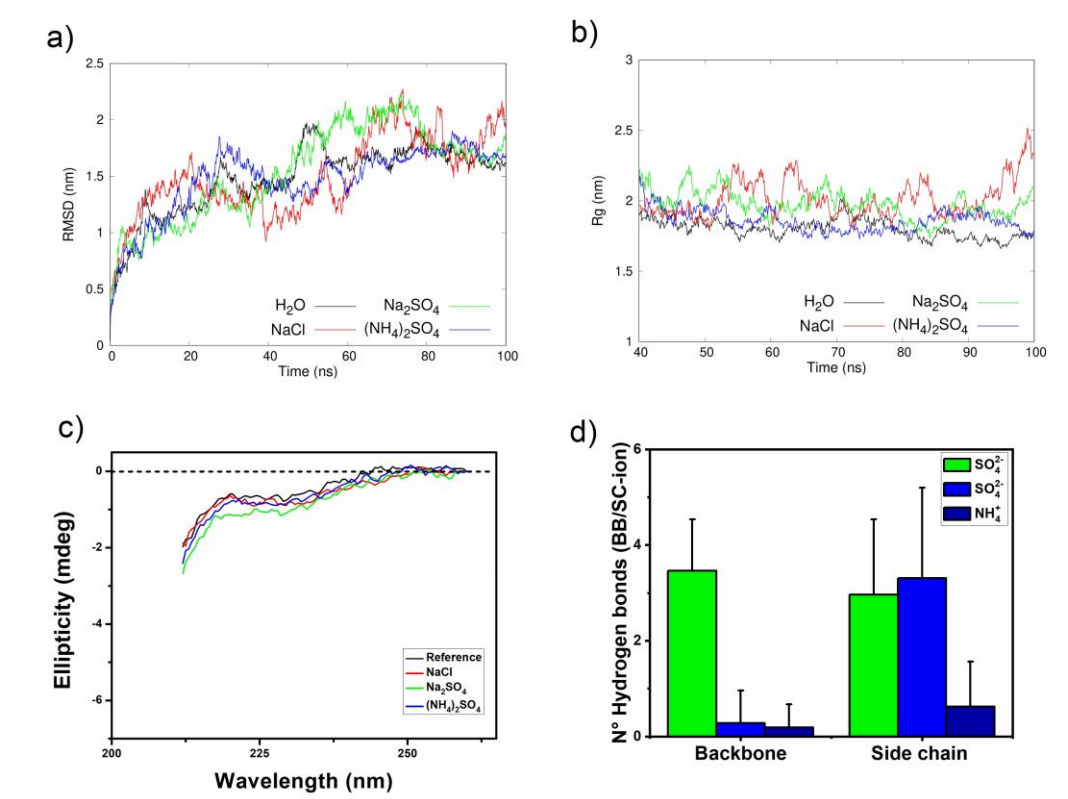

Figure S6. (a) Root mean square deviation (RMSD) of chemically unfolded myoglobin in water and in the presence of each salt at 0.3 M over the 100 ns of simulation. **(b)** Average radius of gyration (Rg) of chemically unfolded myoglobin during the last 60 ns of simulation in the absence or in the presence of each salt. **(c)** Far-UV spectra of urea-unfolded myoglobin with NaCl, $(NH_4)_2SO_4$ and Na_2SO_4 at 0.3 M. **(d)** Average number of hydrogen bonds between myoglobin backbone/sidechain and SO_4^{2-} (green from Na_2SO_4) (blue from $(NH_4)_2SO_4$) or NH_4^+ in U_{Urea} during the last 60 ns of simulation.

Table S1. Reversibility of myoglobin thermal unfolding calculated for each salt condition.

	[Salt] (M)	*Buffer*	*NaCl*	*Na_2SO_4*	*$(NH_4)_2SO_4$*
Reversibility (%)	0.05	69.3	65.6	65.3	71.2
	0.1		70.2	70.0	77.6
	0.15		71.0	70.5	75.0
	0.2		70.2	65.5	69.9
	0.25		72.5	63.9	62.0
	0.3		72.4	62.7	62.1

Table S2. Reversibility of chemical denaturation experiments in buffer and at 0.3 M of all salts.

	Buffer	*NaCl*	*Na_2SO_4*	*$(NH_4)_2SO_4$*
Reversibility (%)	80.0	71.3	79.5	77.8

Table S3. Number of ions and water molecules used in MD simulations.

	System	*Water*	*Cation*	*Anion*	*Urea*	*Total N° of molecules**	*Final volume (nm^3)*
N	Water	10804	2			10806	350.90
	NaCl	10676	66	64		10806	
	Na_2SO_4	10473	130	64		10667	
	$(NH_4)_2SO_4$	10371	130	64		10565	
$U_{Thermal}$	Water	13797				13797	404.91
	NaCl	13637	80	80		13797	
	Na_2SO_4	13372	160	80		13532	
	$(NH_4)_2SO_4$	13208	160	80		13448	
U_{Urea}	Water	15190			3977	19167	868.99
	NaCl	14876	157	157	3977	19167	
	Na_2SO_4	14462	314	157	3977	19090	
	$(NH_4)_2SO_4$	14307	314	157	3977	18755	
U_{Fully}	Water	48456				48456	1508.62
	NaCl	47912	272	272		48456	
	Na_2SO_4	47040	544	272		47856	
	$(NH_4)_2SO_4$	46509	544	272		47325	

*This number of molecules does not include myoglobin

Table S4. T_m (K), ΔH_U (kJ mol^{-1}), C_m (M) and m-values (kJ mol^{-1} M^{-1}) determined for thermal and chemical denaturation with UV-VIS in buffer (or reference) and each salt condition (Average and standard deviation for 3 replica).

	[Salt] (M)	*NaCl*	*Na_2SO_4*	*$(NH_4)_2SO_4$*	*Buffer*
T_m	0.05	351.62 ± 0.11	351.65 ± 0.37	351.92 ± 0.01	352.44 ± 0.28
	0.1	351.12 ± 0.16	350.97 ± 0.27	351.28 ± 0.10	
	0.15	350.84 ± 0.07	350.94 ± 0.19	350.86 ± 0.04	
	0.2	350.78 ±0.01	350.67 ± 0.12	350.39 ± 0.19	
	0.25	350.33 ± 0.09	350.73 ± 0.06	349.84 ± 0.16	
	0.3	350.20 ± 0.13	351.31 ± 0.35	349.70 ± 0.25	
ΔH_U	0.05	528.49 ± 22.18	515.79 ± 13.16	533.94 ± 3.47	547.81 ± 18.33
	0.1	524.99 ± 18.20	491.62 ± 1.29	514.92 ± 4.74	
	0.15	534.80 ± 14.96	527.44 ± 20.56	531.61 ± 4.35	
	0.2	530.86 ± 3.50	504.39 ± 6.13	520.29 ± 6.54	
	0.25	534.68 ± 21.43	504.27 ± 17.43	512.55 ± 16.33	
	0.3	538.28 ± 8.77	500.95 ± 8.37	509.90 ± 3.47	
C_m	0.05	5.31 ± 0.03	5.30 ± 0.01	5.22 ± 0.02	5.48 ± 0.02
	0.1	5.16 ± 0.01	5.35 ± 0.05	5.13 ± 0.03	
	0.15	5.12 ± 0.01	5.63 ± 0.04	5.32 ± 0.03	
	0.2	5.13 ± 0.03	5.66 ± 0.05	5.39 ± 0.01	
	0.25	5.14 ± 0.01	5.89 ± 0.03	5.40 ± 0.02	
	0.3	5.13 ± 0.03	5.88 ± 0.02	5.54 ± 0.04	
m-value	0.05	8.89 ± 0.14	9.41 ± 0.99	9.60 ± 0.25	8.65 ± 0.28
	0.1	9.35 ± 0.22	8.69 ± 0.30	9.48 ± 0.67	
	0.15	8.99 ± 0.84	8.16 ± 0.36	8.69 ± 0.79	
	0.2	8.74 ± 0.30	7.87 ± 0.51	9.77 ± 0.58	
	0.25	8.30 ± 0.59	7.80 ± 0.55	8.10 ± 0.62	
	0.3	8.43 ± 0.70	6.53 ± 0.37	9.44 ± 0.57	

Table S5. Linear fitted values determined for $\Delta\Delta G_U$ (Salt 1- Salt2) vs salt concentration plots (see Fig. 3a, main text).

		Slope	*r^2*
Thermal Unfolding	Na_2SO_4 – NaCl	1.29 ± 0.29	0.856
	Na_2SO_4 – $(NH_4)_2SO_4$	2.16 ± 0.27	0.928
Chemical Unfolding	Na_2SO_4 – NaCl	5.69 ± 0.81	0.941
	Na_2SO_4 – $(NH_4)_2SO_4$	2.94 ± 0.66	0.759