

On the Impact of Post-Translational Succination on Small Ubiquitin-like Modifier 1 Structure: A Dual Approach combining Gas-Phase and Solution Studies

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Abstract

SUMO1 (Small Ubiquitin-like Modifier 1) is a key protein involved in the post-translational modification of a wide range of substrate proteins. SUMOylation plays a pivotal role in regulating various cellular processes, such as protein localization, functional modulation, and complex formation. Notably, SUMO1 contains a cysteine residue in its sequence, making it susceptible to succination, *i.e.* a Michael addition of cysteine onto fumarate, forming a succinated cysteine. This could occur in conditions of elevated intracellular fumarate concentration, a hallmark of metabolic dysregulation. To investigate this hypothesis, we employ a multidisciplinary approach, integrating advanced analytical techniques such as mass spectrometry (including liquid chromatography, ion mobility spectrometry, and hydrogen-deuterium exchange experiments), circular dichroism spectroscopy, and molecular dynamics simulations. We demonstrate that SUMO1 undergoes succination *in vitro*, leading to important conformational changes. These findings provide insights into the susceptibility of SUMO1 to metabolic alterations.

Keywords SUMO1 ; SUMOylation ; Succination ; Mass Spectrometry ; Circular Dichroism ; Molecular Dynamics

SUMOylation is a post-translational modification (PTM) involving the covalent attachment of a Small Ubiquitin-like Modifier (SUMO) protein to substrate proteins. This process, observed in humans with SUMO isoforms SUMO1, SUMO2, SUMO3, and SUMO4¹, occurs through the formation of an isopeptide bond between SUMO and lysine residues on target proteins mediated by a three-step enzymatic cascade². SUMOylation plays a vital role in regulating diverse cellular processes, including protein localization, functional modulation, and complex formation^{2,3}. Its influence extends to over 1,000 identified human proteins (e.g. Hendriks *et al.* identified 40,765 SUMO2 modification sites in 6,747 human proteins)⁴⁻⁶, highlighting its significance in maintaining cellular homeostasis and its involvement in both physiological and pathological conditions.

Another important PTM is non-enzymatic succination (**Figure 1**). During the tricarboxylic acid (TCA) cycle, fumarate is converted into malate by the enzyme fumarate hydratase (FH). However, loss-of-function mutations in the FH gene result in enzyme deficiency, making individuals prone to certain diseases such as Hereditary Leiomyomatosis and Renal Cell Carcinoma (HLRCC)^{7,8}. This deficiency leads to fumarate accumulation in cells, often reaching millimolar concentrations^{9,10}. Excess fumarate can undergo physiologically irreversible reactions with nucleophilic groups, such as the thiol groups of cysteine residues. While the thiol group in free cysteine typically has a pKa of ~9, the pKa of a cysteine residue in a protein can vary greatly depending on its environment. When in proximity of basic amino acids (*i.e.* lysine or arginine), interactions with thiol group lower the pKa of cysteine (up to pKa 2) and increase its nucleophilicity^{11,12}. This enables a Michael addition reaction between fumarate and cysteine, resulting in the formation of 2-(S-succino)cysteine (noted **2SC-protein** in case of succinated proteins⁸), a recognized biomarker of succination¹³.

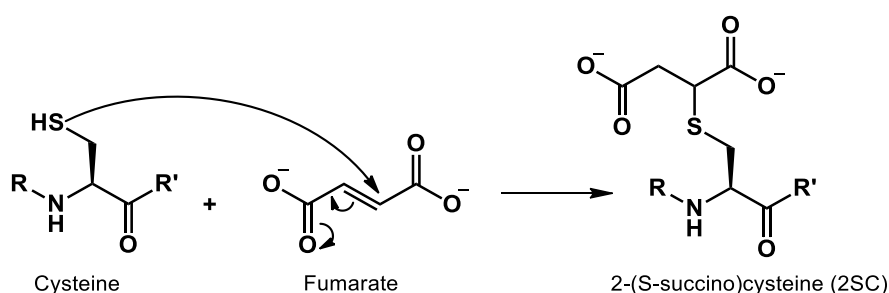


Figure 1: Irreversible succination reaction between cysteine residue and fumarate through a Michael addition leading to 2-(S-succino)cysteine (2SC).

Under conditions of cellular stress, fumarate levels can rise significantly, creating an environment prone to succination reactions. Given that SUMO1 contains a cysteine, it is highly susceptible to succination under such conditions, consistent with observations made for other thiol-containing proteins¹¹. Indeed, although this cysteine is located within the α 1 helix and

partially buried in the hydrophobic core (**Figure 2**), previous studies have demonstrated its reactivity^{14,15}. For instance, Goffinont *et al.* showed that in the UBC9~SUMO1 complex, Cys52 of SUMO1 and Cys138 of UBC9 could be covalently crosslinked, probably, by a dithiothreitol (DTT) molecule, highlighting the accessibility and chemical reactivity of Cys52¹⁴. Moreover, Cys52 contributes to SUMO1 structural integrity through a potential sulfur- π interaction with phenylalanine 64. Supporting this, Drobecq *et al.* found that substituting Cys52 with alanine in the SUMO1 sequence significantly disrupts its thermal stability, secondary structures and biological activity compared to wild-type SUMO1¹⁵. In contrast, Bouchenna *et al.* reported that the same mutation has only a limited effect on SUMO2 stability, despite conservation of the surrounding residues¹⁶.

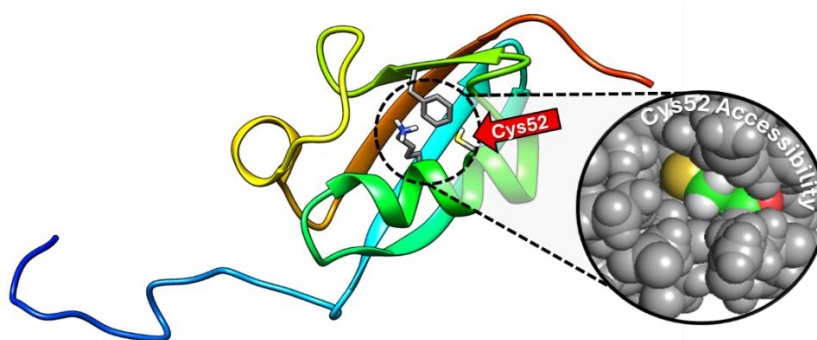


Figure 2: UCSF Chimera rendering of the SUMO1 structure (PDB ID: 1A5R), showing the Cys52 residue located within the α -helix and surrounded by neighbouring amino acids (i.e. Lys48 and Phe64). The inset illustrates the limited accessibility of Cys52, emphasizing its location within the hydrophobic core.

In order to identify and quantify PTMs, mass spectrometry (MS) has become an essential tool^{17,18}. Both bottom-up and top-down proteomics approaches offer unique structural advantages, enabling the sequencing of protein primary structures, localization of PTMs, and quantification of protein abundance through the analysis of peptide and protein fragment ions¹⁹. However, when detailed insights into the three-dimensional (3D) structure of intact proteins and the effects of PTMs are required, native MS provides a distinct advantage²⁰. Native MS enables the analysis of proteins and their complexes in conditions that closely mimic their natural environment, preserving non-covalent interactions and the integrity of protein tertiary and quaternary structures²¹. This approach provides valuable insights into the influence of PTMs on protein structure, stability, and interactions, offering a more physiologically relevant perspective on protein dynamics and regulation²⁰.

Native MS also opens the way for probing the 3D structure of protein ions by ion mobility spectrometry (IMS). IMS separates ions based on their mobility, a property influenced by the ion charge and collisional cross section (CCS), which represents a direct approximation of the ion spatial structure. IMS experiments are advantageously complemented by state-of-the-art molecular dynamics (MD) simulations at the atomistic level. By determining computed CCS

values from conformations sampled during the simulations, MD provides a deeper understanding of the gas-phase structures and conformational dynamics of gaseous protein ions. However, the transition from the solution to the gas phase can sometimes lead to the loss of specific structural details due to protein compaction in the absence of solvent interactions^{22–24}. Native MS can then be complemented by solution-phase techniques such as circular dichroism (CD) spectroscopy and hydrogen-deuterium exchange (HDX) experiments (coupled with MS detection). These complementary methods, when integrated with MD simulations, provide a robust framework for correlating structural and stability changes between gas-phase and solution-phase environments. Together, they enable a more comprehensive understanding of protein dynamics and the influence of PTMs across different states.

By using a multidisciplinary approach integrating advanced analytical techniques such as MS (including liquid chromatography, IMS, and HDX experiments), CD spectroscopy, and MD simulations, we demonstrate that SUMO1 undergoes succination *in vitro*, which leads to conformational changes. Piecing together findings from individual approaches provide new insights into the susceptibility of SUMO1 to metabolic alterations.

Experimental section

Chemicals and proteins. Two different recombinant SUMO1 (>98%) were purchased from Bio-Techne (Minneapolis, MN, USA, MW = 11,000 g.mol⁻¹) and Ubiquigent (Dundee, UK, MW = 11,434 g.mol⁻¹), the first source having become unavailable from the supplier. Both correspond to mature variants of human SUMO1 (UniProt ID: P63165), ending with the diglycine (-GG) motif conserved at residue 97. SUMO1 from Ubiquigent is distinguished by the addition of five amino acid residues, *i.e.* GPGSM, at the N-terminal extremity (flexible tail – See **Table S11**). The proteins were desalted using Sartorius (Goettingen, Germany) Vivaspin 500 (3,000 molecular weight cutoff) filters, centrifugated three times from 50 mM aqueous ammonium acetate (pH 6.8) or phosphate buffer (pH 6.8)²⁵ solutions at 10,000 rpm (3°C). For the MS analysis, the samples were prepared at a protein concentration of 5 μM in 50 mM aqueous ammonium acetate. To perform succination, diethyl fumarate 98% from Thermo Fisher (Waltham, MA, USA) and fumaric acid, 98% from BLD Pharmatech (Shanghai, China), were used without any further purification.

In vitro succination. *In vitro* reactions between SUMO1 (69 μg) and diethyl fumarate/fumaric acid were performed at 1:1000 equivalents to trigger the reaction at 37°C for 24 hours in 50 mM ammonium acetate for MS experiments, and for 48 hours in phosphate buffer for CD analysis. Succinated SUMO1 reaction mixture was ultra-filtrated three times using Sartorius (Goettingen, Germany) Vivaspin 500 (3,000 molecular weight cutoff) filters, and finally recovered and diluted in 50 mM aqueous ammonium acetate (pH 6.8) or phosphate buffer (pH

6.8) to respectively obtain a 5 μM solution in 50 mM aqueous ammonium acetate for MS analysis or a 15 μM solution of SUMO1 in phosphate buffer for CD spectroscopy analysis.

Succination kinetics by LC-MS. Liquid chromatography - mass spectrometry (LC-MS) analyses were performed to monitor the succination reaction over time. An Alliance 2695 HPLC (Waters, UK) apparatus coupled to a Waters QToF API-US mass spectrometer (Manchester, UK) was used, operating in Electrospray Ionization in the positive ion mode (ESI+). HPLC-grade solvents were used with a Thermoscientific Hypercarb (4.6 x 150 mm; 5 μm particle size) column. A linear gradient starting from 70% H_2O (with 0.1% HCOOH) / 30 % acetonitrile going to 100% acetonitrile in 7 minutes was applied.

Reduced L-Glutathione from Sigma Aldrich (Saint-Louis, MO, USA), used as received, was dissolved in ammonium acetate 50 mM to obtain a 5 mM solution and mixed separately with diethyl fumarate/fumaric acid in a 1:5 molar ratio at 37 °C. 20 μL of the stock solution were collected at defined times and diluted to 1 mL to obtain a 100 μM (of GSH) solution and injected in the LC-MS (5 μL). For GSH, the LC-MS ion currents, including all the isotopic compositions, adducts and fragments, were integrated using the integration algorithm, available under MassLynx™ 4.1 Software. A calibration curve was established using GSH solutions at different concentrations (0 to 700 μM) to determine the dynamic range of the LC-MS analysis (see **Figure S11**). This range extends from 0 to 300 μM , ensuring a linear relationship between concentration and signal intensity for kinetics at 100 μM .

Mass Spectrometry experiments. MS experiments were performed on a Waters Synapt G2-Si TWIMS-Q-ToF instrument (Manchester, UK), equipped with an ESI source operated in the positive ion mode. Proteins were analyzed under soft conditions defined as: sampling cone of 10 V, source offset of 50 V, source gas flow of 250 $\text{mL}\cdot\text{min}^{-1}$, desolvation gas flow of 1200 $\text{L}\cdot\text{h}^{-1}$, trap bias voltage of 30 V, and StepWave1 rf offset voltage of 100 V. Temperatures were set as 80 °C for the source and 120 °C for the desolvation gas (N_2). For the IMS experiments, the IMS wave velocity was fixed at 600 $\text{m}\cdot\text{s}^{-1}$ with a 22 V wave height. The gas in the mobility cell is N_2 , at a pressure of 3 mbar. To determine the CCS, a custom calibration procedure was used²⁶, using PEG600, 1,000, 2,000 and 3,350 $\text{g}\cdot\text{mol}^{-1}$ commercially available from Sigma Aldrich (Saint-Louis, MO, USA) and Polylactide (PLA) samples characterized by average molecular weights of 5,500, and 10,000 $\text{g}\cdot\text{mol}^{-1}$. CCS will be abbreviated according to the accepted notation²⁷. The notation is in the form $^{\text{X}}\text{CCS}_{\text{Y}}$, with X corresponding to the experimental/theoretical method used and Y the drift gas. In this study, the experimental CCS are written down as $^{\text{TW}}\text{CCS}_{\text{N}_2\rightarrow\text{He}}$, corresponding to a measure using a Traveling Wave (TW) ion mobility cell filled with nitrogen, but using a calibration based on CCS_{exp} values obtained in helium.

Continuous Flow In-Solution HDX. HDX experiments were conducted in continuous flow conditions using a three-syringe pump set-up (LABM8, Nijmegen, The Netherlands) equipped with HSW Plastipak syringes to fully control the exchange time. Each syringe was filled as follows: (i) the protein solution (30 μ M in 50 mM ammonium acetate, pH 6.5); (ii) aqueous ammonium acetate (50 mM) in H₂O and (iii) ammonium acetate (50 mM) in D₂O (99.9%, pD 6.9). All syringes were connected to a tubular reactor whose volume was estimated to 13.6 μ L, directly connected to the ESI inlet of the mass spectrometer. The residence time in the flow reactor was determined at 4.5 min. The MS parameters were similar to those described above. The HDX experiments were performed as follows : (i) the protein solution (1 μ L.min⁻¹) and the ammonium acetate solution in H₂O (2 μ L.min⁻¹) and, afterwards, (ii) the protein solution (1 μ L.min⁻¹) and the ammonium acetate solution in D₂O (2 μ L.min⁻¹) were co-infused to obtain the 'full-H' and the 'HDX' mass spectra, respectively. For the HDX mass spectra recording, steady states were considered to be reached when no modifications in the mass spectra were detected after 2 minutes of infusion. To calculate the mass shift, the m/z distribution of each charge state is converted to mass shifts, as proposed by Simmons and Konermann²⁸: Mass shift = $(m/z_{HDX} - m/z_{noHDX}) \times Z$.

Circular dichroism measurements. CD measurements were recorded using a Chirascan™ Plus CD Spectrophotometer from Applied Photophysics. The measurements were carried out using 1 mm suprasil quartz cells from Hellma Analytics. The spectra were recorded between 190 and 260 nm, with a bandwidth of 1 nm and a time per point of 1.5 s. The buffered water solvent (10 mM potassium phosphate, 50 mM Na₂SO₄) reference spectrum was used for the background measurement and was automatically subtracted from the CD spectra of the samples. Temperature-ramping experiments were recorded for the 15 μ M protein solution in phosphate buffer at pH 6.8. The mean residue ellipticity at 225 nm was monitored from 20 to 90 °C, and data were fitted with a two-state folding curve to obtain the T_m of SUMO1 and 2SC-SUMO1. Thermodynamic unfolding properties were computed using the protocol established by Greenfield *et al.*²⁹ and described in the supporting information.

Theoretical determination of Collisional Cross Section and SASA. The SUMO1 structure was uploaded from the protein data bank (PDB - 1A5R, model 1³⁰), modified by taking off terminal amino acids to obtain the Bio-Techne SUMO1 sequence, as experimentally determined by MS (see **Table SI1**). Then the distribution of positive and negative charges across titratable sites (yielding a net charge of +6 in the gas phase and -4 in the solution phase) was calculated with the Virginia Tech H++ software³¹. MD simulations at 300 K were performed to get structural information at the atomic level. First, a 1 μ s simulation was performed in a truncated octahedral solvent box constituted by 14,665 water molecules and Na⁺/Cl⁻ ions, reaching a 50 mM NaCl concentration, and ensuring electroneutrality of the system. The final

snapshot from this simulation was then extracted and subjected to a 2 μ s vacuum simulation (External dielectric constant set to 1). This approach was used to mimic the ESI process, from the droplet to the vacuum environment. Simulations were carried out with the AMBER suite of programs³², using the ff19SB force-field for the protein³³, GAFF2.1 for the DEF moiety³⁴, and the OPC model for water³⁵. The last 500 conformations were extracted from the vacuum simulation each 0.5 ns and used to compute theoretical CCS values in Helium through the Trajectory Method (TM)³⁶ via the Collidoscope program (**Figure 3**). The theoretical CCS were annotated in the form $^{TM}CCS_{He}$, corresponding to values calculated using the TM with helium gas. The Solvent-accessible surface area (SASA) with the respective molecular volume (V_{SASA}) were obtained and averaged on the last 10 frames of the dynamics with Materials Studio 2022 “Atom Volumes & Surfaces” module³⁷. For the SASA determination, to “probe” the surface of the proteins, a helium atom was used as the “solvent” for the gas phase experiments, as defined in the algorithm by its atomic radius while a water molecule was used as the solvent for the solution-phase structures. The software defines the probe as a sphere with a Van der Waals radius of 1.4 Å for both water and helium.

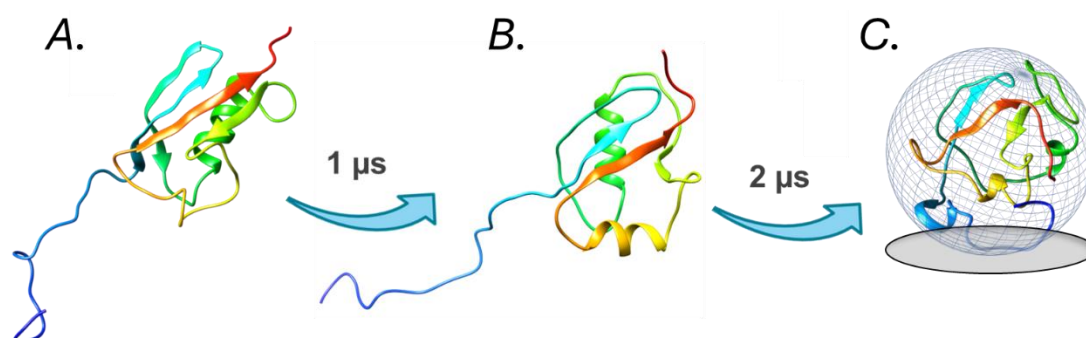


Figure 3: UCSF Chimera rendering of SUMO1 6+. (A) Solution NMR structure from pdb 1A5R, (B) Last conformation after 1 μ s simulation in explicit solvent, (C) Last conformation after 2 μ s of simulation in vacuum. CCS were calculated on these gas-phase conformations.

Results and discussion

1. Kinetics of succination of glutathione as a model compound

To evaluate the reactivity of thiolated amino acids towards succination in typical native MS conditions, *i.e.* in 50 mM ammonium acetate³⁸, we first determine the succination kinetics of a free cysteine, *i.e.* the Michael addition of cysteine onto fumarate, under these specific pH and ionic strength conditions (pH 6.8). Succination kinetics were assessed by LC-MS using Glutathione (GSH) as a model substrate, and both diethyl fumarate (DEF) or fumaric acid (FA) as succination agents, see **Figure 4**. To study succination, an agent that enables a fast and efficient reaction is required. Both FA and DEF are electrophilic compounds capable of inducing cysteine succination and are therefore compared in this kinetic study. As shown in **Figure S12**, GSH is detected as $[GSH+H]^+$, $[GSH+Na]^+$ and $[GSH+K]^+$ when the ammonium acetate solution

is subjected to ESI(+) conditions. Upon addition of five equivalents of DEF at 37°C in 50 mM ammonium acetate, these m/z signals are shifted 172 u higher. Since ESI is known to produce non-specific adducts (same mass as the covalent species), irrelevant for solution chemistry, the $[GS-DEF+H]^+$ ions (m/z 480) have been further exposed to collision-induced dissociation (CID) experiments to establish the covalent nature of the DEF•GSH association. The CID mass spectra presented in **Figures S13/S14** confirm the covalent addition of GSH onto DEF or FA.

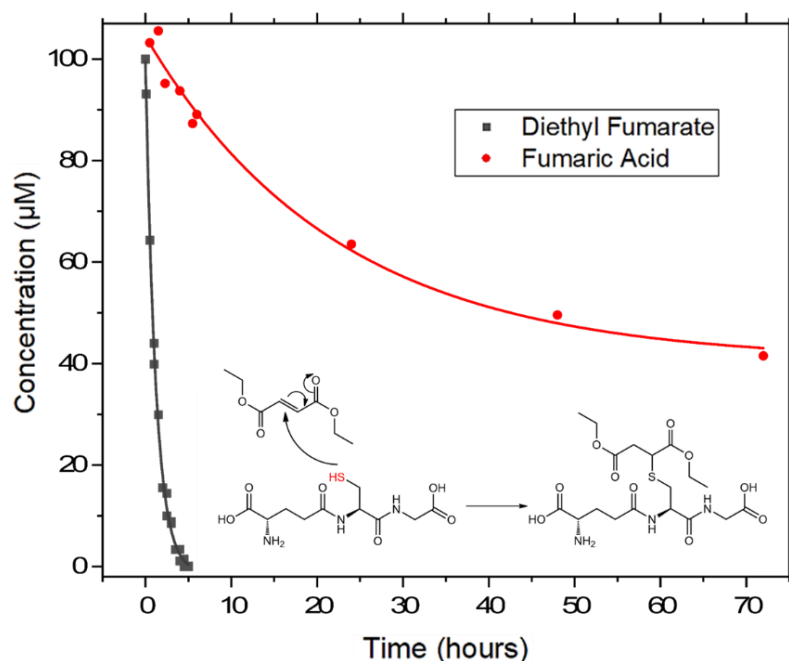


Figure 4: Succination of GSH by DEF (black curve, $R^2 = 0.99$) and FA (red curve, $R^2 = 0.98$) in ammonium acetate (50 mM, 1:5 GSH:DEF/FA molar ratio, 37 °C): time evolution of the unreacted GSH concentrations as monitored using LC-MS experiments, fitted by a first order law.

Figure 4 shows the time evolution of the free GSH concentrations when reacting one equivalent of GSH with five equivalents of DEF or FA in ammonium acetate 50 mM at 37°C. The GSH concentrations were measured upon LC-MS experiments using a calibration curve created with commercially available GSH standard (see experimental section). DEF is clearly more reactive toward GSH since residual GSH is hardly detected already after 5 hours with DEF, whereas after 55 hours only 50% of GSH have been consumed by FA. This result is probably due to the greater electrophilicity of the double bonds of fumarate esters compared to fumarate⁸. Furthermore, it has already been shown that in Michael additions, fumarate esters are up to 100-fold more reactive than fumarate¹⁰. Consequently, DEF proves to be advantageous for probing the SUMO1 succination, ensuring quicker and more efficient reactions³⁹. Hence, this succination agent is then used to explore SUMO1 succination.

2.SUMO1 succination by diethyl fumarate in ammonium acetate

Figure 5a presents the ESI(+) mass spectrum recorded for a SUMO1 (Bio-Techne, USA) solution (5 μ M) in 50 mM ammonium acetate. Three different charge states were detected at m/z 1376, 1573 and 1834, corresponding respectively to the 8+, 7+ and 6+ ions. Globular biomolecules typically adopt a gas-phase charge just below the “Rayleigh limit” charge⁴⁰, as defined by $Z_R = 0.078 \times MW^{1/2}$, where MW is the molecular weight (g.mol⁻¹), leading to a predicted dominant charge state ~8+ for SUMO1. SUMO1 being a globular protein is thus likely to conserve its native conformation in the gas phase when electrosprayed from a 50 mM AA solution (see below for IMS experiments).

SUMO1 (50 μ M) and DEF (50 mM) were mixed at 37 °C in 50 mM ammonium acetate for 24 hours. The reaction mixture was then diluted to obtain a 5 μ M protein solution and infused into the ESI source. The recorded mass spectrum, shown in **Figure 5b**, features m/z shifts 21.5, 24.6, and 28.7 for 8+, 7+, and 6+ ions respectively. These shifts correspond to a mass increase of 172 u, which is consistent with the addition of one DEF molecule onto the protein. Interestingly, the relative abundance between unmodified and succinated SUMO1 (noted **2SC-SUMO1**, we use the term 2SC-SUMO1 for the DEF-succinated protein, although ‘2SC’ traditionally refers to fumarate-derived succination) is charge state dependent, with a higher proportion of 2SC-SUMO1 ions at higher charge state. This could suggest that succination induces a partial unfolding of the protein tertiary structure, making more basic sites accessible for protonation upon ionization. To evaluate this possible conformational rearrangement in solution, protein conformational dynamics were investigated by HDX experiments.

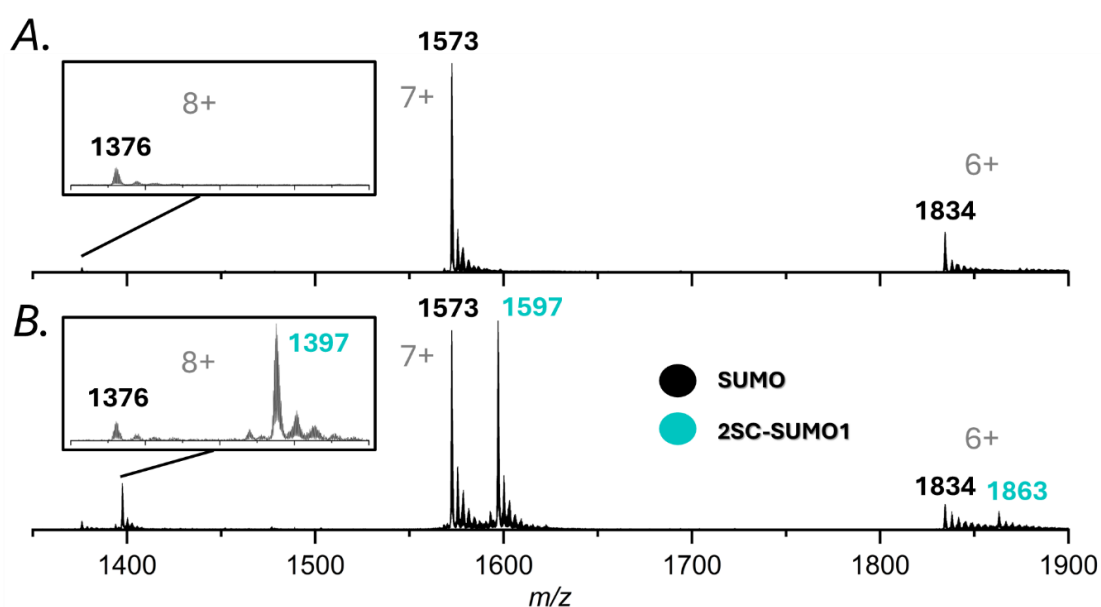


Figure 5: ESI-MS analysis of SUMO1 succination by DEF (Waters Synapt G2-Si TWIMS-Q-ToF): (A) ESI(+) mass spectrum of SUMO1 solution 5 μ M in 50 mM ammonium acetate and (B) ESI(+) mass spectrum of the SUMO1 (5 μ M) / DEF in 50 mM ammonium acetate after 24 hours at 37 °C. Black labels correspond to SUMO1 and blue ones to 2SC-SUMO1.

3. Hydrogen-Deuterium Exchange experiments

HDX-MS experiments take advantage of labile hydrogen atoms exposed to the solvent. By transferring a protein from H₂O into a D₂O environment, these labile hydrogen atoms can be exchanged with deuterium atoms, thereby providing insights on protein conformational dynamics⁴¹. HDX-MS analyses are advantageously used to obtain information on protein structure and protein conformational changes induced by PTM⁴². The impact of succination on the tertiary structure of SUMO1 should be here associated with a different number of exchanged hydrogen atoms between unmodified SUMO1 and 2SC-SUMO1. The protein is constituted by 101 amino acids and 190 labile protons (96 backbone amide, 91 side-chain and 3 termini hydrogen atoms)⁴³.

We used an original continuous flow apparatus, described in the experimental section and in supporting information (**Figure SI5**), to maintain the exchange time at 4.5 min prior to ESI-MS analysis. **Figure 6** (as well **Figures SI6** and **SI7**) presents the results of the H/D exchange by showing the mass shift (in Da) corresponding to deuterium intake for all the detected charge states ($z = 6+$ to $8+$) for SUMO1 (left panel) and 2SC-SUMO1 (right panel).

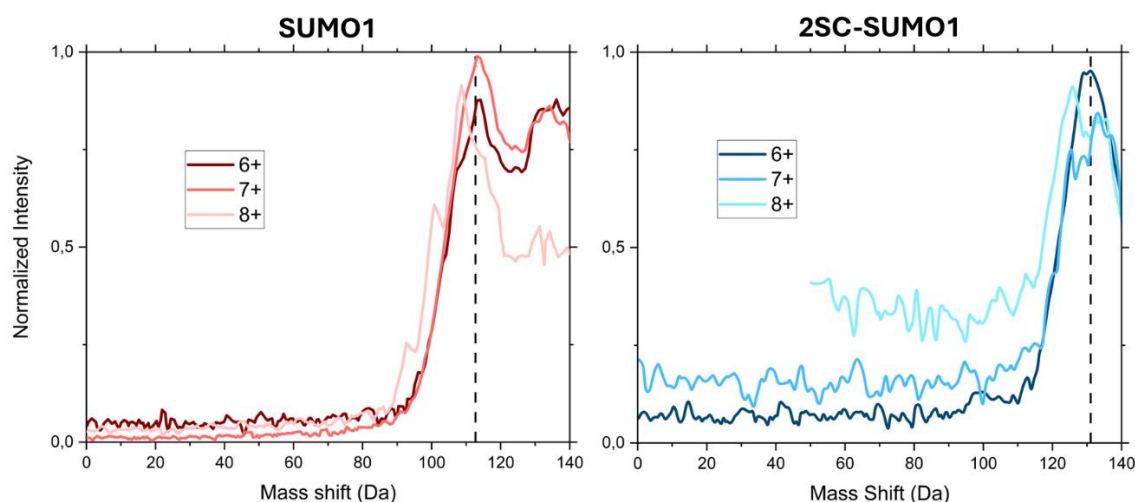


Figure 6: Evolution of the mass shifts [Mass shift = $(m/z_{\text{HDX}} - m/z_{\text{noHDX}}) \cdot z$] in HDX-MS for all the charge states based on the work of Gabelica et al.⁴⁴. SUMO1 is represented with red curves with an average mass shift of 114 (the maximum at 137 Da represents a sodium adduct), and 2SC-SUMO1 is represented with blue curves with an average shift of 132 Da (the 8+ curve was cropped due to low signal/noise ratio).

Regardless of the charge state, 2SC-SUMO1 is characterized by an 18-hydrogen atom higher deuterium uptake compared to unmodified SUMO1, *i.e.* a ~13% increase (**Figure 6**). This suggests that, for each protein, the three observed charge states are produced from a similar solution-phase conformation, as the deuterium uptake is not charge state dependent. Moreover, these results indicate that the succination induces a conformational rearrangement of SUMO1 in solution, resulting in an increased surface accessible to the solvent (see below).

This corroborates the charge state dependence of the succinated-to-non succinated ion abundance ratio, observed in the mass spectra in **Figure 5**. The increased surface accessible to the solvent of 2SC-SUMO1 gives access to more basic sites, thereby favoring the production of higher charge states during the ESI process.

4. Circular Dichroism spectroscopy experiments

Figure 7A compares the CD spectra of unmodified and 2SC-SUMO1 in phosphate buffer. The spectra reveal that the secondary structures constituting SUMO1 are globally preserved upon succination, since the CD profiles are nearly superimposable and identical to the CD spectrum reported by Chatterjee *et al.*⁴⁵. The CD spectra of SUMO1 exhibit two negative bands, one peak at 205 nm and a shoulder 225 nm. Upon succination, the shoulder at 225 nm (attributed to α -helices, see below) vanishes, indicating a weak structural modification.

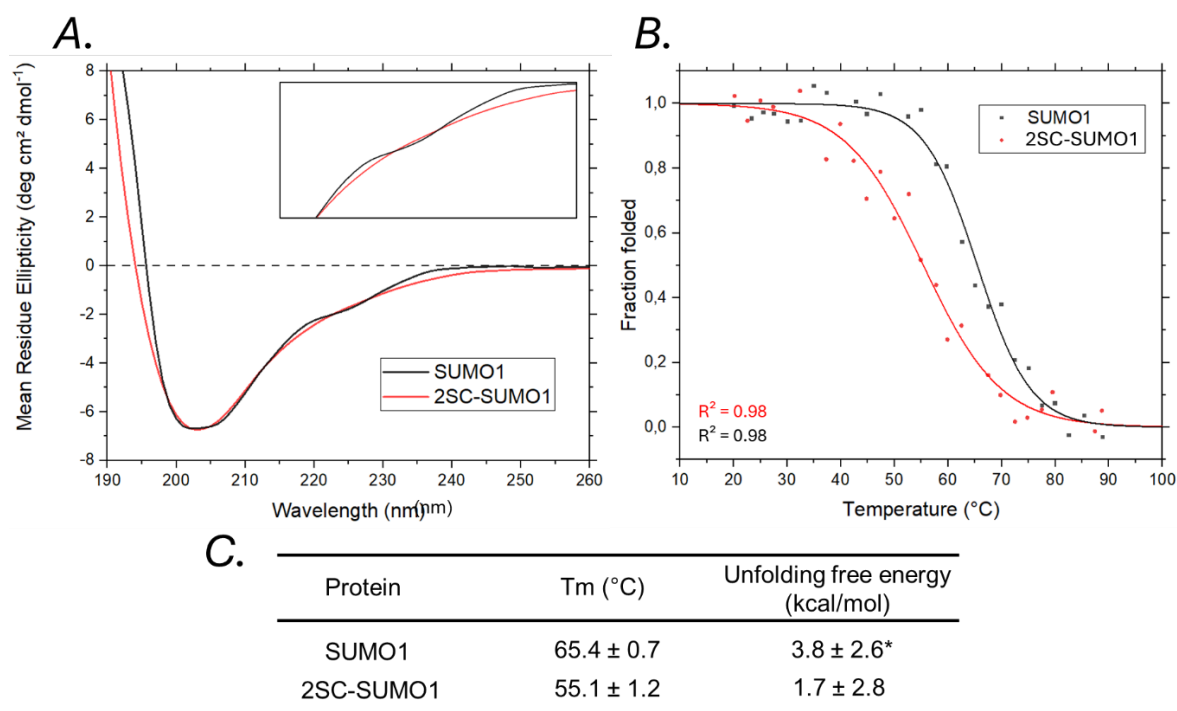


Figure 7: (A) UV CD spectra show a globally conserved tertiary structure when SUMO1 is succinated to 2SC-SUMO1. The inset represents a zoom on the shoulder (225 nm) region. (B) temperature melts for SUMO1 and 2SC-SUMO1. The change in mean ellipticity at 225 nm is normalized and plotted against temperature. (C) T_m and unfolding standard free energy for SUMO variants at 38 °C. (*) 3.72 kcal/mol from Chatterjee *et al.*⁴⁵

Using the software CDSSTR^{46–48} from the Dichroweb website⁴⁹, we have quantified the proportions of secondary structures (α -helix, β -sheet...) for both SUMO1 and 2SC-SUMO1 (**Table SI2**). The only notable change is the diminution of the proportion of α -helices from 13% to 9% upon succination. Let us remind that the Cys52 residue is located within an α -helix

(**Figure 2**). Thermal denaturation experiments, monitored using CD spectroscopy by monitoring the signal at 225 nm (or across all wavelengths presenting a transition, see **Figure SI8**), were performed to compare the melting temperature (T_m) of native and succinated SUMO1 (**Figure 7B**). The results showed a significant decrease in T_m from 65 °C for SUMO1 to 55 °C for the succinated SUMO1, indicating a destabilization of SUMO1 tertiary structure. This observation is likely due to the fact that in SUMO1, the Cys52 residue is initially oriented towards the inside of the helix (**Figure 2**). Upon succination, the residue is likely reoriented outwards, which correlates with a reduction in the hydrogen bond network within the helix. This suggests a potential interplay between cysteine reorientation and the weakening of secondary structure hydrogen bonds.

Thanks to this two-state folding curve, thermodynamical properties of the unfolding can be determined (as explained in supporting information) and are shown on **Figure 7C**. The difference of unfolding free energies at 38 °C is 2.1 kcal.mol⁻¹. This positive value indicates that the unfolding of the unmodified protein is more energy-demanding than the succinated form; meaning that the succination decreases the stability of SUMO1 (see below).

5. Molecular Dynamics simulations

The solution phase structures of SUMO1 and 2SC-SUMO1 have been modelled starting from the NMR structures of SUMO1 reported in the Protein Data Bank (PDB ID: 1A5R, a discussion regarding the NMR structure is provided in **Figure SI9**). The charge state of the solution phase proteins at pH 7 was defined as 4- based on the isoelectric point calculated by Virginia Tech H++ software³¹. MD simulations in explicit solvent have been conducted on SUMO1 and 2SC-SUMO1 and the last frame of each 1 μ s trajectory is presented in **Figure 8**. The comparison between these structures reveals that most secondary structures are preserved in 2SC-SUMO1, in agreement with CD data. However, the Cys52 residue located on the helix is reoriented outwards when succinated, partially de-structuring the end of the α -helix. This is in line with the previous observation by CD of a reduced helix proportion. Moreover, the average distance of the Cys52 residue from the protein center-of-mass on the last microsecond of the MD trajectories (**Table 1**) tends to increase upon succination, confirming the cysteine reorientation. The 2SC-SUMO1 structure appears to be less compact than the SUMO1 one, since it is characterized by an increased radius of gyration, as a result of a reduced number of intramolecular H-bonds (**Table 1**) by the reorientation of the cysteine. Since the SUMO1 protein is mainly stabilized by H-bonds, the decreased number of hydrogen bonds in 2SC-SUMO1 aligns well with the reduced stability observed in thermal denaturation experiments.

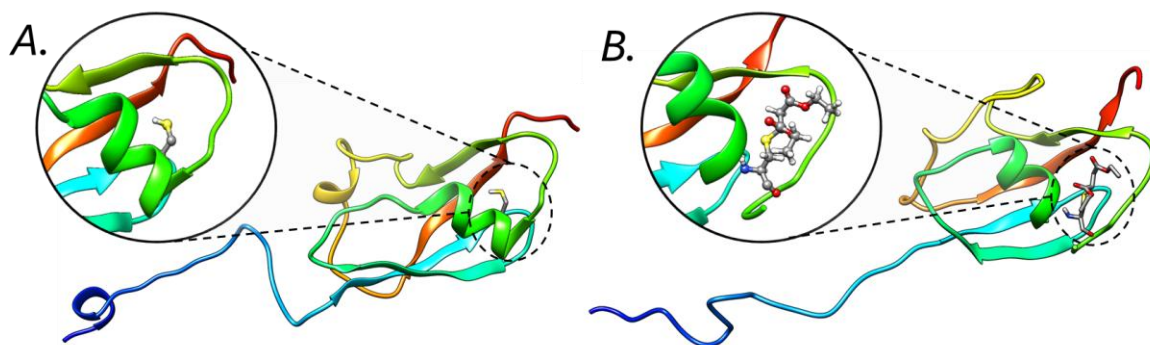


Figure 8: Last frame of (A) SUMO1 and (B) 2SC-SUMO1, extracted from the 1 μ s simulation in explicit solvent at 300K. After 1 μ s, the tertiary structure is looser when succinated, while the secondary structures are preserved. Cys52 residues are highlighted in the insets.

To approach the surface accessibility of the proteins, the total area of the protein envelope can be theoretically estimated by calculating the SASA, which can be obtained from MD data by considering a water molecule as the probe in the algorithm. The values presented in **Table 1** indicate that SASA tends to slightly increase upon succination. Such an unfolding is in line with the HDX results, which indicated a higher proportion of exchanged hydrogen atoms, *i.e.* solvent-accessible, upon succination. The number of exchanged hydrogen atoms can also be estimated based on the MD results. To do so, on the last 250 frames of the MD simulation, the number of water molecules located within 2.5 Å (corresponding to a strong/moderate H-bond⁵⁰) of each labile hydrogen was counted. The average value was calculated for each labile hydrogen, and denoted as H_{prox} , *i.e.* hydration proximity parameter, which can be used to evaluate whether the corresponding H atom is accessible and therefore considered as exchangeable. Graphical representation of the H_{prox} parameter (**Figure SI10**) shows that all the labile hydrogens separate into 2 distinct groups, centered to H_{prox} of 0.1 (not exchanged) and 0.8 (exchanged), respectively, with a H_{prox} threshold of 0.45 between the two hydrogen groups; a hydrogen atom with a value of $H_{prox} > 0.45$ was considered as exchanged. This value corresponds to the minimum of the bimodal distribution (see **Figure SI10**) and matches the experimental number of 114 exchanged hydrogen atoms for SUMO1. This same threshold was then applied to succinated SUMO1 to ensure consistency. The results of this analysis are also presented in **Table 1**. The values are in good agreement with those obtained by HDX-MS (114 for SUMO1 and 132 for 2SC-SUMO1), again revealing an unfolding (destabilization) of the structure upon succination.

Table 1. MD of SUMO1 and 2SC-SUMO1 in solution.

	SASA ^a (n=3) ^b	Theoretical number of exchangeable hydrogen (n=3)	Cysteine distance from center-of- mass (n=2)	Averaged number of H-bonds (n=3)	Radius of gyration (n=3)
SUMO 1	7517 ± 185 Å ²	114 ± 4	10.8 ± 1.3 Å	34 ± 2	14.7 ± 0.5 Å
2SC-SUMO1	7750 ± 215 Å ²	129 ± 1	13.8 ± 0.1 Å	29 ± 0	16.7 ± 0.3 Å

^aSASA with water molecule (probe size = 1.4 Å); ^b Number of replicates

6. Ion Mobility Spectrometry experiments

IMS experiments were conducted to establish whether the structure modifications of SUMO1 upon succination detected in solution are also observable in the gas phase on protein ions. Structure modifications are established by monitoring the momentum transfer cross section (commonly called “collisional cross section”, CCS²⁷) of the gaseous ions that characterizes the effective compactness and roughness of the ions in the gas-phase. We anticipated that the unfolding suggested by the HDX experiments upon succination should be reflected by a higher CCS value. The 6+ charge state was selected based on the analysis of the arrival time distribution (ATD) of the SUMO1 ions, shown in **Figure S11**. The ATD profiles indicate that the 6+ charge state better describes the protein native structure. Indeed, for the 7+ charge state, a strong structure heterogeneity is highlighted by the presence of numerous IMS signals, pointing to a partial coulombic unfolding of the 7+ ions⁵¹. As far as the 8+ ions are concerned, we attribute the narrow mobilogram detected to totally unfolded ions, due to the high charge state. Such a gas phase unfolding is clearly triggered by the presence of additional protons, from 6+ to 8+ ions, and is not inherited from the solution phase, as supported by the HDX results.

Figure 9 displays ATD of the 6+ of SUMO1 and 2SC-SUMO1 ions. These curves are quite similar with the apex of the two curves being slightly shifted by 0.2 ms. This arrival time difference translates into a $^{TW}CCS_{N_2 \rightarrow He}$ difference of ~ 20 Å² (the methodology to determine $^{TW}CCS_{N_2 \rightarrow He}$ is described in the experimental section, and the calibration curve can be found in supporting information, **Figure S12**). Experimental CCS are displayed in **Table 2**, together with the theoretical $^{TM}CCS_{He}$ values calculated from *in vacuo* MD simulations. The $^{TM}CCS_{He}$ are in fair agreement

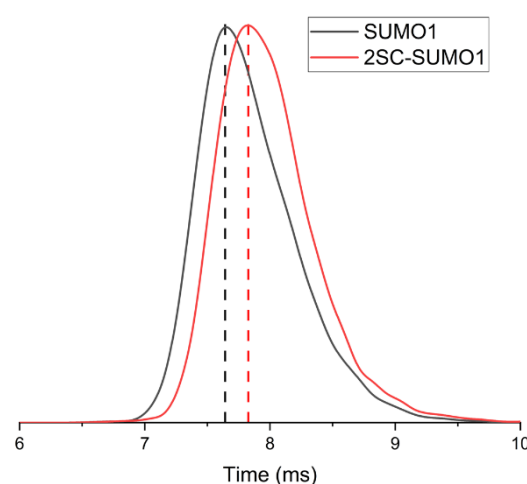


Figure 9: ATD of the SUMO1 and 2SC-SUMO1 6+ ions. These curves are almost superimposable with a weak +0.2 ms shift upon succination. This difference translates into a CCS difference of ~20 Å².

with the experimental ones, with less than the 5% deviation generally encountered for protein ions⁵². Moreover, they indicate that the succination slightly affects the $^{TM}CCS_{He}$ value, with a $\sim 25 \text{ \AA}^2$ increase, $\sim 2\%$, corresponding to the influence of DEF ($23 \pm 1 \text{ \AA}^2$). The determined CCS are also in good agreement with the relationship established by Ruotolo *et al.* between CCS and molecular weight for globular protein ions⁵³, i.e. $CCS = 2.435 \times M^{2/3} = 1,204 \text{ \AA}^2$ (MW = $11,000 \text{ g mol}^{-1}$).

Table 2. IMS and MD simulation data for SUMO1 and 2SC-SUMO1 ions in the gas phase (see text and experimental section for details)

	$^{TW}CCS_{N_2 \rightarrow He}^a$ (n=4) ^b	$^{TM}CCS_{He}^c$ (n=3)	SASA ^d	V_{SASA} (n=3) ^e
SUMO 1	$1,124 \pm 5 \text{ \AA}^2$	$1,170 \pm 5 \text{ \AA}^2$	$4,702 \pm 16 \text{ \AA}^2$	$19,326 \pm 11 \text{ \AA}^3$
2SC-SUMO1	$1,143 \pm 5 \text{ \AA}^2$	$1,196 \pm 5 \text{ \AA}^2$	$4,755 \pm 22 \text{ \AA}^2$	$19,689 \pm 5 \text{ \AA}^3$

^a Experimental CCS; ^b Number of replicates; ^c Theoretical CCS; ^d SASA with Helium atom (probe size = $1.4 \text{ \AA}$); ^e Molecular volume delimited by the SASA.

As presented in **Figure 10**, the final MD snapshots confirm that the ions adopt a globular shape in the gas phase. For the spherical gas phase ions, CCS may be approximated as the projected surface area ($CCS = \pi.R_{CCS}^2$) of a hard sphere⁵⁴, with the total surface area of the ion envelope being given by $SASA = S_{CCS} = 4.\pi.R_{CCS}^2$. For a perfect sphere, i.e. without any surface roughness, the ratio between SASA and CCS should thus be equal to 4. Several geometrical parameters, including SASA and V_{SASA} , calculated in Helium are reported in **Table 2**, with once again no significant variation between gaseous SUMO1 and 2SC-SUMO1 (6+ ions), that could have implied conformational changes in the SUMO1 ion structure.

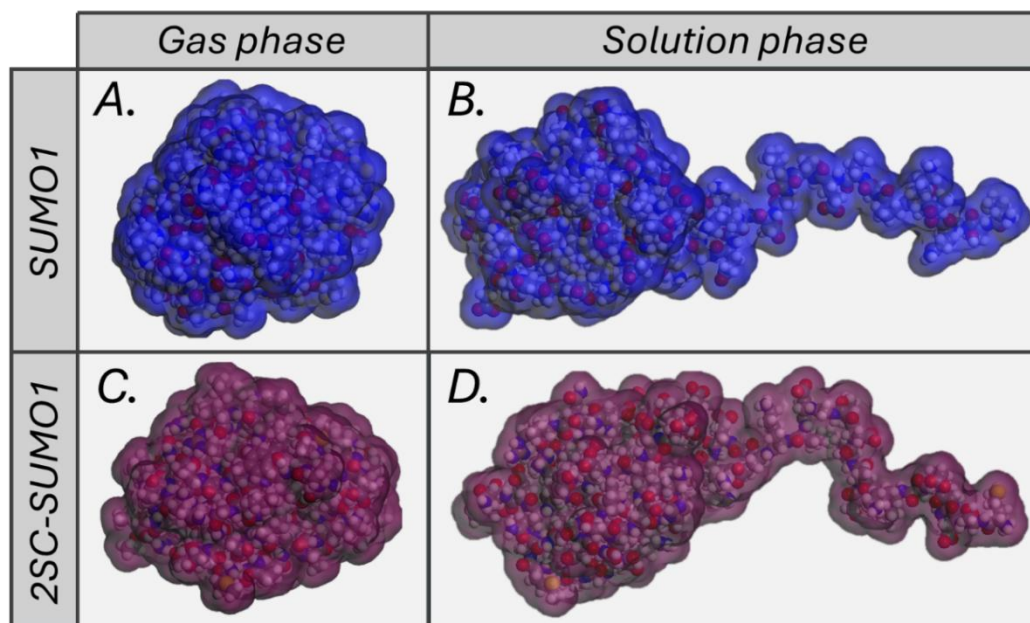


Figure 10: Last conformations for gas-phase (A and C) and solution phase (B and D) MD simulations. The protein (ion) envelopes are delimited by the SASA and include the V_{SASA} . The blue structures represent SUMO1 ions (6+ in the gas phase and 4- in solution), while the purple structures represent 2SC-SUMO1 ions (6+ in the gas phase and 4- in solution).

For both SUMO1 and 2SC-SUMO1 6+ ions, a 4.1 ratio value is obtained, confirming that the ions are nearly spherical in the gas phase. Following this conclusion, the surface area, volume, and densities can be calculated based on the experimental CCS. For SUMO1 6+ ions, a radius of $R_{CCS} = 18.9 \text{ \AA}$ is determined, which can then be used in the equation $V_{CCS} = (4/3) \cdot \pi \cdot R_{CCS}^3$ to compute the corresponding solid sphere volume, yielding $V_{CCS} = 28,347 \text{ \AA}^3$. This volume is subsequently used in the density equation⁵⁵: $\rho_{CCS} = 10^{24} \cdot M / V \cdot N_A$ where ρ_{CCS} represents the protein density based on the CCS ($\text{g} \cdot \text{cm}^{-3}$), M is the molecular weight ($\text{g} \cdot \text{mol}^{-1}$), V is the molecular volume ($\text{\AA}^3$), and N_A is the Avogadro number (mol). The calculated values are presented in **Table 3** for SUMO1 only since the succination does not really affect the density. Notably, S_{CCS} is found to align closely with the theoretical SASA (see **Table 2**), further supporting the conclusion that these ions adopt a spherical conformation in the gas phase. Furthermore, when the densities of both SUMO1 and 2SC-SUMO1 6+ ions are calculated using CCS, a value of $0.64 \text{ g} \cdot \text{cm}^{-3}$ is obtained, which is consistent with the $0.6 \text{ g} \cdot \text{cm}^{-3}$ reported by Bush *et al.* for globular proteins⁵³.

Table 3. Comparison of structural and density parameters of SUMO1 in the gas and solution phases. No CCS parameters are provided for the solution phase, as CCS cannot be approximated as the projected surface area of a sphere.

	S_{CCS}^a	V_{CCS}^b	ρ_{CCS}^c	V_{SASA}^d	ρ_{SASA}^e
Gas Phase	4,496 $\text{\AA}^2$	28,347 $\text{\AA}^3$	0.64 $\text{g} \cdot \text{cm}^{-3}$	$19,326 \pm 11 \text{ \AA}^3$	0.95 $\text{g} \cdot \text{cm}^{-3}$
Solution Phase	/	/	/	$22,964 \pm 55 \text{ \AA}^3$	0.80 $\text{g} \cdot \text{cm}^{-3}$

^a CCS-based Surface; ^b CCS-based Volume; ^c CCS-based density; ^d Molecular volume delimited by the SASA; ^e Densities calculated from the molecular volume delimited by SASA.

Comparing the effective density of the protein in the solution phase to that in the gas phase provides insight into structural changes occurring during transfer to vacuum. To account for surface roughness and internal cavities, the SASA associated volume is used. The resulting densities are found to be $0.95 \text{ g} \cdot \text{cm}^{-3}$ in the gas phase and $0.80 \text{ g} \cdot \text{cm}^{-3}$ in solution, indicating that greater compaction occurs in the gas phase. Interestingly, this gas-phase density is found to be higher than that derived from the SASA-based calculation. This discrepancy arises because the considered volumes provide a more realistic representation of ion roughness, whereas CCS approximates the ion as a perfect sphere⁵⁴. The transition from solution to gas phase results in structural compaction due to charge solvation effects⁴⁰. In other words, such a folding may obscure the actual impact of succination on SUMO1 structure as detected in solution.

Conclusions and perspectives

In this study, we aimed to investigate the impact of spontaneous SUMO1 succination on its tertiary structure. SUMO1, which contains a cysteine residue, is susceptible to succination via Michael addition of cysteine to fumarate. To assess whether this PTM may affect the tertiary structure of SUMO1, we employed a combination of solution-phase and gas-phase methods. The experimental findings were further supported by molecular modeling simulations conducted in both explicit solvent and gas-phase conditions.

The increased number of exchanged hydrogen atoms upon succination, as demonstrated upon HDX-MS experiments, is associated with a partial unfolding of the protein structure rendering additional acidic hydrogen atoms solvent-accessible in 2SC-SUMO1 compared to SUMO1. The succination is not resulting in a severe loss of the secondary structures, as shown by the good similarity of the CD spectra, highlighting only a slight reduction of the helix contribution from 13% to 9% upon succination. MD simulations performed in explicit solvent on SUMO1 and 2SC-SUMO1 (4-) ions revealed that the main structural modification upon succination is the Cys52 residue reorientation, partially disorganizing the α -helix extremity and the tertiary structure, in agreement with the CD results. Also, MD simulations showed that the SASA underwent a 3% expansion upon succination, with a higher number of labile hydrogen atoms becoming exchangeable, from 114 to 129, in good agreement with the HDX experiments. These results are partially confirmed using MS-based methods. First of all, the SUMO1 succination has been detected by ESI-MS and the covalent nature of the DEF attachment onto SUMO-1 has been confirmed using tandem MS experiments. SUMO1 and 2SC-SUMO1 proteins are detected as 6+, 7+, and 8+ ions, with the abundance ratio of 2SC-SUMO1 to SUMO1 ions increasing as the charge state rises. We suspect that this is also related to the larger SASA upon succination, allowing higher charge states to be reached. However, when exposing the SUMO1 and 2SC-SUMO1 6+ ions to IMS experiments, the structural differences detected in solution vanish due to the ion compaction upon transfer to the vacuum, as attested by a higher effective density, from 0.8 to 0.95 g.cm⁻³, when passing from the solution to the gas phase, respectively.

All these results indicate that succination induces conformational changes. In the study by Drobecq *et al.*¹⁵, it was shown that modification of the SUMO1 Cys52 residue induces conformational changes, as this cysteine is essential for maintaining the 3D structure of SUMO1. The study also demonstrated that such a modification impairs the biological function of the protein. Therefore, the succination of SUMO1 could potentially affect its interaction with other proteins and its functional role. For instance, in cardiomyocytes, alterations in SUMO1 structure due to succination could influence its ability to stabilize and modulate SERCA2a

(Sarcoplasmic/Endoplasmic Reticulum Ca^{2+} ATPase) a calcium ion pump critical to the function of healthy cardiomyocytes. It facilitates the reuptake of calcium ions into the sarcoplasmic reticulum, a process essential for normal heart muscle relaxation⁵⁶. Notably, SERCA2a activity and expression levels are tightly regulated in coordination with SUMO1 levels^{57,58}. Studies have revealed that both SERCA2a SUMOylation and SUMO1 concentrations are significantly reduced in failing hearts^{57,58}. Furthermore, restoring SUMO1 expression has been shown to preserve SERCA2a levels and improve cardiac function, emphasizing the therapeutic potential of targeting the SUMO1-SERCA2a axis in cardiac dysfunction^{58,59}. SUMO1 succination could then potentially contribute to cardiotoxicity, especially under conditions of elevated fumarate levels, such as those observed in certain metabolic disorders. To gain a deeper understanding of these interactions, further experiments are necessary to investigate how SUMO1 succination impacts the interaction with the E1/E2 enzyme complex, the enzymes catalyzing SUMOylation, and SERCA2a. To validate these *in vitro* findings, *in vivo* studies are currently being conducted in cardiac cells to assess the physiological relevance of SUMO1 succination and its contribution to cardiotoxicity, which could pave the way for a quantitative approach.

Supporting information

Protein sequences; IMS-MS, MS and MS/MS spectra; calibration curves; HDX-MS deconvolution and MD data; thermal denaturation curves by CD; and CD-based thermodynamic calculations.

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