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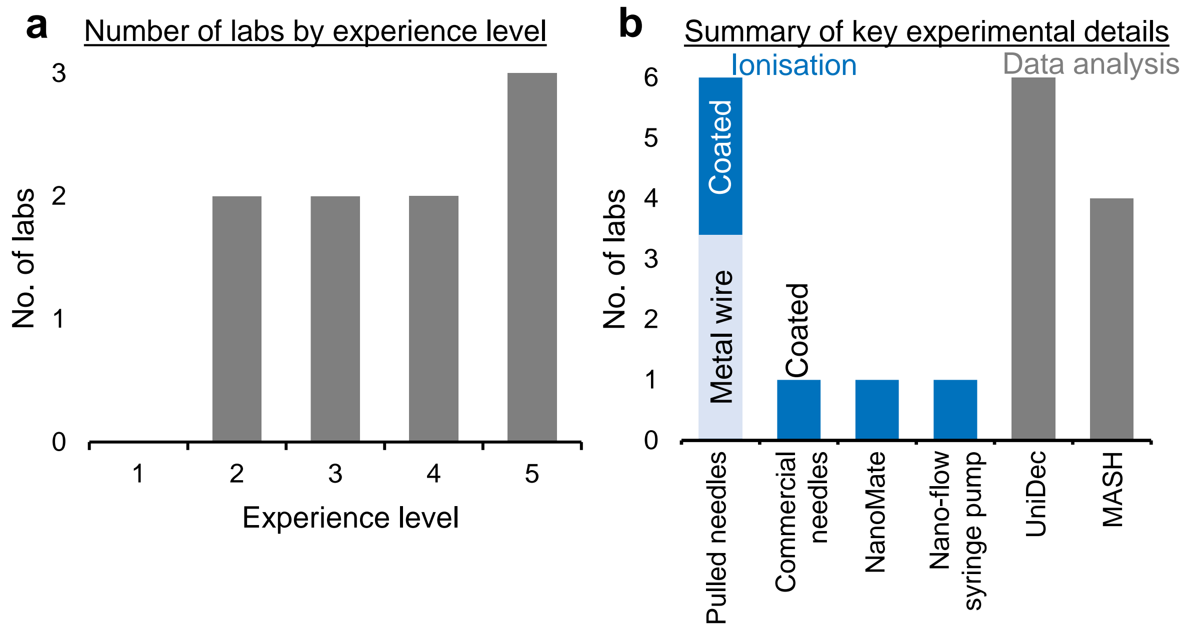
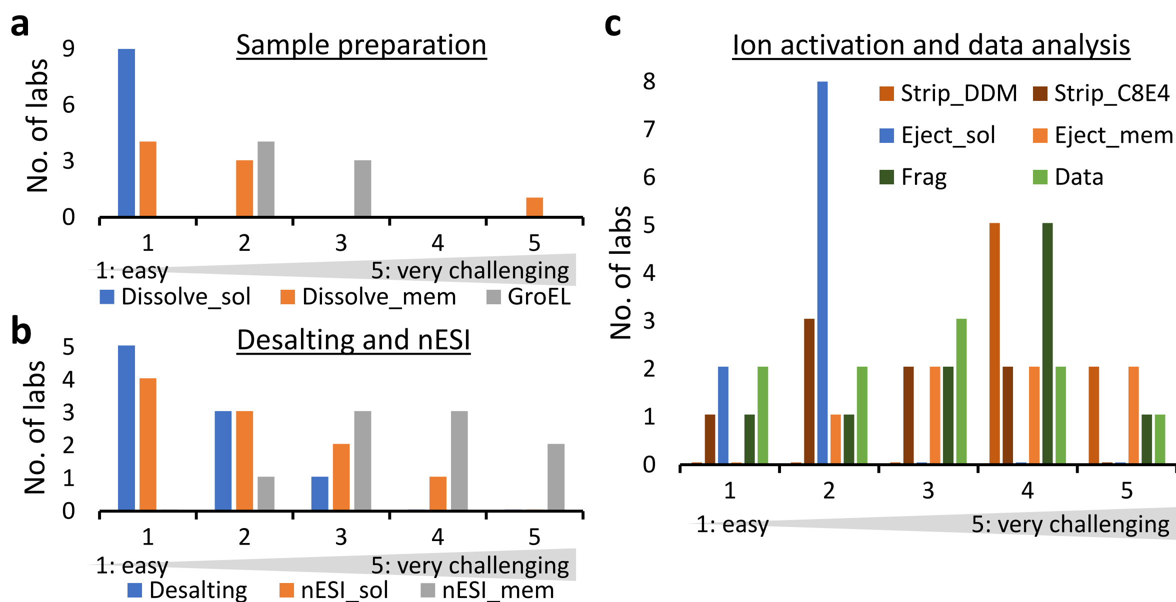
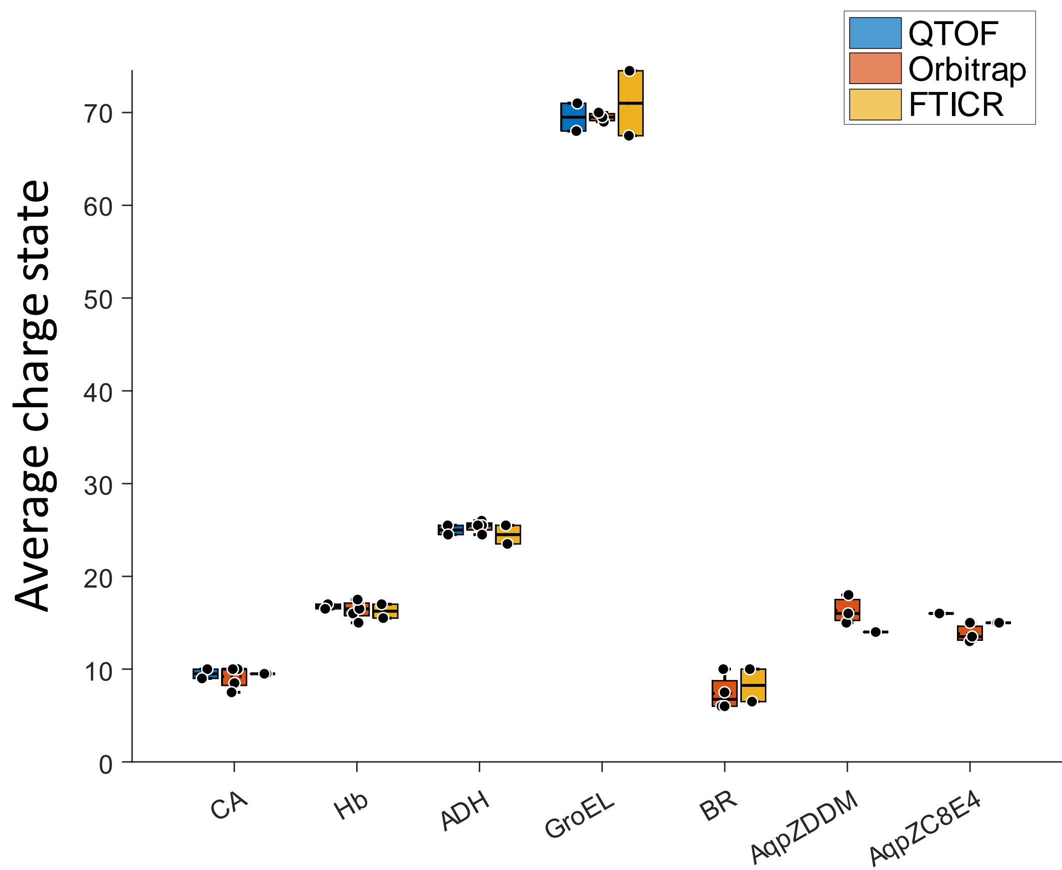


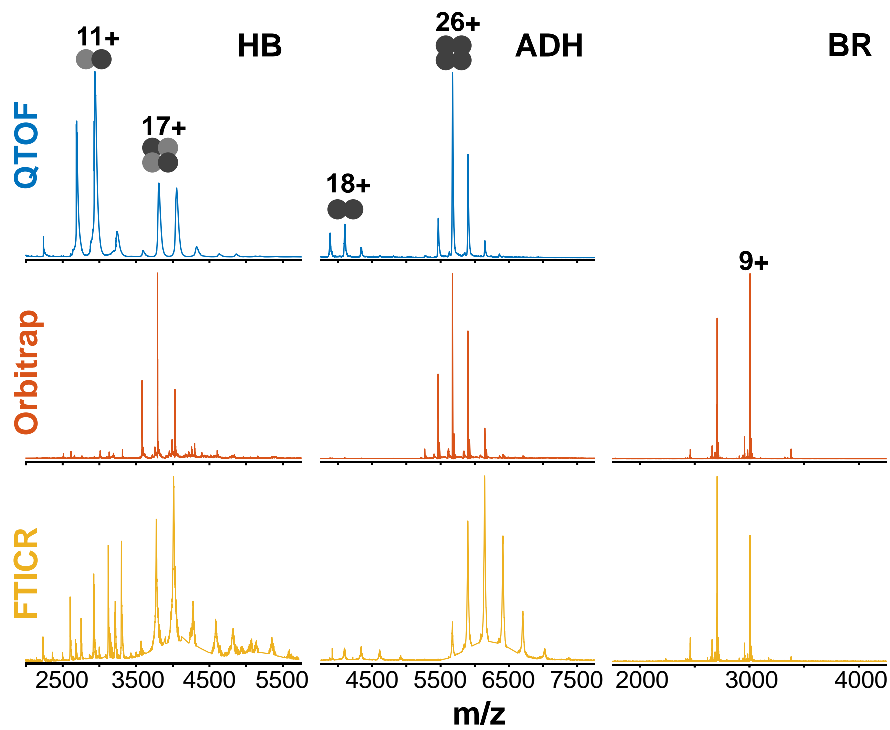
Supplementary Figure S1. Histograms with (**a**) the self-assessed experience in native and top-down MS (1 = least experienced; 5 = most experienced), and (**b**) key experimental details regarding ionisation and data analysis methods by number of labs.



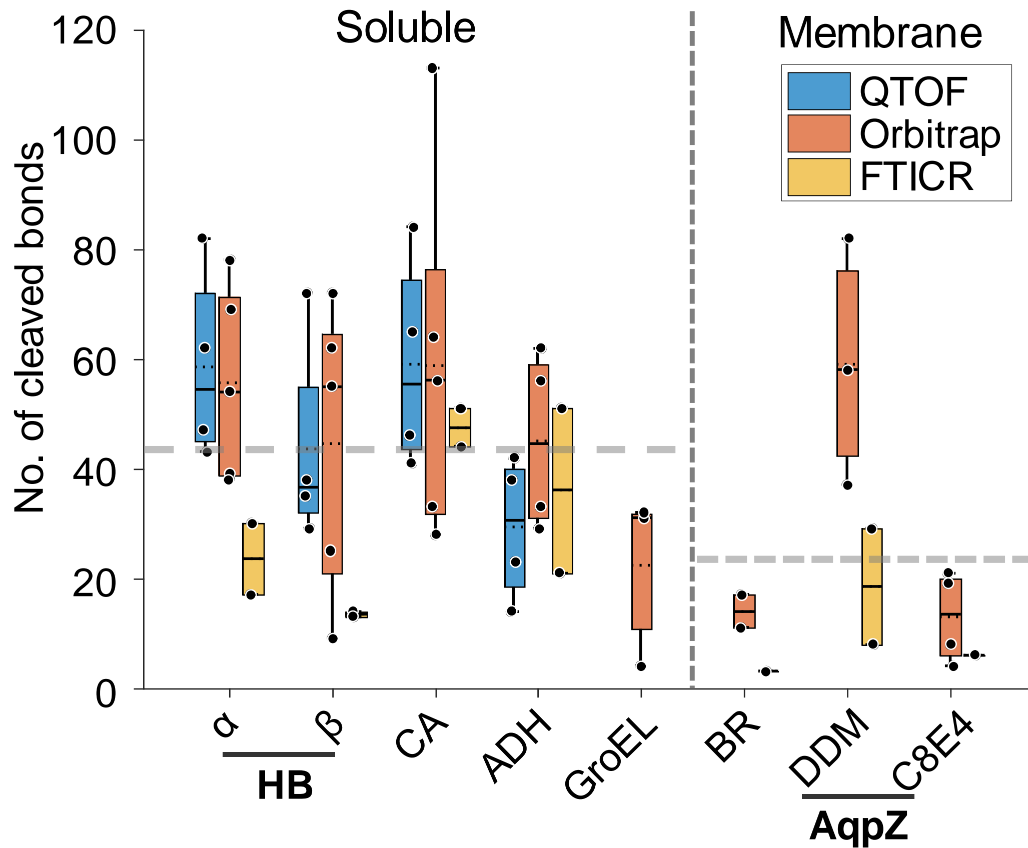
Supplementary Figure S2. Subjective participant experience of difficulty of different aspects of the experiments, *i.e.*, **(a)** Aspects of sample preparation: solubilising lyophilised powder (soluble protein), solubilising lyophilised powder (membrane protein), GroEL sample preparation. **(b)** Aspects of desalting and nESI in general: protein desalting/buffer exchange, obtaining a stable nano-ESI spray for soluble proteins, obtaining a stable nano-ESI spray for membrane proteins. **(c)** Aspects of ion activation and data analysis: removing detergent to release intact membrane protein or complex (DDM), removing detergent to release intact membrane protein or complex (C8E4), monomer ejection (soluble protein), monomer ejection (aquaporin Z), obtaining extensive backbone fragmentation, and data analysis. Note the difference in detergent stripping between C8E4 (fairly easy) and DDM (rather challenging) in panel (c). The relevant post-study participant survey questionnaire can be found elsewhere in the **Supplementary Survey S1**.



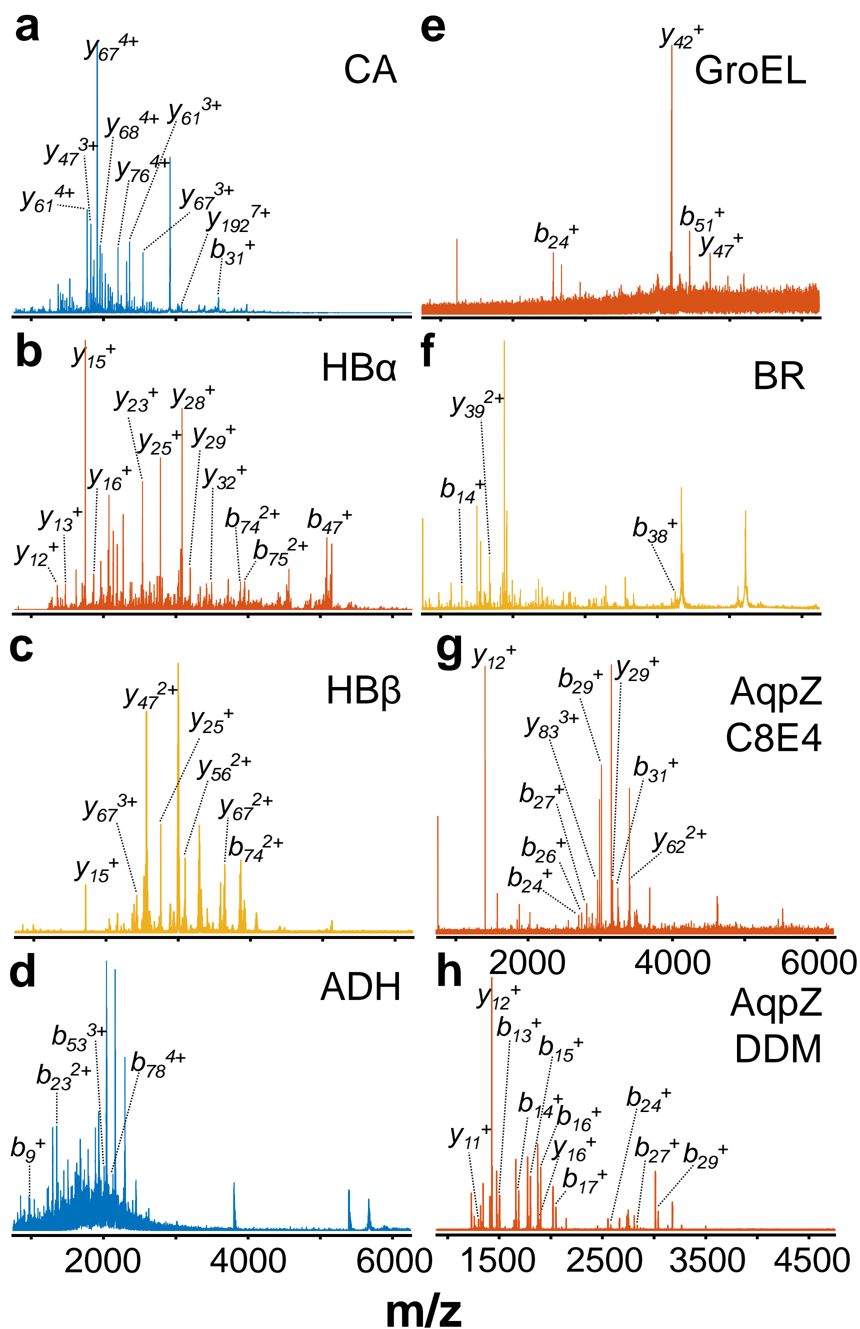
Supplementary Figure S3. Average observed charge states per protein and per instrument type in native MS. Sample size n for the respective boxes: CA/QTOF 2; CA/Orbitrap 5; CA/FTICR 2; HB/QTOF 2; HB/Orbitrap 5; HB/FTICR 2; ADH/QTOF 2; ADH/Orbitrap 4; ADH/FTICR 2; GroEL/QTOF 2; GroEL/Orbitrap 3; GroEL/FTICR 2; BR/Orbitrap 4; BR/FTICR 2; AqpZ_DDM/Orbitrap 3; AqpZ_DDM/FTICR 1; AqpZ_C8E4/QTOF 1; AqpZ_C8E4/Orbitrap 3; AqpZ_C8E4/FTICR 2. The boxplot shows the minimum and maximum values as whiskers, the mean value is indicated by a dotted line, and the solid line represents the median. The boxes indicate 25% of the upper and lower quartile respectively. Individual data points are indicated as black dots.



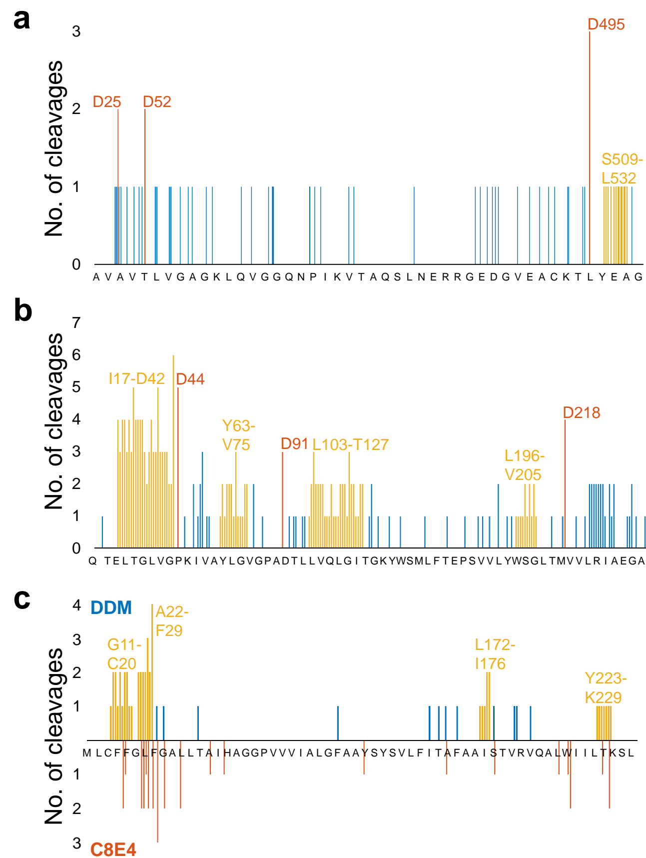
Supplementary Figure S4. Native mass spectra of haemoglobin (HB), ADH, and bacteriorhodopsin (BR) in OG acquired on different instruments. Spectra on the first, second, and third row were acquired on QTOF, Orbitrap, and FTICR instruments, respectively.



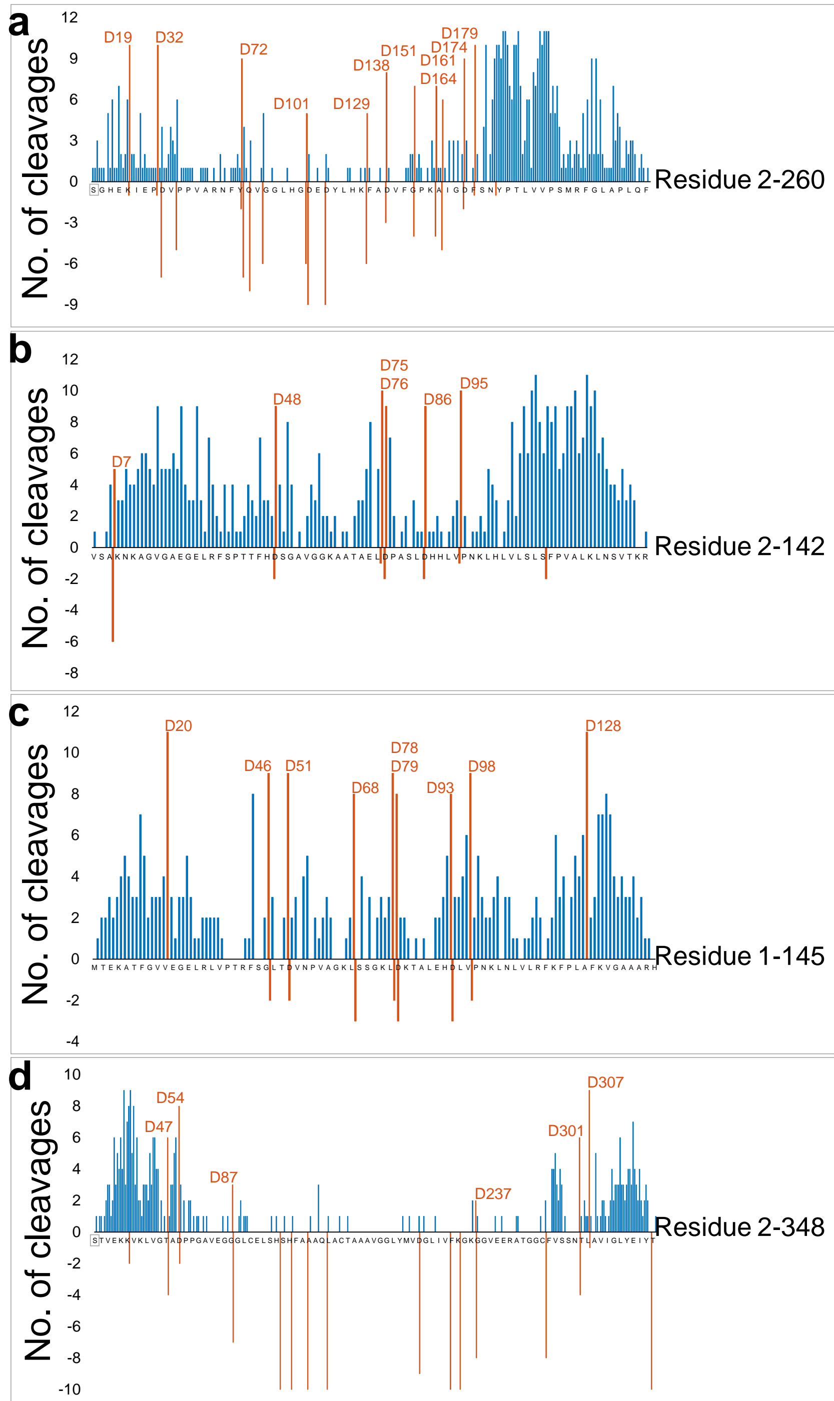
Supplementary Figure S5. Boxplots of the obtained number of cleavages by protein and instrument type. Sample size n for the respective boxes: CA/QTOF 4; CA/Orbitrap 5; CA/FTICR 2; HBα/QTOF 4; HBα/Orbitrap 5; HBα/FTICR 2; HBβ/QTOF 4; HBβ/Orbitrap 5; HBβ/FTICR 2; ADH/QTOF 4; ADH/Orbitrap 4; ADH/FTICR 2; GroEL/Orbitrap 3; GroEL/FTICR 2; BR/Orbitrap 3; BR/FTICR 2; AqpZ_DDM/Orbitrap 4; AqpZ_DDM/FTICR 1; AqpZ_C8E4/Orbitrap 2; AqpZ_C8E4/FTICR 1. Grey dashed lines in the left- and right-hand panels show the average number for water-soluble and membrane proteins, respectively. The boxplot shows the minimum and maximum values as whiskers, the mean value is indicated by a dotted line, and the solid line represents the median. Individual data points are indicated as black dots.



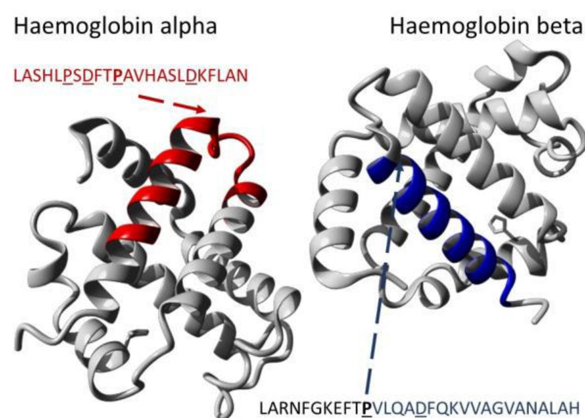
Supplementary Figure S6. Representative fragment spectra of **(a)** CA (Synapt XS, Lab 8), **(b)** HB alpha subunit (Orbitrap UHMR, Lab 2), **(c)** HB beta subunit (Solarix FTICR, Lab 5), **(d)** ADH (QTOF Water G2Si, Lab 1), **(e)** GroEL (Orbitrap UHMR, Lab 5), **(f)** bacteriorhodopsin (Solarix FTICR, Lab 3), **(g)** AqpZ (in C8E4, Orbitrap UHMR, Lab 2), and **(g)** AqpZ (in DDM, Orbitrap UHMR, Lab 2).



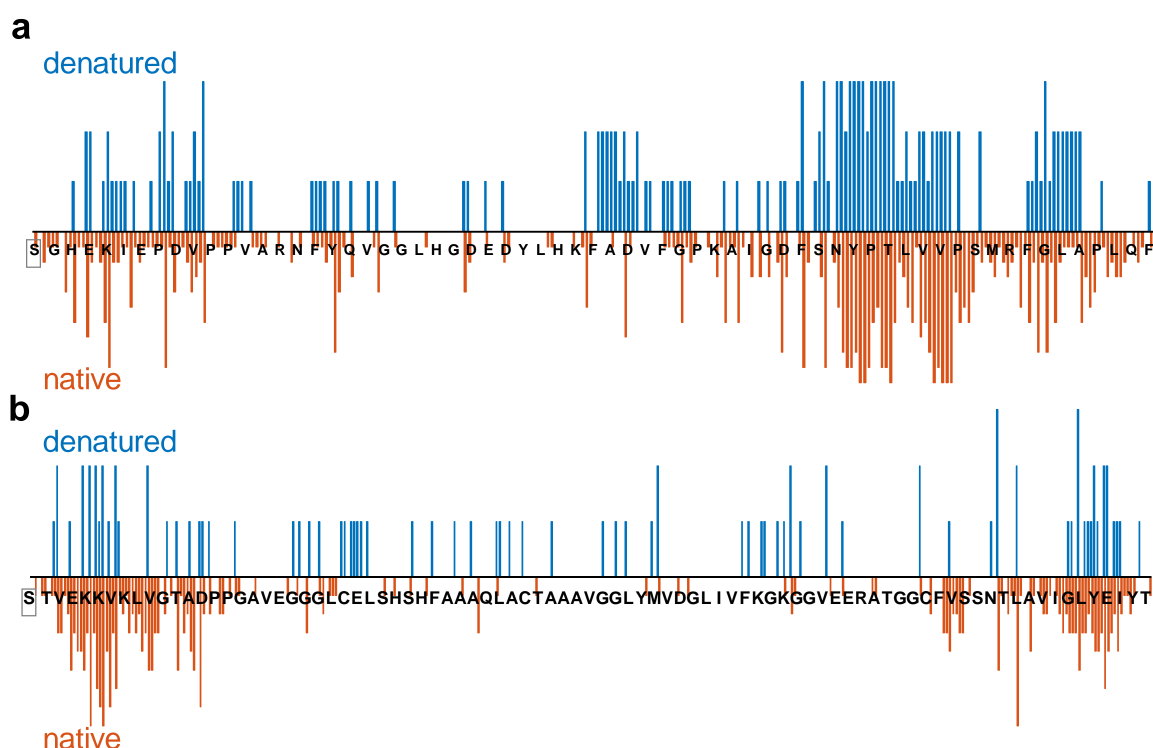
Supplementary Figure S7. Number of observations of site-specific cleavage in (**a**) GroEL, (**b**) bacteriorhodopsin (detergents used: C8E4 or octyl glucoside), and (**c**) aquaporin Z (upper panel – DDM as detergent; lower – C8E4). For (**a**) and (**b**) the residue-specific cleavage sites are highlighted in orange, and for all three panels the most common cleavage regions in yellow.



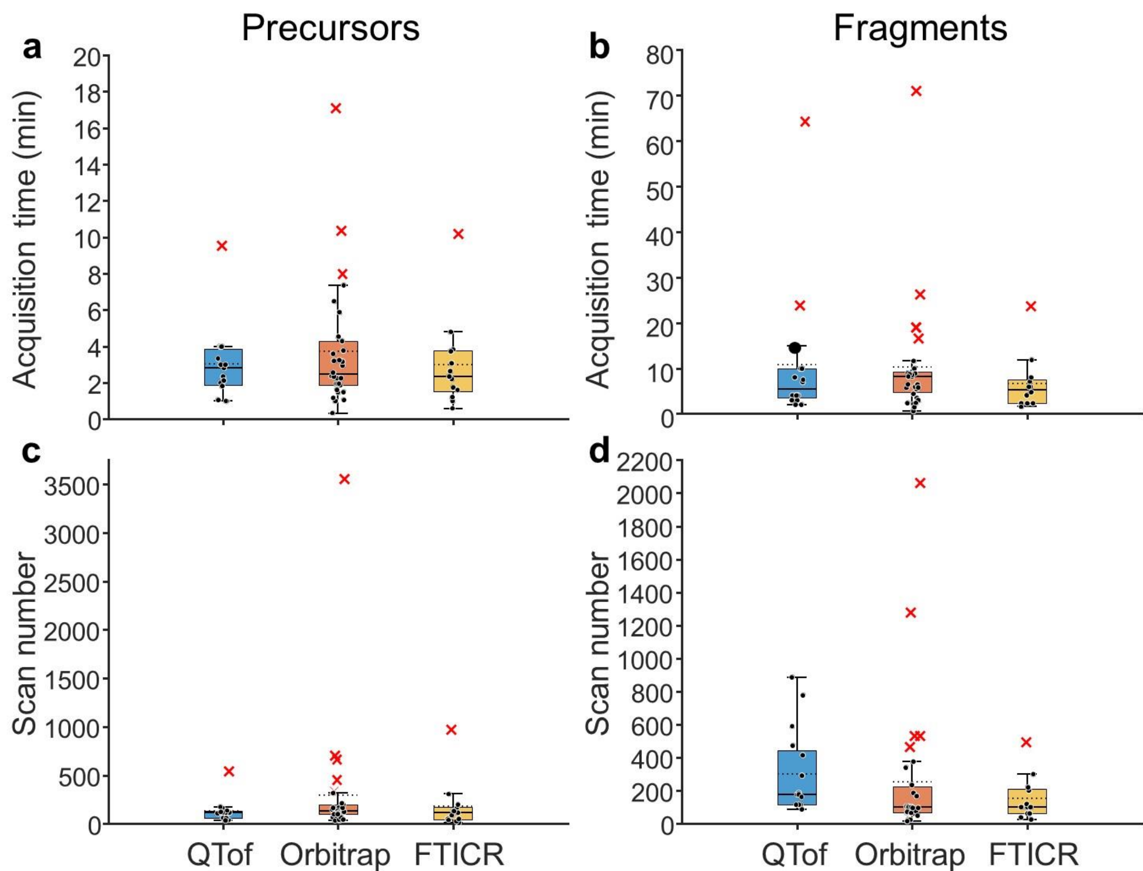
Supplementary Figure S8. Number of observations of site-specific cleavage in (**a**) carbonic anhydrase, (**b**) alpha subunit and (**c**) beta subunit of haemoglobin, and (**d**) alcohol dehydrogenase. Aspartic acid residues are highlighted in orange, and missed cleavages C-terminal to aspartic acid are shown as negative values (*e.g.*, for in panel a, residue D19 has bars with values of +10 and -1, meaning that in 10 datasets cleavage C-terminal to this residue was observed, while in 1 dataset it was not). Amino acid residues highlighted with a grey box were acetylated.



Supplementary Figure S9. Fragmentation 'hotspots' of HBα and HBβ. In both monomers, the main 'hotspot' is located near the C-terminus. In the beta subunit, this region of efficient fragment generation (coloured in blue) contains one Asp and one Pro residue (underlined in the sequence), while for the alpha subunit ('hotspot' in red), it extends slightly further toward the N-terminus and contains two Asp and two Pro residues.



Supplementary Figure S10. Comparison of preferred cleavage sites in native and denatured top-down for **(a)** CA and **(b)** ADH. The bars are normalized by the absolute number of datasets, *i.e.*, 3 datasets for the denatured analysis (upper panels) and 11 (CA) and 10 (ADH) datasets for the native analysis (bottom panels).



Supplementary Figure S11. Boxplots of acquisition time per instrument type for (a) precursor (sample size n: QTOF 15; Orbitrap 30; FTICR 13) and (b) fragment spectra (sample size n: QTOF 16; Orbitrap 31; FTICR 12), and number of scans averaged per instrument type for (c) precursor (sample size n: QTOF 15; Orbitrap 30; FTICR 13) and (d) fragment spectra (sample size n: QTOF 16; Orbitrap 31; FTICR 12). All boxplots show the minimum and maximum values as whiskers, the mean value is indicated by a dotted line, and the solid line represents the median. The boxes indicate 25% of the upper and lower quartile respectively. Individual data points are indicated as black dots, with outlier values in red. Values are considered outliers if they are 1.5-times larger or smaller than the upper or lower quartile, respectively. The single data points per instrument type for the visualisation can be found in **Supplementary Table 1-3**.

QTOF			
Precursor		Fragments	
Acquisition **time (min)**	Scan number	Acquisition time (min)	Scan number
2.8	163	8.03	474
1	58	3	176
3.35	40	10	187
4	118	4	118
1.05	61	7	414
1	58	2	117
3	35	7.5	89
4	118	4	118
2.35	137	15	886
2	117	2	117
9.7	571	24.5	290
4	118	4	118
1.82	107	10	590
3	176	3	176
2.12	124	65	777
		4	163

Supplementary Table S1: Acquisition time and scan number values for precursor and fragment spectra acquisition on QTOF instruments reported in this study. For respective visualization as a boxplot see **Supplementary Figure S11**.

Orbitrap			
Precursor		Fragments	
Acquisition **time (min)**	Scan number	Acquisition time (min)	Scan number
10.53	738	5.7	65
1.94	100	8.83	100
0.33	168	9.37	108
1	32	6.49	187
8.16	168	8.16	168
3.2	157	2.4	27
2.94	150	8.89	99
1.05	137	3.49	376
3.78	68	19.74	550
3.16	35	10	96
2.36	116	2.2	25
1.96	100	8.99	100
1.46	124	0.62	71
1.87	84	19.74	550
3.24	168	11.63	107
4.54	200	5.5	63
1.48	45	8.88	100
1.16	65	6.09	77

7.37	362	71.69	2080
1.94	100	5.9	68
6.47	3589	1.51	17
5.88	166	27.01	1296
2.24	110	8.48	96
2.33	100	8.74	100
3.59	693	8.26	235
17.27	483	6.44	74
4.3	211	4.4	50
2.25	100	3	340
2.57	316	17.27	483
1.61	45	5.78	66
		2.42	96

Supplementary Table S2: Acquisition time and scan number values for precursor and fragment spectra acquisition on Orbitrap instruments reported in this study. For respective visualization as a boxplot see **Supplementary Figure S11**.

FTICR			
Precursor		Fragments	
Acquisition **time (min)**	Scan number	Acquisition time (min)	Scan number
3.83	307	4.75	301
2.2	51	11.84	202
2.35	162	2.3	62
1	12	5.91	100
3.06	134	2.3	62
3.74	200	5.91	100
2.63	114	4.1	39
10.35	1000	7.08	120
1.74	88	7.98	222
0.59	25	2.33	100
1.6	40	1.56	27
1.2	50	24.32	512
4.8	131		

Supplementary Table S3: Acquisition time and scan number values for precursor and fragment spectra acquisition on FTICR instruments reported in this study. For respective visualization as a boxplot see **Supplementary Figure S11**.

Protein	UniProt ID	Sigma catalogue no.	Sequence	Modifications (common)	Modifications (rare)
Carbonic anhydrase	P00921	C2624	SHHWGYGKHNGP EHWHKDFPIANGE RQSPVDIDTKAVV QDPALKPLALVYGE ATSRRMVNNGHSF NVEYDDSQDKAVL KDGPLTGTYRLVQF HFHWGSSDDQGS EHTVDRKKYAAELH LVHWNTKYGDFGT AAQQPDGLAVVG VFLKVGDANPALQ KVLDALDSIKTKGK STDFPNFDPGSLLP NVLDYWTYPGSLT TPPLLESVTWIVLKE PISVSSQQMLKFRT LNFNAEGEPELLML ANWRPAQPLKNR QVRGFPK	-M1; Acetylation S2; 2x non-covalent Zn ligands	R57Q variant; Phosphorylatio n
Haemoglobin alpha subunit	P01966	H2500	VLSAADKGNVKAA WGKVGGHAAEYG AEALERMFLSFPTT KTYFPHFDLSHGSA QVKGHGAKVAAAL TKAVEHLDDLPGAL SELSDLHAHKLRVD PVNFKLLSHSLLVTL ASHLPSDFTP	-M1; Haem-b ligand	
Haemoglobin beta subunit	P02070	H2500	MLTAEEKAAVTAF WGKVKVDEVGGE ALGRLLVVYPWTQ RFFESFGDLSTADA VMNNPKVKAHGK KVLDSFSNGMKHL DDLKGTFAALSELH CDKLHVDPENFKLL GNVLVVVLARNFG KEFTPVLQADFQKV VAGVANALAHRYH	Haem-b ligand	

Alcohol dehydrogenase	P00330	A3263	SIPETQKGVIFYESH GKLEYKDIPVPKPK ANELLINVKYSGVC HTDLHAWHGDWP LPVKLPLVGGHEGA GVVVGMGENVKG WKIGDYAGIKWLN GSCMACEYCELGN ESNCPHADLSGYT HDGSFQQYATADA VQAAHIPQGTDLA QVAPILCAGITVYK ALKSANLMAGHW VAISGAAGGLGSLA VQYAKAMGYRVLG IDGGEGKEELFRSIG GEVFIDFTKEKDIVG AVLKATDGGAHGV INVSVSEAAIEASTR YVRANGTTVLVGM PAGAKCCSDVFNQ VVKSISIVGSYVGN RADTREALDFFARG LVKSPIKVVGLSTLP EIYEKMEKGQIVGR YVVDTSK	-M1; Acetylation S2; 2 non-covalent Zn ligands per subunit	V58T variant; I151V variant

GroEL	P0A6F5	C7688	AAKDVKFGNDARV KMLRGVNVLADAV KVTLGPKGRNVVL DKSFGAPTITKDGV SVAREIELEDKFEN MGAQMVKEVASK ANDAAGDGTTTAT VLAQAIITEGLKAV AAGMNPMDLKRG IDKAVTAAVEELKA LSVPCSDSKAQVGT ISANSDETVGKLIAE AMDKVGKEGVITV EDGTGLQDELDVV EGMQFDRGYLSPY FINKPETGAVELESP FILLADKKISNIREM LPVLEAVAKAGKPL LIIAEDVEGEALATL VVNTMRGIVKVAA VKAPGFGDRRKAM LQDIATLTGGTVISE EIGMELEKATLEDL GQAKRVVINKDTT TIIDGVGEEAAIQG RVAQIRQQIEEATS DYDREKLQERVAKL AGGVAVIKVGAAT EVEMKEKKARVED ALHATRAAVEEGV VAGGGVALIRVASK LADLRGQNEDQNV GIKVALRAMEAPLR QIVLNCGEEPSVVA NTVKGGDGNYGY NAATEEYGNMID MGILDPTKVTRSAL QYAASVAGLMITTE CMVTDLPKNDAAD LGAAGGMGG	-M1	
Bacteriorhodopsin	P02945	B0184	QAQITGRPEWIWL ALGTALMGLGTLYF LVKGMGVSDPDAK KFYAITTLVPAIAFT MYLSMLLGYGLTM VPFGGEQNPIYWA RYADWLFTTPLLLL DLALLVDADQGTIL ALVGADGIMIGTGL VGALTKVYSYRFV WWAISTAAMLYIL YVLFFGFTSKAESM RPEVASTFKVLRNV TVVLWSAYPVVWL IGSEGAGIVPLNIET LLFMVLDVSAKVGF GLILLRSRAIFGEAE APEPSAGDGAAAT SD	-Propeptide(1-13); N6-(retinylidene)lysine K229	Pyrrolidone carboxylic acid Q1/Q14; -D262 (C-Terminus)

Aquaporin Z	P60844	N/A	MFRKLAAECFGTF WLVFGGCGSAVLA AGFPELGIGFAGVA LAFGLTVLTMAFAV GHISGGHFNPAVTI GLWAGGRFPAKEV VGYVIAQVVGGIVA AALLYLIASGKTGFD AAASGFASNGYGE HSPGGYSMLSALV VELVLSAGFLLVIHG ATDKFAPAGFAPIA IGLALTLIHLISIPVT NTSVNPARSTAVAI FQGGWALEQLWF FWVVPIVGGIIGGLI YRTLLEKRD	N-terminal formylation; oxidations

Supplementary Table S4: Proteins used in the study with their UniProt ID, sequence, and rare and commonly (*i.e.*, (near-)universally) reported modifications.

Residue	Carbonic anhydrase		Haemoglobin alpha subunit		Haemoglobin beta subunit		Alcohol dehydrogenase	
	#	%cleavages	#	%cleavages	#	%cleavages	#	%cleavages
A	17	6.4	20	34.5	16	27.8	35	2.3
R	9	15.2	3	21.2	4	13.6	8	10.0
N	13	27.3	3	33.3	7	29.9	11	8.2
D	19	58.4	8	81.8	9	82.8	16	28.1
C	0		0		1	45.5	8	1.3
E	11	44.6	5	65.5	8	38.6	20	18.0
Q	12	7.6	1	0	3	21.2	9	6.7
G	20	8.6	9	30.3	11	14.0	44	4.8
H	11	7.4	10	41.8	6	22.7	10	16.0
I	5	36.4	0		0		21	15.7
L	26	24.1	20	31.4	17	20.9	24	11.7
K	18	20.2	11	28.1	13	22.4	24	20.8
M	3	9.1	1	18.2	2	9.1	6	5.0
F	11	9.1	7	42.9	10	19.1	8	2.5
P	19	20.1	6	31.8	4	4.5	13	10.8
S	16	11.9	13	22.4	5	0.0	21	8.6
T	14	29.9	8	29.5	6	13.6	14	4.3
W	7	29.9	1	54.5	2	22.7	5	16.0
Y	8	26.1	3	18.2	2	9.1	14	11.4
V	20	21.4	12	39.4	18	26.8	36	11.4

Supplementary Table S5. Number of each residue type in the sequences of carbonic anhydrase, haemoglobin, and ADH, along with the overall percentage of observed cleavages C-terminal to each residue type across our dataset. For example, carbonic anhydrase has 19 aspartic acid residues, so across 11 datasets, a maximum of 209 cleavage events could have been observed. In practice, 122 (58.4%) such events were observed. Aspartic acid residues are highlighted in orange, given the significantly higher-than-average percentage of D-X cleavages we observed.

	Correct database	*B. subtilis* (decoy)	*B. subtilis* + target sequence
Carbonic anhydrase (bovine)	1.42E-18	1.81E+00	1.36E-18
Haemoglobin-α (bovine)	5.31E-45	6.64E+00	5.28E-45
Haemoglobin-β (bovine)	2.57E-42	7.28E+00	1.28E-42
ADH (yeast)	1.83E-24	5.46E-01	1.93E-24
GroEL (*E. coli*)	Not found	Not found	Not found
BR (*H. salinarum*)	2.24E-22	6.18E+00	6.12E-22
Aquaporin Z (*E. coli*)	2.01E-06	4.37E+01	2.03E-06

Supplementary Table S6. Protein identification using the Discovery Mode of the MASH Native software (all data from Laboratory 5, acquired on an Orbitrap UHMR). Each search was run three times: First against the correct database (successful identifications except for GroEL; E-values shown in table), then against the *B. subtilis* database as a decoy (negative control; only spurious hits found with high E-values), and finally against a modified *B. subtilis* database where we manually added the correct protein sequences, resulting again in successful identifications (with GroEL again being the exception), with similar E-values to the first search.

	Instrument	Tune parameters source	Tune parameters instrument	fragmentation parameters	General information
1	Waters Synapt G2Si	spray voltage 0.7 - 1.3 kV; source temp 50 °C	Sampling cone 50-200 V; Trap gas setting 3-8 mL/min	Trap CID	most settings were kept default
2	Q-Exactive HF quadrupole-Orbitrap (ultrahigh mass range research modifications)	spray voltage 1.5 kV	-150 V in-source trapping (IST); capillary temperature 200 °C. gas pressure 7; resolution 15000	150 V HCD to isolated monomer, In-source activation (capillary temp 300 °C; gas pressure 3); resolution 240000	
3	Bruker 12 T SolariX FT-ICR	TriVersa NanoMate source; spray voltage 1.8 - 1.9 kV; gas pressure 0.4 - 0.6 psi; dry gas 120 °C	Funnel 1 150 V; Skimmer 1 130 V Funnel RF amplitude 300 Vpp; ion accumulation 700 ms; Collision cell frequency 1.4 MHz; Collision RF amplitude 600 Vpp; Transfer Optics time of flight 3 ms; Transfer Optics frequency 2 MHz; Transfer Optics RF Amplitude 450 Vpp; Sweep excitation power 40%; Gas control 40%	broadband CID without isolation; 60 and 70 V	
4	Waters Synapt G2Si	spray voltage 1.0 kV; source temp 80 °C	Sampling cone 40-100 V; source offset 40 V	Sampling cone 100 V; Trap CID 130 V; quadrupole MS profile was adjusted to highest transmission efficiency (MS1 500, MS2 1000, MS3 2000, Dwell time 25, Ramp time 25)	
5a	Bruker Solarix FT-ICR	spray voltage 0.9 - 1.5 kV	Native: Skimmer voltage at 150V; Capillary Exit at 280V; Deflector Plate at 280V; Funnel 1 at 200V; CAD at 0V; Collision Voltage (entrance) at -15V; Resolution at 32K Complex up: Skimmer voltage at 180V; Capillary Exit at 280V; Deflector Plate at 230V; Funnel 1 at 200V; CAD at 15V;	Skimmer voltage at 180V; Capillary Exit at 280V; Deflector Plate at 230V; Funnel 1 at 200V; CAD at 30V; Collision Voltage (entrance) at -22V; Resolution at 4M	parameters were kept as soft as possible

			Collision Voltage (entrance) at -15V; Resolution at 128K	
5b	Orbitrap UHMR	spray voltage 1 - 2 kV	Resolution 12500	Resolution 100000, HCD 27V
6	Q Exactive™ UHMR Hybrid Quadrupole-Orbitrap™	spray voltage 1.6- 2 kV	Native complex: Electrospray voltage: 2.0 kV; In-source CID: 0 eV; Microscans: 5; Resolution: 1,563; AGC: 1e6; Maximum Inject Time: 50 ms; Detector m/z mode: High m/z; Ion transfer m/z mode: High m/z; Trapping gas pressure: 10.0; In-source trapping: ON, -25 eV; Extended trapping energy: 50 eV Complex up: Electrospray voltage: 1.6 kV; In-source CID: 0 eV; Microscans: 5; Resolution: 1,563; AGC: 1e6; Maximum Inject Time: 100 ms; Detector m/z mode: High m/z; Ion transfer m/z mode: High m/z; Trapping gas pressure: 5.0; In-source trapping: ON, -30 eV; Extended trapping energy: 50 eV	Isolation window: 2500-4250 *m/z;* Electrospray voltage: 1.6 kV*;* In-source CID: 0 eV*;* HCD activation energy: 25 NCE*;* Microscans: 5*;* Resolution: 100,000*;* AGC: 1e6*;* Maximum Inject Time: 100 ms*;* Detector *m/z* mode: Low *m/z;* Ion transfer *m/z* mode: Low *m/z;* Trapping gas pressure: 2.0*;* In-source trapping: ON, -30 eV, Extended trapping energy: 50 eV
7	Q Exactive UHMR Hybrid Quadrupole-Orbitrap	spray voltage 1.2- 1.8 kV	Capillary temperature=250C; S-lens RF=200V; SID=5V; IST=0 (280 to eject monomer subunits); HCD gas=1.0; HCD NCE=0 (for MS1)	In-source fragmentation 280 V; All Ion Fragmentation; HCD 140 NCE, Resolution 280000

8a	Waters Synapt XS	spray voltage 1.0 - 1.6 kV	Cone voltage 50-100 V; Source temperature 30 °C; Trap gas flow 4-8 mL/min; Stepwave1RFoffset 250; Stepwave2RFoffset 600; tuned for high mass resolution	Cone voltage 150 V; Monomer isolation; Trap CID	most settings were kept default
9	Agilent 6545XT SLIM QtoF	150 nl/min Agilent nano source	critical parameters are gas temperature, fragmentor, skimmer, Vcap, collision energy voltages.	All ion fragmentation; Critical parameters are gas temperature, fragmentor, skimmer, Vcap, collision energy voltages.	

Supplementary Table S7: Selected tuning parameters for fragmentation of ADH for all lab/instrument combinations in this study.

Supplementary Protocol S1:

Protocol issued to participants at the start of the study

1. Objective:

Determine the mass of a range of standard proteins and complexes in their most native form. Use this information to determine stoichiometry, including potential ligands or modifications if possible. In a second step, eject monomers from the complexes (if applicable) and perform tandem MS to obtain detailed sequence information from these natively ionised proteins. Specific questions to be answered are: (1) Can a robust, 'universal' set of protocols and benchmarks be produced for sequencing of monomers from natively ionised protein complexes? (2) Given a common set of sample preparation methods and standard proteins, how much will results vary when the experiments are carried out by ten or so different labs? (3) How much of this variation can be attributed to specific factors such as instrument used, prior experience of operators, or factors inherent to specific proteins?

2. General protocol:

a. Perform native MS of the protein standards listed below, following the protocols detailed in Sections 4.2 and 4.3. Note the deconvoluted mass, peak width (as this can be indicative of residual salt, solvent, or ligand binding), and charge states observed.
b. Use the obtained mass to calculate the stoichiometry (or stoichiometries) of the observed complex(es) and note any 'excess' or 'missing' mass that might be due to modification or ligand binding.
c. For monomeric samples (carbonic anhydrase; bacteriorhodopsin) directly apply tandem MS after native ionisation to obtain sequence information. The choice of fragmentation method (CID/HCD/ECD/UVPD/…) is left up to each participant; however, we ask that, if possible, at least one purely collision-based (ISD/CID/HCD) experiment be performed as a baseline, to facilitate comparison between instruments/labs.
d. For complexes, apply (collisional) activation to eject highly charged monomers, and fragment these in turn to obtain sequence information. This should be done in a true MS^3 approach if the instrument allows it; alternatively, two MS^2 experiments might have to be performed – one in which the complex is isolated and monomer ejection is demonstrated, and a second in which monomers are ejected prior to precursor selection, followed by MS^2 (essentially pseudo-MS^3) of the monomer(s) which were demonstrated to originate from the complex in the first MS^2 experiment. Again, for fragmentation of the monomer, the choice of method is up to each participant, although we ask for at least one collision-based experiment for each complex.
e. Provide an annotated fragmentation spectrum and a fragmentation map summarising the observed fragments for each monomer.

3. Samples:

a) From Sigma (we request that each participating lab purchase these directly from Sigma in order to minimise variability due to shipping and storage):
    a. Carbonic anhydrase (Sigma part number C2624; UniProt accession code P00921)
    b. Haemoglobin (H2500; P01966 (alpha) and P02070 (beta))
    c. ADH (A3263; P00330)
    d. GroEL (C7688; P0A6F5)

e. Bacteriorhodopsin (B0184; P02945)

b) Supplied directly by Initiative:

Aquaporin Z (Uniprot accession code P60844)

i. 2.3 µM in 200 mM ammonium acetate + 0.5% C8E4 (50 µL)
ii. 3.4 µM in 200 mM ammonium acetate + 0.025% DDM (50 µL)

Note: Although they should be mostly accurate, small discrepancies between canonical sequences from UniProt and experimental observations have been reported in some previous top-down MS studies and so this possibility must be taken into account during data analysis.

4. Sample preparation and native electrospray ionisation:

We strongly recommend reviewing the protocols detailed in [Donnelly *et al.*, *Nature Methods*, 2019, **16**, 587-594] as a starting point for sample desalting, native ionisation, and default settings for a range of commercial mass spectrometers, in particular Protocol 2a, 2b, 4b, and Supplementary Protocols 2a, 2b, and 4b.

For membrane proteins specifically, we recommend also reviewing the guidelines and procedures in [Laganowsky *et al.*, *Nature Protocols*, 2013, **8**, 639-651].

4.1. Background

In 2016, Leney and Heck (*JASMS*, **28**, 5-13) formulated a definition of native mass spectrometry as '*the mass spectrometric analysis of biomolecules that are prior to their ionization in their most native-like state*'. Ideally, this would require a buffered solution with appropriate ionic strength and concentration of species such as Na^+, K^+, Ca^{2+}, Cl^-, *etc*. In practice, production of gas-phase ions is achieved by (nano-)electrospray ionisation, which is incompatible with non-volatile salts such as those present in traditional biochemistry buffers. Instead, ammonium acetate is typically used to provide the required ionic strength – this decomposes during the ESI process, resulting in well-desolvated protein ions. A benefit specific to the ammonium ion is its similar radius to Na^+, allowing it to displace this particular metal cation, which is usually abundant and otherwise difficult to remove from intact proteins. Some degree of ion activation in the ion source or initial vacuum stages of the instrument might be required for desolvation and declustering, although excessive activation needs to be avoided, *i.e.*, minimal activation voltages to achieve acceptable spectra should be used.

4.1.1. Sample storage

We recommend that protein solutions be used relatively quickly after making them. If experiments cannot be performed or finished on the same day, storing solutions for up to a week at 4 °C should not result in significant degradation. Aliquoting and freezing (ideally at -80 °C) water-soluble protein samples is possible; however, this is not recommended for detergent-containing membrane protein solutions, as it might result in aggregation. The time between making the sample solution and performing MS analysis should be noted, as well as storage conditions.

4.1.2. Charge state distributions in native MS:

In native MS, a narrow range of protein charge states is typically observed, with these charge states being lower than those obtained in ESI under denaturing conditions of proteins of similar mass. For example, the haemoglobin (64 kDa) and ADH (148 kDa) tetramers tend to occur in a range of 4-5 charge states, centred around 16+ (4000 *m/z*) and 26+ (5700 *m/z*) respectively. It should be noted that the exact charge states observed can differ somewhat between instruments, but the general pattern should hold. Observation of groups of peaks at significantly higher or lower *m/z* can indicate inadvertent monomer ejection, which typically results in a highly charged monomer at low *m/z*, and a residual charge-stripped (n-1)mer at higher *m/z* than the original complex. This behaviour indicates that conditions are too harsh and lowering of at least one acceleration voltage might be required.

4.1.3. Desalting:

In addition to the elimination of non-volatile salts from native MS buffers, it is important to remove residual salt from protein samples before electrospray. This is because, during sample preparation, a 'salting out' step is nearly always used, resulting in a significant amount of Na^+ remaining in the sample. Protein desalting can be achieved in a number of ways, including size-exclusion chromatography, buffer exchange columns (*e.g.* BioRad P6) or concentrator columns (*e.g.* Amicon centrifugal filters). Choice of column is dependent on the mass of the protein or complex, and this must be taken into account to ensure efficient desalting without excessive sample loss. Regardless of the method used, care must be taken to wash the system with ammonium acetate solution prior to protein desalting. For the latter two methods, several rounds of desalting might be needed to desalt certain samples.

4.1.4. Membrane proteins

Membrane proteins require detergent to mimic their native environment and stabilise them in aqueous solution. Using standard, 'gentle' instrument settings for native MS, it is not expected that protein signal will be observed. This is because a mixture of empty and protein-containing micelles is sprayed under these conditions. Significantly higher activation voltages are required in the source (and possibly collision cell if in-source activation is insufficient) to strip detergent molecules away and liberate the membrane protein, yielding again the narrow charge state distribution typical for native MS. If no protein signal is observed using a particular detergent, a possible cause is that the instrument used is unable to provide sufficient collisional activation to break up adducts of the detergent with the membrane protein. In this case, use of an alternative that requires less activation energy can potentially ameliorate the issue.

4.2. Instructions for native ionisation of soluble proteins (carbonic anhydrase, haemoglobin, ADH, GroEL)

We recommend use of 200 mM aqueous ammonium acetate as a working solution for native MS, and static nano-ESI in positive ion mode using borosilicate glass capillaries.

After dissolving the lyophilised proteins in aqueous ammonium acetate, they will likely require desalting to produce useful signal in ESI. We recommend that participants use whatever desalting

method they are most comfortable with. Depending on the protein and amount of non-volatiles in the sample, multiple rounds of desalting might be needed to achieve good spectra.

Typical final protein concentrations in native MS are around 10 µM (monomer concentration). While sensitivity varies somewhat between instruments and complexes, we recommend this as a starting point.

GroEL requires additional sample preparation steps, as detailed in Section 4.4.1.

4.3. Instructions for native ionisation of membrane proteins (bacteriorhodopsin, aquaporin Z)

We recommend dissolving proteins in 200 mM aqueous ammonium acetate with 2 x critical micelle concentration (CMC) of detergent as a working solution for native MS, and static nano-ESI in positive ion mode using glass capillaries.

We recommend the use of tetraethylene glycol monooctyl ether (C8E4; Anatrace part number T350; CMC = 0.25% or 8.1 mM) as an optimal detergent that requires a relatively low activation energy to remove (*vide supra*). n-Dodecyl β-D-maltoside (DDM; Sigma part number D4641; CMC = 0.0087% or 0.17 mM) can also be used but generally requires higher activation energy to remove and may give a lower signal/noise ratio.

Desalting will again be necessary; while this can be done using a variety of methods, care must be taken that the detergent micelles are not diluted or, conversely, concentrated in the process, as this can lead to significant protein destabilisation or peak broadening, respectively. See [Laganowsky *et al.*, *Nature Protocols*, 2013, **8**, 639-651] for more details.

4.4. Additional protein-specific instructions

4.4.1. GroEL sample preparation:

The 800 kDa GroEL 14mer requires a more elaborate sample preparation protocol compared to the three smaller soluble proteins. We recommend the protocol as used by several groups and summarised by Campuzano and Giles (*Nanoproteomics*, Springer, 57-70). In this protocol, buffer A consists of (100 mM ammonium acetate, 50 mM KCl, 0.5 mM EDTA, 1 mM ATP, and 5 mM $MgCl_2$)

a. Dissolve 1 mg of GroEL in 1 mL of refolding buffer A, and vortex gently at room temperature for 1 h.
b. Add 240 µL of cold methanol, previously chilled on ice, to the 1-mg/mL solution of GroEL. Gently vortex this ∼20% (v/v) of methanol solution for an additional 1 h at room temperature
c. Add 600 µL of cold acetone, previously chilled on ice, to the ∼20% (v/v) methanol/GroEL solution. Upon addition of the cold acetone, the solution becomes cloudy. This is the intact GroEL precipitating out of solution. This is normal and is part of the purification–refolding procedure.
d. Centrifuge the cloudy solution for 10 min at 8,000 × g
e. Remove the supernatant, and resuspend the pellet in 160 µL of refolding buffer A to make a stock solution of 8 µM, based on the 14-mer mass of 802 kDa. Store this concentrated stock solution at −80°C for up to 1 year

f. To prepare a working GroEL solution, this stock solution requires buffer exchanging and diluting to 1 µM.

4.4.2. Aquaporin Z sample preparation:

Samples in ammonium acetate are ready to inject directly and do not require additional sample preparation.

5. Data analysis

A variety of software packages and protocols exist for native and top-down MS data analysis. Rather than recommend any specific method, we encourage participants to use the method(s) they are most familiar with. An overview of some excellent relevant software packages can be found on the website of the Consortium for Top-Down Proteomics (https://www.topdownproteomics.org/resources/software/). For manual spectrum analysis, Protein Prospector offers a user-friendly interface for generating a list of possible fragments based on a user-supplied sequence (http://prospector.ucsf.edu/prospector/cgi-bin/msform.cgi?form=msproduct).

Supplementary survey S1: Post-study participant survey questions

Please indicate how challenging you found the following steps to be

(Scale of 1 to 5; 1 = very easy; 5 = very challenging; N/A = not attempted)

1) Solubilising lyophilised powder (soluble protein)

2) Solubilising lyophilised powder (membrane protein)

3) GroEL sample preparation

4) Protein desalting/buffer exchange

5) Obtaining a stable nano-ESI spray for soluble proteins

6) Obtaining a stable nano-ESI spray for membrane proteins

7) Removing detergent to release intact membrane protein or complex (DDM)

8) Removing detergent to release intact membrane protein or complex (C8E4)

9) Monomer ejection (soluble protein)

10) Monomer ejection (Aquaporin Z)

11) Obtaining extensive backbone fragmentation

12) Data analysis