Covalent Cross-Linking as an Enabler for Structural Mass Spectrometry

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Table S1. Synapt G2-Si HDMS (Waters) - Instrument default setup.

Capillary (ESI)	1.6 kV
Sampling cone	5 V
Source offset	1 V
Source temperature	80 °C
Desolvation temperature	100 °C
Cone gas flow	50 $L.h^{-1}$
Desolvation gas flow	500 $L.h^{-1}$
Nebulizer	3 bar
Trap collision energy	2 V
Transfer collision energy	2 V
Trap/transfer gas pressure (Ar)	2.2×10^{-2} mbar (2 $mL.min^{-1}$)
He cell gas pressure (He)	3.4 mbar (180 $mL.min^{-1}$)
IM cell gas pressure (N_2)	2.75 mbar (90 $mL.min^{-1}$)
Trap DC entrance	3 V
Trap DC bias	35 V – 100 V
Trap DC exit	0 V
IMS DC Entrance	20 V
He Cell DC	50 V
He Exit	20 V
IMS DC Bias	3 V
IMS DC exit	0 V
Transfer DC entrance	5 V
Transfer DC exit	15 V
IM wave speed	1000 $m.s^{-1}$
IM wave height	40 V
Trap wave speed	311 $m.s^{-1}$
Trap wave height	4 V
Transfer wave speed	380 $m.s^{-1}$
Transfer wave height	4 V

Table S2. timsTOF (Bruker) - Instrument default setup.

Capillary (ESI)	4.5 kV
End Plate Offset	2.5 kV
Dry gas	8 L/min
Dry temp	220 °C
Δ1	-20 V
Δ2	-270 V
Δ3	18 V
Δ4	250 V
Δ5	0 V
Δ6	70 V
Funnel 1/2 RF	300 Vpp
Collision Energy	10 eV
$1/K_0$ start	0.71 V.s/cm^2
$1/K_0$ end	1.78 V.s/cm^2
Ramp Time	300 ms
Accumulation Time	20 ms – 300 ms
Tunnel In	2.57 mbar
Tunnel Out	8.06×10^{-1} mbar

Table S3. Synapt G2-Si HDMS (Waters) - List of calibrants.

Molecule	z	m	m/z	Ω(He)
LVVSTQTALA	2	1001.6	501.8	237
QTALVELLK	2	1013.6	507.8	243
HLVDEPQNLIK	2	1304.7	653.4	284
TVMENFVAFVDK	2	1398.8	700.4	298
SLHTLFGDELCK+CAM	2	1418.8	710.4	297
YICDNQDTISSK+CAM	2	1442.6	722.3	294
LGEYGFQNALIVR	2	1478.8	740.4	306
DDPHACYSTVFDK+CAM	2	1553.6	777.8	310
MPCTEDYLSLILNR+CAM	2	1723.8	862.9	337
YNGVFQECCQAEDK+CAM	2	1746.8	874.4	318
DDPHACYSTVFDK+CAM	3	1553.7	518.9	321
KVPQVSTPTLVEVSR	3	1638.9	547.3	335
DAFLGSFLYEYSRR	3	1722.9	575.3	372
DAIPENLPPLTADFAEDKDVCK+CAM	3	2457.3	820.1	429
Ubiquitin[b]	7	8568	1225	1580
Ubiquitin	8	8568	1072	1622
Ubiquitin	9	8568	953	1649
Ubiquitin	10	8568	858	1732
Ubiquitin	11	8568	778	1802
Bradykinin[d]	1	1059	1060	245
Myoglobin (equine)[e]	13	16952	1305	3136
Myoglobin (equine)	14	16952	1212	3143
Myoglobin (equine)	15	16952	1131	3230
Myoglobin (equine)	16	16952	1061	3313
Myoglobin (equine)	17	16952	998	3384
Myoglobin (equine)	18	16952	943	3489
Myoglobin (equine)	19	16952	893	3570
Myoglobin (equine)	20	16952	849	3682
Myoglobin (equine)	21	16952	808	3792
Myoglobin (equine)	22	16952	772	3815

[a] Bush, M. F., Campuzano, I. D. G. & Robinson, C. V. Ion mobility mass spectrometry of peptide ions: Effects of drift gas and calibration strategies. Anal. Chem. 84, 7124–7130 (2012).

[b] Valentine, S. J., Counterman, A. E. & Clemmer, D. E. Conformer-dependent proton-transfer reactions of ubiquitin ions. Journal of the American Society for Mass Spectrometry 8, 954–961 (1997).

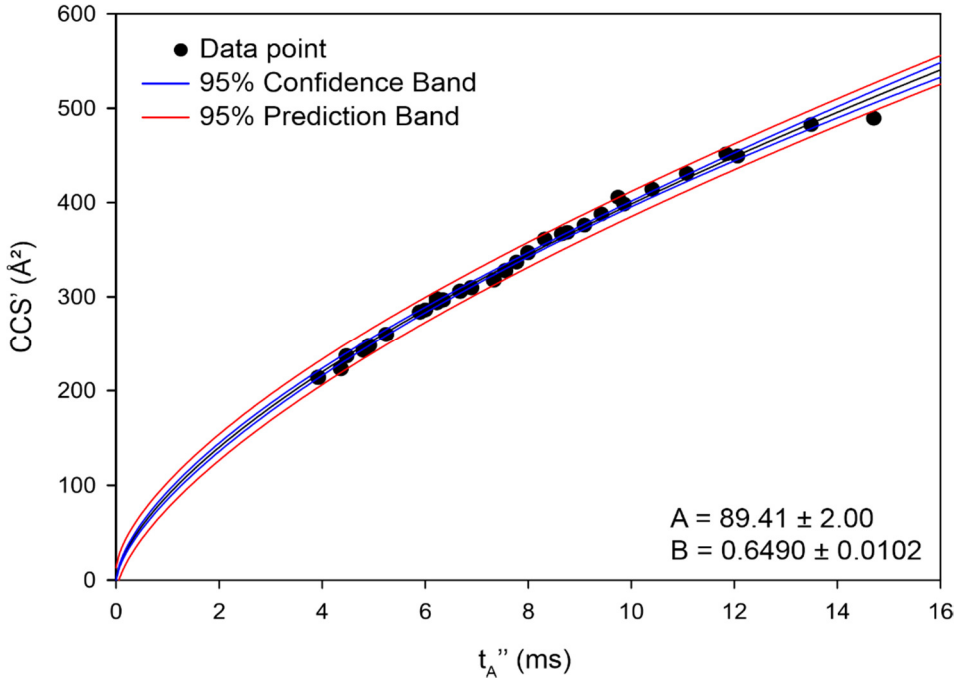
[c] Chen, Y. L., Collings, B. a. & Douglas, D. J. Collision cross sections of myoglobin and cytochrome c ions with Ne, Ar, and Kr. J. Am. Soc. Mass Spectrom. 8, 681–687 (1997).

[d] Counterman, a. E. et al. High-order structure and dissociation of gaseous peptide aggregates that are hidden in mass spectra. J. Am. Soc. Mass Spectrom. 9, 743–759 (1998).

[e] Shelimov, K. B. & Jarrold, M. F. Conformations, unfolding, and refolding of apomyoglobin in vacuum: An activation barrier for gas-phase protein folding. J. Am. Chem. Soc. 119, 2987–2994 (1997).

Figure S1. Synapt G2-Si HDMS (Waters) - Ion mobility calibration.

Calibration curve to convert arrival time t_d measured in nitrogen into collision cross section Ω:

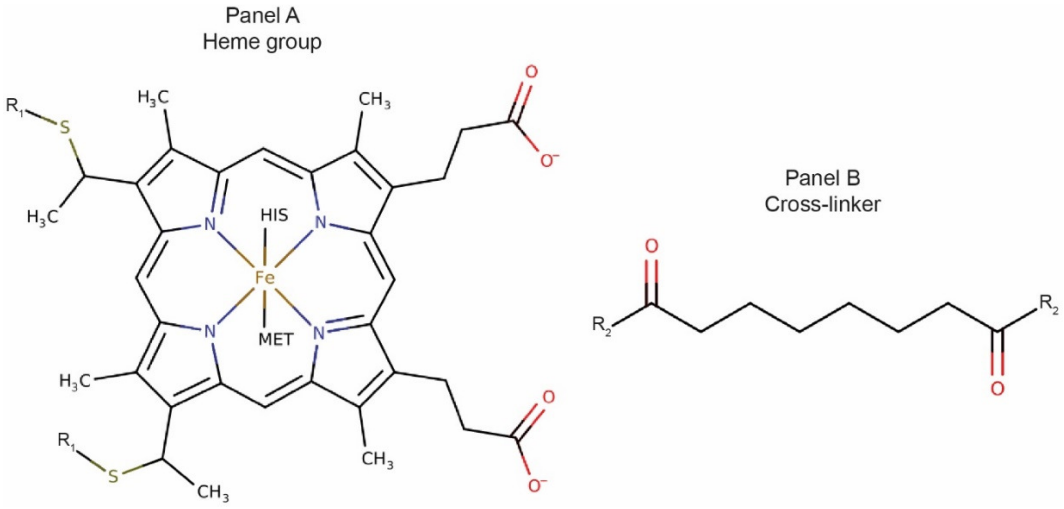


The corrected drift time t_d" is calculated as $t_d - c\frac{\sqrt{\frac{m}{z}}}{1000} - \frac{L_{transfer}}{v_{transfer}}$, where c is equal to 1.57, m/z is the mass-to-charge ratio, $L_{transfer}$ is the length of the transfer cell (130 mm) and $v_{transfer}$ is the wave speed in the transfer cell (380 m.s^{-1} in our case). Reported collision cross sections Ω are converted into Ω' using equation $\Omega' = \Omega.\frac{\sqrt{\mu}}{z}$, where μ is the reduced mass. The fit relies on a power relationship $y = A.x^B$. Using an IMS wave speed of 1000 m/s, a wave height of 40V and a nitrogen pressure of 2.75 mbar in the mobility cell, we obtained the following parameters:

A = 89.41 ± 2.00 and B = 0,6490 ± 0,0102

The prediction error (Ω interval defining 95% chance of finding the true value using the calibration curve, red bands) is 3.5 %, the confidence error (Ω interval defining 95% chance of finding the value for repeated measurements of molecules belonging to the calibration data set, blue bands) is 1.1 %.

Figure S2. Representation of the heme group and BS3 cross-linker and force field parametrization.



[a] Gattuso, H.; Duchanois, T.; Besancenot, V.; Barbieux, C.; Assfeld, X.; Becuwe, P.; Gros, P. C.; Grandemange, S.; Monari, A. Interaction of iron II complexes with B-DNA. Insights from molecular modeling, spectroscopy, and cellular biology, Front. Chem. **2015**, *3*, 1-12.

[b] Charge-Transfer versus Charge-Separated Triplet Excited States of $[Re^{I}(dmp)(CO)_3(His124)(Trp122)]^+$ in Water and in Modified *Pseudomonas aeruginosa* Azurin Protein, Chem. Eur. J. **2019**, M Marazzi, H Gattuso, M Fumanal, C Daniel, A Monari. Chemistry–A European Journal, *25*, 2519-2526.

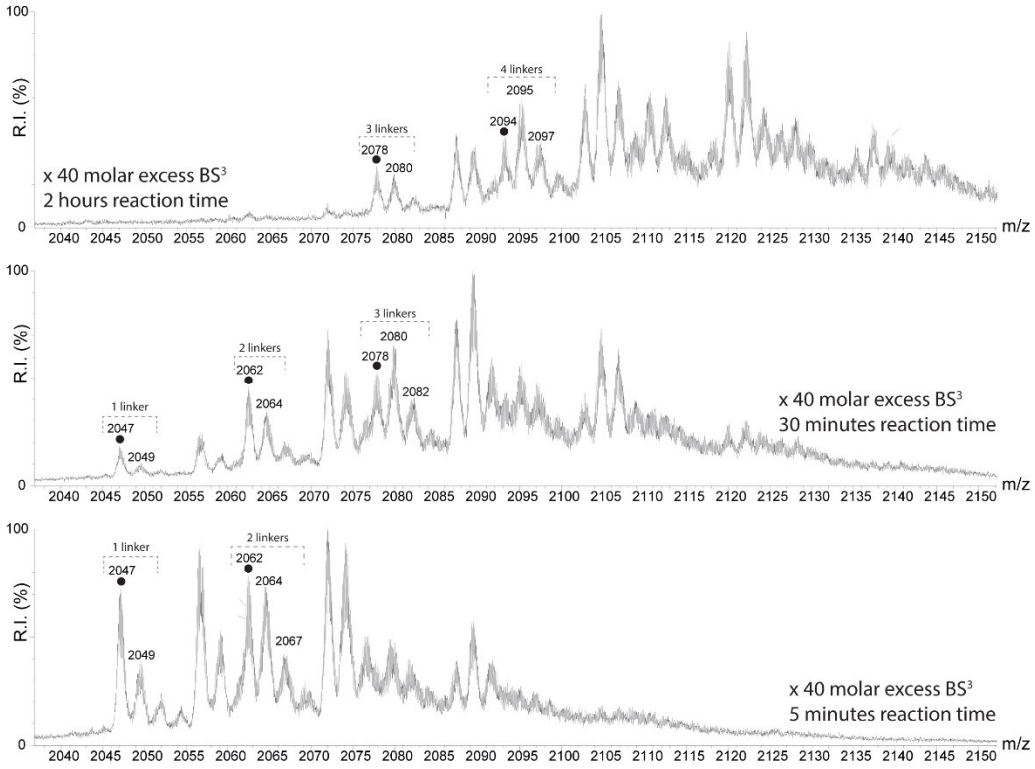
To ensure accurate geometry optimization and charge distribution, the side chains of bound His and Met and of both bound Cys were included in the modeled system for the heme group and the N-hydroxysulfosuccinimide leaving groups of the linker were replaced with $-NHCH_3$ groups. The two covalently linked Cys residues were first capped with $R_1 = -CH_3$ groups (Figure S2, Panel A) and geometry optimization were performed for both molecules at he DFT/B3LYP level of theory with the LANL2DZ basis set for the heme residue and 6-31G* for the cross-linker. Partial atomic charges were computed at the same level of theory using the standard RESP procedure. Then, the R_1 units were removed and their partial charges were redistributed on the heme group. Both bound and unbound force field parameters were extracted from the Amber14SB force field for bonds, angles and dihedrals. For parameters involving the iron atom,

parameters were manually fitted to obtain reasonable geometry fluctuations around the optimized structure similarly to previous studies on octahedral environment around heavy metals such as Fe and Re.[a,b]

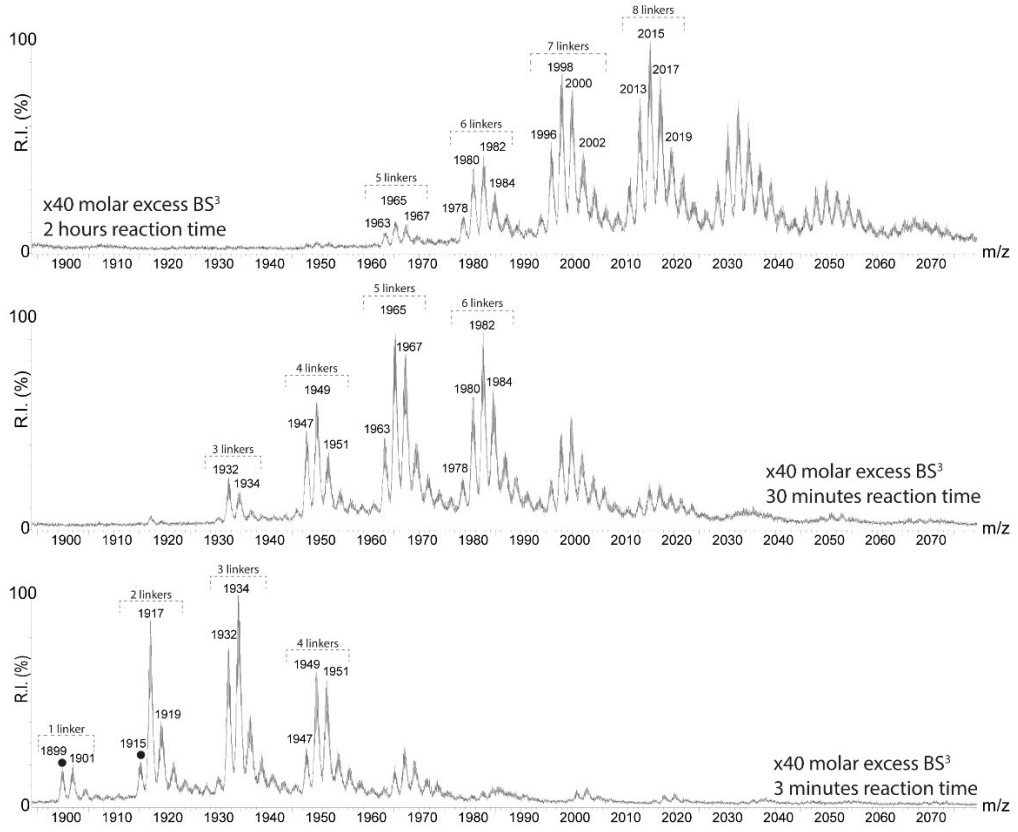
Similarly, the N-hydroxysulfosuccinimide leaving groups of the cross-linker were replaced with R_2 = -$NHCH_3$ moities (Figure S2, Panel B) for optimization and atomic charge calculations. They were subsequently removed and partial atomic charges were redistributed on all other atoms. Additionally, a new residue, characterized by the same canonical Amber force field parameters as the Lys residue, was defined in Amber libraries to allow the binding of cross-linkers.

Figure S3. CL/ML products for β-lactoglobulin (z = +9) and myoglobin (z = +9).

(a) β-lactoglobulin

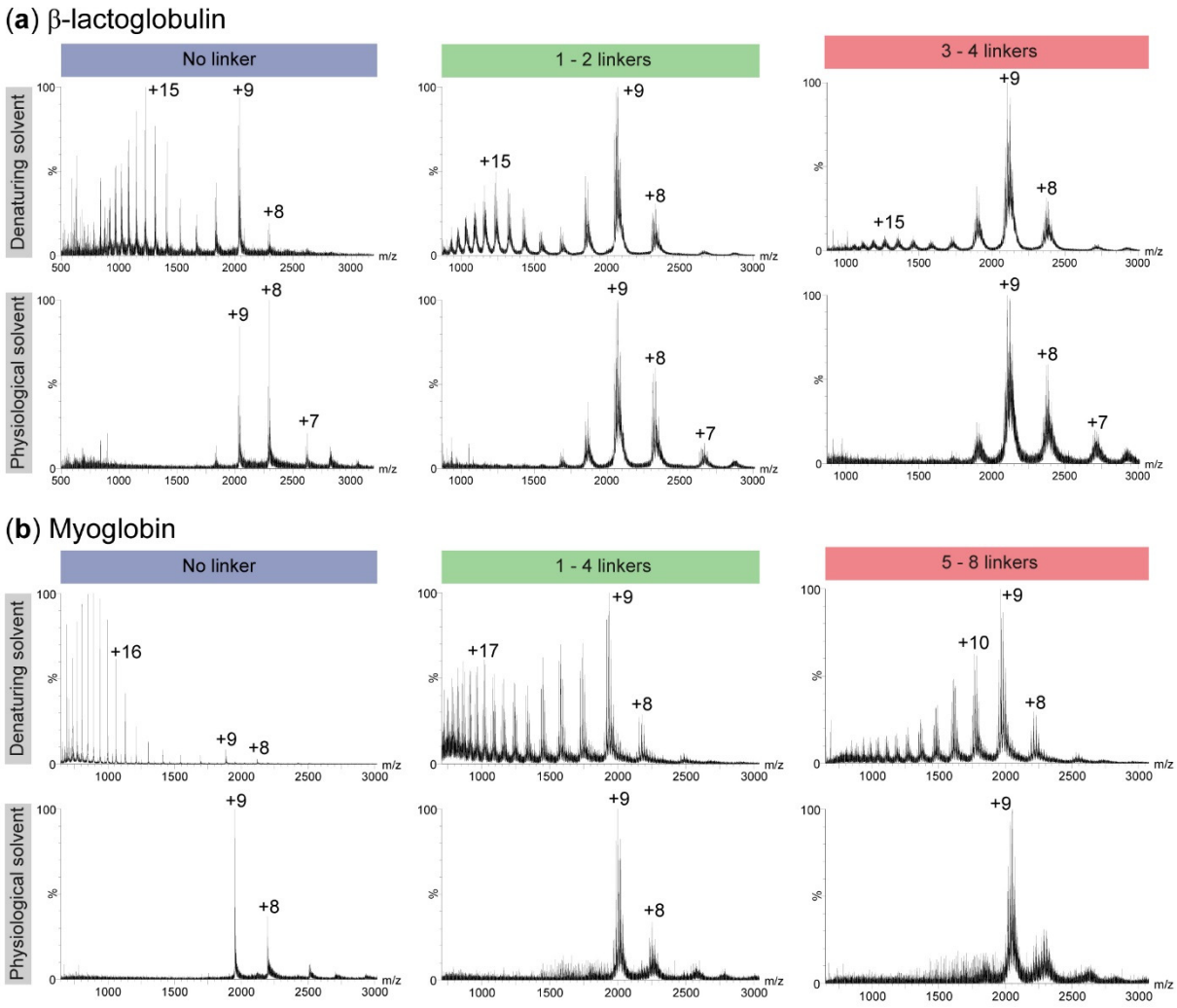


(b) myoglobin



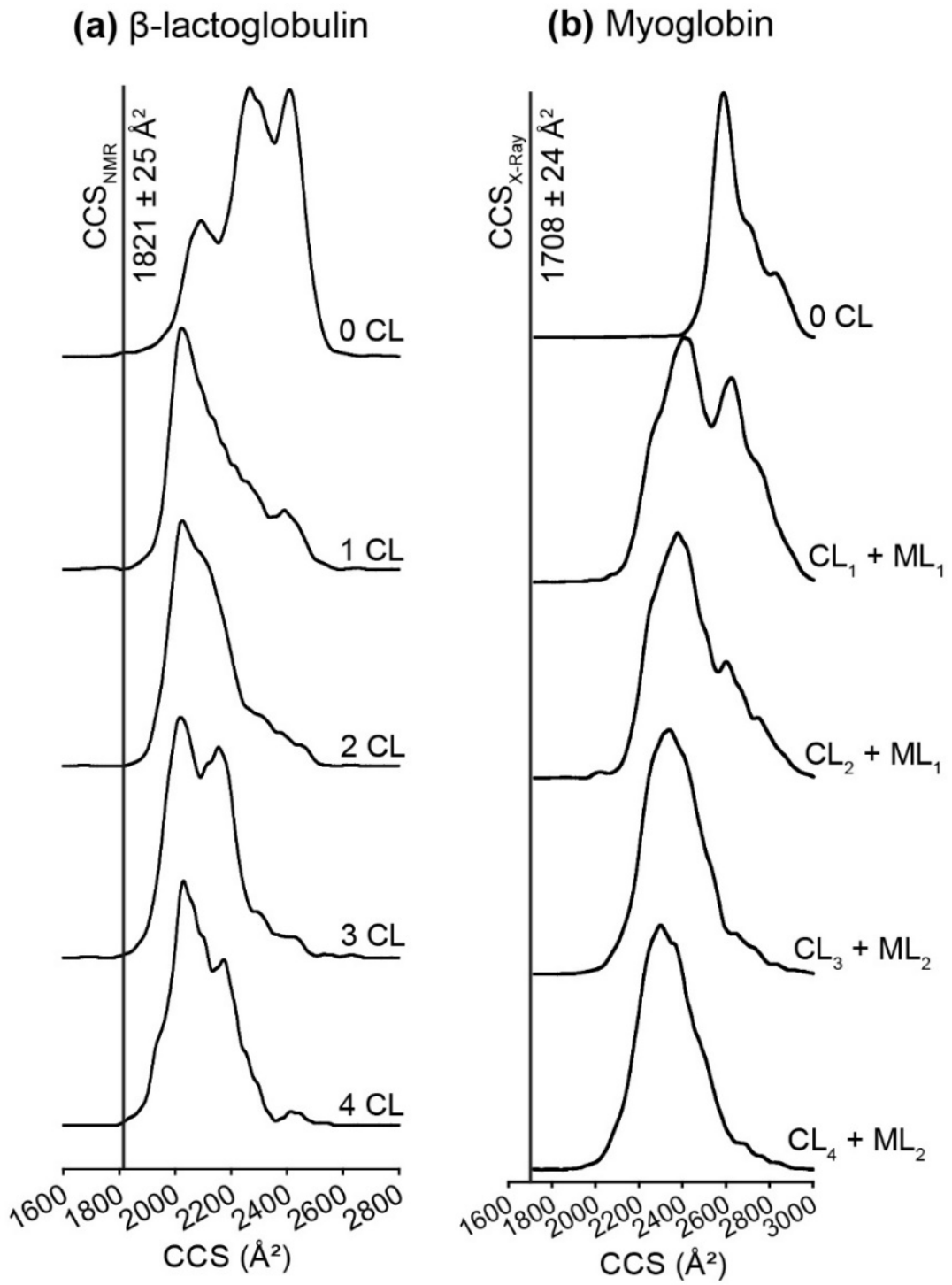
The black circles correspond to 100% CL. Data were acquired in Q-TOF mode, IM off.

Figure S4. CSDs of (non-)cross-linked β-lactoglobulin and myoglobin.



Data were acquired in Q-TOF mode, IM off mode.

Figure S5. $^{TW}CCS_{N2\rightarrow He}$ distributions of β-lactoglobulin (z = +9) and myoglobin (z = +9).



Ions are sprayed from physiological solvent (100:0 buffer:MeOH) and a trap bias voltage of 60 V is used.

Figure S6. Positions of the BS^3 linkers on the starting geometry of cytochrome *c* used for MD.

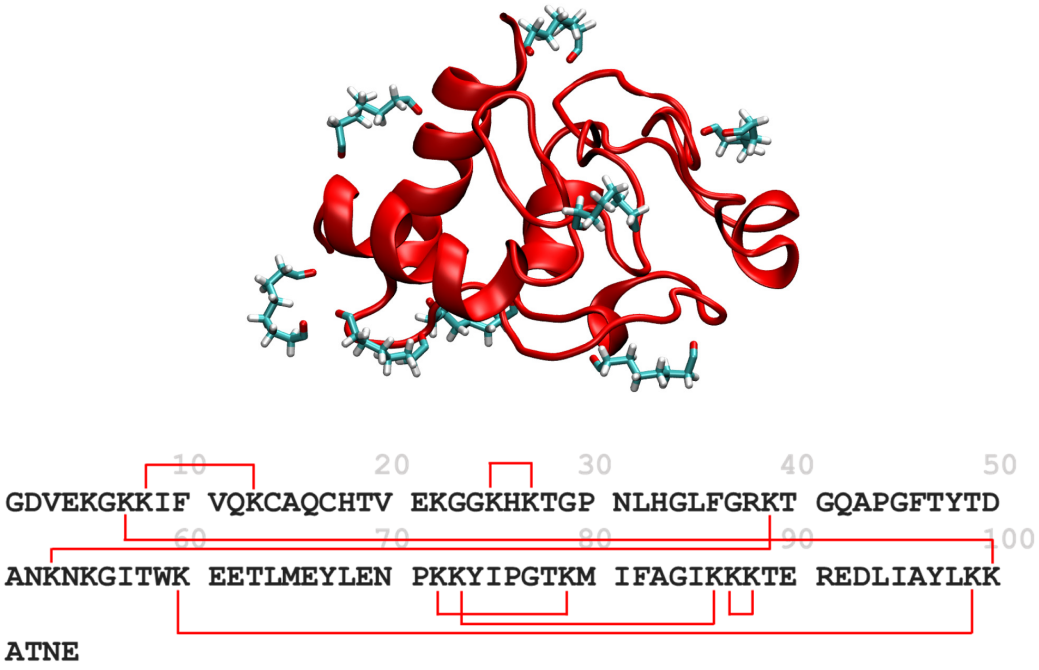


Table S4. List of the reported BS3 cross-linked residues for cytochrome *c*.

Cross-link	Reference
K7 – K100	a
K13 – K8	b, c
K25 – K27	a, b, c, d, e
K39 – K53	b, e
K72 – K79	b
K86 – K73	b
K87 – K88	e
K99 – K60	Absence of steric constrains

[a] Xu, H.; Hsu, P.-H.; Zhang, L.; Tsai, M.-D.; Freitas, M. A., J. Proteome Res. **2010**, *9*, 3384–3393.

[b] Pearson, K. M.; Pannell, L. K.; Fales, H. M., Rapid Commun. Mass Spectrom. **2002**, *16*, 149–159.

[c] Young, J. L.; Lackner, L. L.; Nunnari, J. M.; Phinney, B. S., J. Proteome Res. **2007**, *6*, 3908–3917.

[d] Dihazi, G. H.; Sinz, A., Rapid Commun. Mass Spectrom. **2003**, *17*, 2005–2014.

[e] Seebacher, J.; Mallick, P.; Zhang, N.; Eddes, J. S.; Aebersold, R.; Gelb, M. H., J. Proteome Res. **2006**, *5*, 2270–2282.

Figure S7. Positions of the electric charges on the initial geometry of cytochrome *c* used for MD.

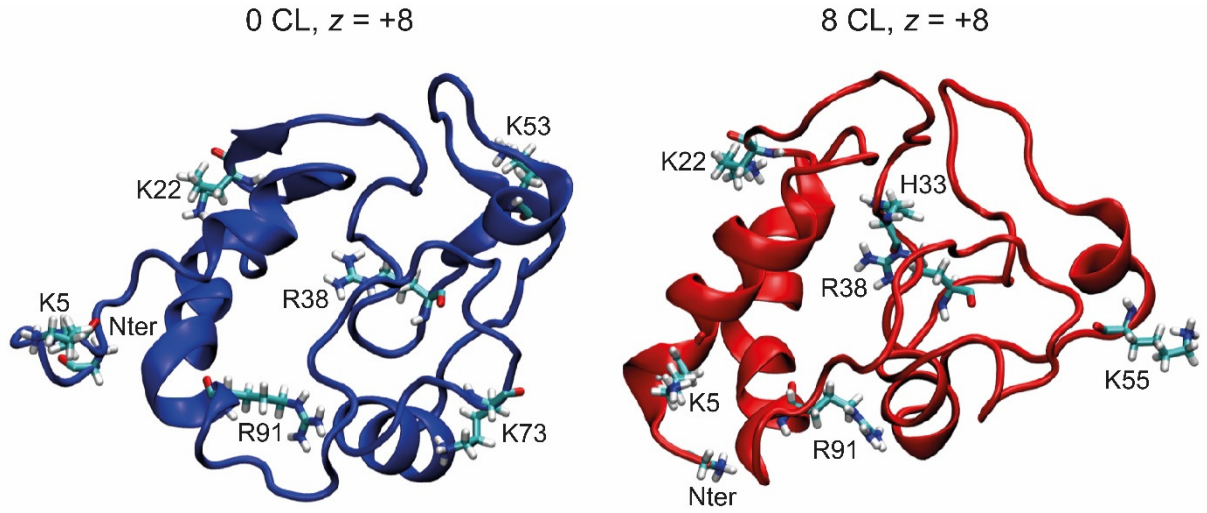
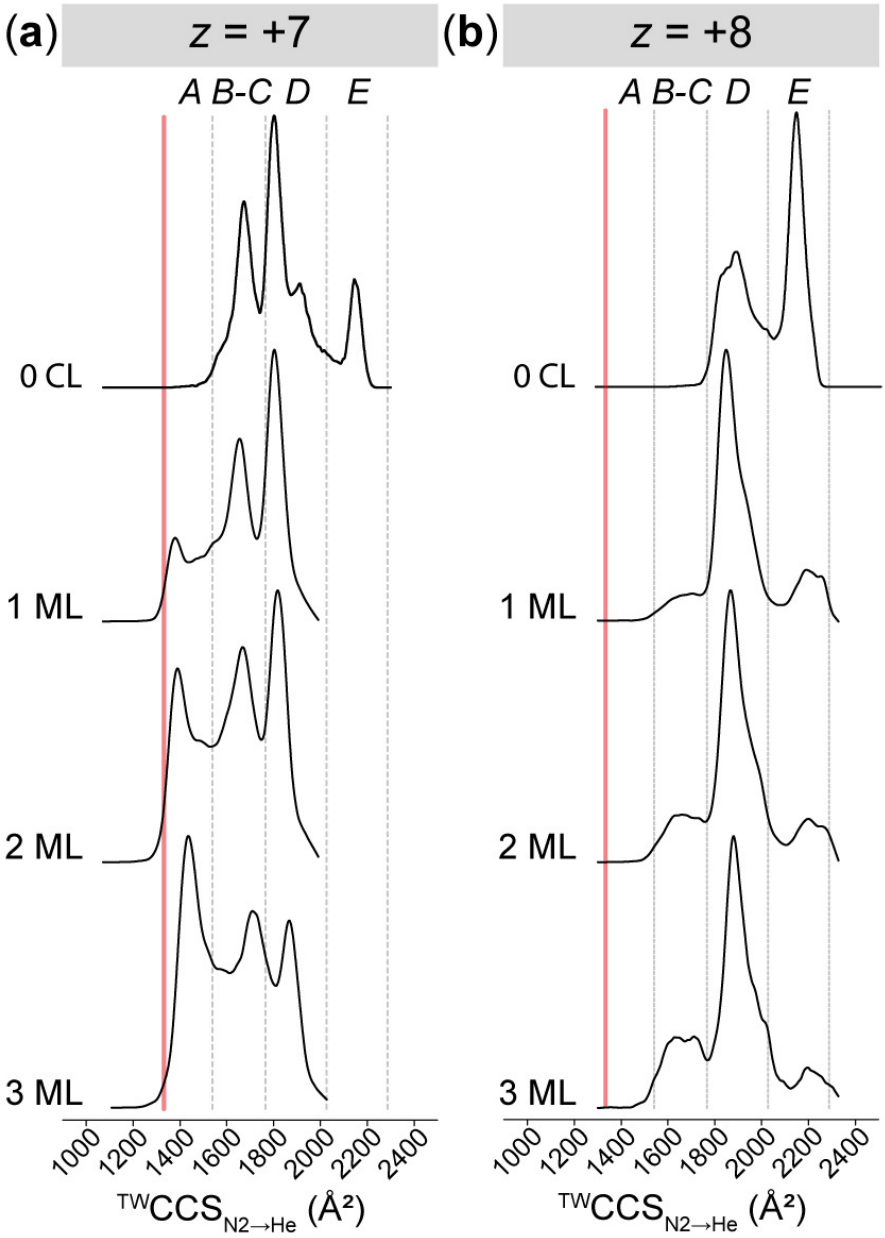


Table S5. List of the positions of the electric charges on the initial geometry of cytochrome *c* used for MD.

0 CL	**8 CL**
N_{ter}	N_{ter}
K5	K5
K22	K22
K53	K55
K73	H33
R38	R38
R91	R91
Heme (+1)	Heme (+1)

Figure S8. $^{TW}CCS_{N2\rightarrow He}$ distributions of cytochrome *c* carrying from 0 ML to 3 ML.



Ions are sprayed from physiological solvent (100:0 buffer:MeOH) and a trap bias voltage of 60 V is used.