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Jennifer Bouchenna, Magalie Sénéchal, Hervé Drobecq, Nicolas Stankovic-Valentin, Jérôme Vicogne, et al.. The Role of the Conserved SUMO-2/3 Cysteine Residue on Domain Structure Investigated Using Protein Chemical Synthesis. *Bioconjugate Chemistry*, 2019, 30 (10), pp.2684-2696. 10.1021/acs.bioconjchem.9b00598 . hal-02349318

HAL Id: hal-02349318

<https://hal.science/hal-02349318>

Submitted on 30 Nov 2020

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The Role of the Conserved SUMO-2/3 Cysteine Residue on Domain Structure Investigated Using Protein Chemical Synthesis

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ABSTRACT

While the semi or total synthesis of ubiquitin or polyubiquitin conjugates have attracted a lot of attention the last decade, the preparation of small-ubiquitin like modifier (SUMO) conjugates is much less developed. We describe hereinafter some important molecular features to consider when preparing SUMO-2/3 conjugates by chemical synthesis using the native chemical ligation and extended methods. In particular, we clarify the role of the conserved cysteine residue on SUMO-2/3 domain stability and properties. Our data reveal that SUMO-2 and 3 proteins behave differently to the Cys→Ala modification with SUMO-2 being less impacted than SUMO-3 likely due to a stabilizing interaction occurring in SUMO-2 between its tail and the SUMO core domain. While the Cys→Ala modification has no effect on the enzyme-catalyzed conjugation, it shows a deleterious effect on the enzyme-catalyzed deconjugation process, especially with the SUMO-3 conjugate. Whereas it is often stated that SUMO-2 and SUMO-3 are structurally and functionally indistinguishable, here we show that these proteins have specific structural and biochemical properties. This information is important to consider when designing and preparing SUMO-2/3 conjugates, and should help making progress in the understanding of the specific role of SUMO-2 and/or SUMO-3 modifications on protein structure and function.

KEY-WORDS

Protein chemical synthesis, SUMO conjugates, SUMO-2, SUMO-3, desulfurization, circular dichroism

INTRODUCTION

Protein synthesis¹⁻³ is a powerful mean for accessing proteins harboring a large variety of small post-translational modifications such as methylation,⁴ acetylation,^{5,6} phosphorylation^{5,7} or sulfation.^{8,9} Its increasing capacity to address the production of proteins decorated by post-translational modifications of much higher complexity stimulates the creativity of protein chemists worldwide.¹⁰⁻²⁴ In particular, chemical synthesis has been used to produce ubiquitin (Ub),^{12,25-27} polyubiquitin,^{12-14,22,24,28-30} small ubiquitin-like modifiers (SUMO)^{19,20,31} and conjugates thereof^{10,11,21,23,29,32-35} (for reviews see^{36,37}) and as such has permitted to investigate in a new way how these modifications modulate protein structure and function.

Among the five human SUMO isoforms identified so far, SUMO-1-3 are the most studied since they are constitutively expressed in all eukaryotic cells (Fig. 1A).^{38,39} SUMO-2 and 3 are highly homologous (~95%) but are only 45% identical to SUMO-1. SUMO proteins are linked to the side-chain of exposed lysine residues (Lys) through an isopeptidic bond involving the C-terminus of the modifier. While ubiquitination is principally a signal for protein degradation by the proteasome, SUMO modification can change the localization, the stability, or the activity of the target proteins by modulating protein-protein interactions and by competing or intersecting with other lysine modifications such as ubiquitin.^{40,41} Many proteins are the target of SUMO modification.⁴² The SUMOylation and deSUMOylation machinery, by being implicated in the regulation of many cellular mechanisms, is now considered as a promising target for the development of drugs against diseases such as cancer.⁴³⁻⁴⁵

Contrary to SUMO-1, SUMO-2 and 3 isoforms contain an internal SUMOylation site within their N-terminal tail situated at lysine 11. This feature makes possible the production of polySUMO-2/3 chains *in vivo*. Therefore, understanding how SUMOylation and, in particular, how polySUMO-2/3 modification modulates protein structure and function can strongly benefit from the production of homogeneous and branched SUMO-2/3 conjugates, as this is currently pursued with synthetic ubiquitin scaffolds. Such scaffolds can nicely complement studies performed with linear and recombinant polySUMO constructs.^{46,47}

While methods enabling the chemical synthesis of polyubiquitin chains have been extensively developed during the last decade, the reports describing the synthesis of SUMO conjugates are few and far between.^{10,11,21,23,32-35} This situation arises probably in part from the difference in length between Ub (76 AA) and SUMO domains (92-96 AA), the latter being beyond the limits of conventional SPPS: the SPPS of Ub has been reported by Ramage and co-workers about 30 years ago,²⁶ when an efficient SPPS approach to SUMO has been disclosed only recently.⁴⁸ A second and important difference between Ub and SUMO proteins is also the presence of a central cysteine (Cys) residue in the core of all SUMO isoforms (Fig. 1A).⁴⁹

Ub conjugates can be produced by reacting an Ub thioester domain with a target peptide featuring a γ ^{50,51} or δ -mercaptolysine^{13,14,52} residue according to the native chemical ligation (NCL⁵³, for a recent review see ²). Appending a Cys residue to the side-chain of the target lysine is an interesting alternative to the use of mercaptolysines.^{54,55} The thiol group from mercaptolysine or Cys is subsequently removed by desulfurization (Scheme 1).^{56,57} Since Ub features an central alanine residue in its sequence (Ala46), Ub (Ala46Cys) analogue can also

be assembled from two peptide segments by NCL and converted afterwards into native Ub by global desulfurization.

The central Cys residue in SUMO domains is ideally located and facilitates their assembly by NCL. However, the production of SUMO conjugates requires an adaptation of desulfurization protocols if a native SUMO domain has to be obtained.^{19,20,23} Indeed, the desulfurization of the thiol amino acid used to produce the isopeptidic bond, *i.e.* a mercaptolysine or Lys(Cys), can potentially result in the desulfurization of the SUMO domain. This is especially the case when desulfurization is conducted in denaturing conditions, *e.g.*, in concentrated guanidine hydrochloride (Gn·HCl), since all thiols are equally exposed. Interestingly, the possibility of a selective desulfurization of SUMO-1-3 domains in native conditions was reported by Geurink *et al.*³³ and confirmed afterwards by Drobecq *et al.* with the preparative synthesis of SUMO-1 conjugates (Scheme 1).²³ The basis of this selectivity is the masking of the internal Cys thiol provided by the hydrophobic core of the folded SUMO domain, whereas the thiol from the residue used to produce the isopeptidic bond is solvent exposed and accessible to the desulfurization reagents.

Nevertheless, the selective desulfurization protocol discussed above might not preclude the desulfurization of the Cys residues present in the SUMOylated polypeptide that are accessible to the solvent. Moreover, the popularity of 6 M Gn·HCl as a medium for protein synthesis originates from its capacity to significantly improve the solubility of polypeptides. Therefore, another pitfall of performing a desulfurization in native conditions can be the limited solubility of the reactants. In this context, knowing the relative contribution of the internal Cys thiol to SUMO-2/3 domain structure and functionality can significantly influence the design of SUMO conjugates and the way they are produced.

While the importance of the internal Cys residue on SUMO-1 thermal stability and biochemical properties has been investigated,²³ this information is not yet available for SUMO-2 or SUMO-3. The differences between SUMO-1 and SUMO-2/3 in amino acid composition and in the Cys thiol environment within the protein hydrophobic core of the SUMO domain hampers any generalization and stimulated the investigations reported hereinafter (Fig. 1B, C). In particular, we used circular dichroism (CD) to compare SUMO-2/3 and desulfurized SUMO-2/3 secondary structure and thermal stability. These proteins were also compared using SUMO biochemical conjugation assays, including FRET-based conjugation experiments. We also determined the kinetics for the SENP1 and SENP2-catalyzed cleavage of synthetic conjugates

featuring a wild type or desulfurized SUMO-2/3 domain. In addition of showing the importance of the internal Cys residue on SUMO-2/3 secondary structure and recognition by deconjugation enzymes, this work establishes that SUMO-2 and 3 behave differently to the Cys→Ala perturbation.

Figure 1. A) Protein sequences from Homo sapiens (human) SUMO isoforms hSUMO-1 (Accession number P63165), hSUMO-2 (P61956), hSUMO-3 (P55854), hSUMO-4 (Q6EEV6) and hSUMO-5 (G2XKQ0) have been aligned using MegAlign Pro™ (Lasergen© DNASTar®) with MAFFT algorithm (BLOSUM30 scoring matrix). Sequences have been associated according to their phylogenic proximities. Color background of amino acids corresponds to the chemical nature of the side chain (aromatic – yellow, acidic – red, basic – blue, nonpolar – orange, polar – green). B) NMR structure of SUMO-1, Protein Data Bank (pdb) entry 1A5R.⁵⁸ C) NMR structure of SUMO-2 (pdb entry 2N1W). For B,C some side-chains of hydrophobic residues surrounding the internal Cys are highlighted.

Scheme 1. Chemical synthesis of Ub and SUMO-conjugates by the combined use of NCL and desulfurization

RESULTS AND DISCUSSION

Chemical synthesis of SUMO-2/3 proteins

Wild-type and desulfurized SUMO-2 and 3 proteins (SUMO-2 C48A and SUMO-3 C47A) were required for performing comparative studies by circular dichroism and by SUMO biochemical conjugation assays. The synthesis of wild-type and desulfurized SUMO-2/3 core domain was also undertaken to further investigate the role of the SUMO tail in protein folding.

The chemical synthesis of the different SUMO proteins was undertaken as described in Scheme 2. The presence in SUMO proteins of a Cys residue in central position enabled their assembly in two pieces. The segments corresponding to the sequence of SUMO-2, SUMO-3 and SUMO-2/3 core on both sides of the central cysteine residue were produced by 9-fluorenylmethyloxycarbonyl (Fmoc) SPPS using bis(2-sulfanylethyl)amino (SEA) ChemMatrix® resin^{19,20} for segments **1a-c** and 4-hydroxymethylphenoxyacetyl (HMPA) ChemMatrix® resin for segment **2**. The use of Fmoc-Asp(OrBu)-(Dmb)Gly-OH dipeptide unit for introducing the Asp and Gly residues indicated in bold in Scheme 2 was found to be mandatory for suppressing aspartimide formation during SPPS, as already noticed by other groups.^{31,48} SEA-mediated ligation⁵⁹ of segments **1a-c** and **2** proceeded efficiently at pH 5.5 in

the presence of 4-mercaptophenylacetic acid (MPAA) to produce the target SUMO proteins. While SEA-mediated ligations are usually carried out at 37°C, we performed the assembly of the different SUMO proteins at a lower temperature of 25°C, since higher temperatures resulted in the appearance of aspartimide side-products. Using these conditions, SUMO-2, SUMO-3 and SUMO-2/3 core proteins were isolated in good yield (60-83% after HPLC purification). Typical HPLC-MS and SDS-PAGE analyses of synthetic SUMO-2 and 3 proteins are shown in Fig. 2. For the latter analysis, the synthetic proteins are compared to SUMO-2 and SUMO-3 recombinant products (see Notes i, ii).

Scheme 2. Chemical synthesis of SUMO-2, SUMO-3 and SUMO-2/3 core proteins

Figure 2. Analysis of synthetic SUMO-2 and SUMO-3 proteins. A) UPLC-MS analysis of synthetic SUMO-2; B) UPLC-MS analysis of synthetic SUMO-3; C) SDS-PAGE analysis of SUMO-2, SUMO-3, desulfurized SUMO-2 and 3 proteins (i.e. SUMO-2 C48A and SUMO-3 C47A respectively), and recombinant SUMO-2 and 3 proteins (i.e. *r*₁-SUMO-2 and *r*₁-SUMO-3 respectively, see Notes i, ii). The gel was loaded with 1 μ g of each protein and stained using Coomassie blue R250, MW = molecular weight markers).

The successful preparation of the different SUMO proteins enabled the preparation of SUMO-2 C48A, SUMO-3 C47A and SUMO-2/3 core C48A by *tris*(2-carboxyethyl)phosphine (TCEP)-induced radical desulfurization of the internal Cys residue in denaturing conditions (Scheme 3, see Note iii).⁵⁷ The presence of methionine in the desulfurization mixture proved to be important for minimizing the oxidation of the methionine residues during the reaction. The SDS-PAGE analysis of SUMO-2 and 3 Cys→Ala variants is shown in Fig. 2C in comparison with wild-type synthetic and recombinant SUMO-2 and 3 proteins (for other analyses, see Supporting Information).

Scheme 3. Preparation of SUMO-2 C48A, SUMO-3 C47A and SUMO-2/3 core C48A analogues by desulfurization

Chemical synthesis of SUMO-2 and 3 conjugates

The chemical synthesis of SUMO-2, SUMO-3 and desulfurized conjugates derived thereof was undertaken for studying the impact of the Cys→Ala modification on the deconjugation reaction catalyzed by Sentrin-specific protease 1 (SEN1⁶⁰) or 2 (SEN2^{61,62}), which are human SUMO-1, 2 and 3 specific deconjugating enzymes.^{43,63}

The conjugates were prepared starting from SEA peptides **1a,b** as described in Scheme 4. Briefly, peptide thioesters **3** derived from 3-mercaptopropionic acid (MPA) were first prepared from SEA peptides **1** by SEA-thiol exchange in mildly acidic conditions (pH 4.0).⁶⁴ The one-pot assembly of the conjugates started with the production of SUMO-SEA intermediates **5** by coupling peptide segments **3** and **4** according to the NCL reaction. This step was performed in

the absence of TCEP to keep the SEA group in its latent form as a cyclic disulfide.^{20,65} Once formed, the SUMO-SEA domain **5** was activated by adding TCEP to the ligation mixture and ligated in one-pot with the target peptide **6**. The latter corresponds to the N-terminal tail of SUMO-2, which includes the SUMO-2 SUMOylation site VKTE (see Figure 1A). Typical HPLC-MS analyses of SUMO-2 and 3 conjugates **7a,b** are shown in Fig. 3A,B.

The successful synthesis of SUMO 2 and 3 conjugates **7a,b** set the stage for the desulfurization reactions, which were performed under native conditions, i.e., in the absence of Gn·HCl (Scheme 5, left), or in denaturing conditions that is in 6 M Gn·HCl (Scheme 5, right). Importantly, the native conditions resulted in the selective desulfurization of the Cys thiol linking the SUMO domains to the target peptide and hence in the production of conjugates **8a,b**, while denaturing conditions furnished the fully desulfurized products **9a,b**. The selectivity of the desulfurization reaction in native conditions for the Cys residue between SUMO domain and the target lysine was confirmed by proteomic analysis of conjugates **8a,b** (see Supporting Information). The SDS-PAGE analysis of the different conjugates is shown in Fig. 3C. All the conjugates migrate to an apparent molecular weight of ~20 kDa, while wild-type SUMO-2 and 3 proteins migrate at around 15 kDa.

Scheme 4. Synthesis of SUMO-2 and 3 conjugates 7a,b

Scheme 5. Selective or global desulfurization of SUMO conjugates 7a,b. Production of selectively desulfurized SUMO conjugates 8a,b and of fully desulfurized conjugates 9a,b

Figure 3. Analysis of synthetic SUMO-2 and SUMO-3 conjugates. A) HPLC-MS analysis of synthetic SUMO-2 conjugate **7a**; B) HPLC-MS analysis of synthetic SUMO-3 conjugate **7b**; C) SDS-PAGE analysis of synthetic SUMO-2 and SUMO-3 proteins and of the different conjugates derived thereof, i.e. **7-9a** and **7-9b** respectively (1 μ g of each protein, Coomassie blue R250 staining, MW= molecular weight markers).

Role of the internal Cys residue on SUMO-2 and 3 secondary structure and stability

The successful synthesis of the different SUMO 2 and 3 variants set the stage for the analysis by CD of the impact of the Cys→Ala modification on the secondary structure and thermal stability of the SUMO-2 and SUMO-3 domains. The CD spectra of synthetic and recombinant SUMO-2 and 3 proteins are highly similar and the same for the thermal denaturation curves (see Supporting Information, Fig. S28 and S29). The CD spectra of wild-type synthetic SUMO-2 and 3 are highly similar. The α -helical content of ~15% found for synthetic and recombinant SUMO-2 and 3 proteins is identical to the CD α -helical content recently reported for recombinant SUMO-2.⁶⁶ Determined α -helical content is also consistent with that extracted from 20 different pdb entries describing the structure of SUMO-2 or SUMO-3 (see Supporting Information, Table S1).

Figure 4 shows the far-UV CD spectra at 25 °C, the deduced α -helical content according to the method of Greenfield & Fasman⁶⁷ and the evolution of the mean residue ellipticity at 222 nm, $[\theta]_{MRW222\text{ nm}}$, as a function of the temperature for synthetic SUMO-2 and 3 proteins and their Cys→Ala variants. The data show that the Cys→Ala modification induced only subtle changes in the far-UV CD spectrum of the SUMO-2 protein with a decrease of protein α -helical content of ~1% ($15.2 \pm 0.12\%$ ($n = 10$) for SUMO-2 vs $14.1 \pm 0.23\%$ ($n = 12$) for SUMO-2 C48A). The thermal denaturation curves for SUMO-2 and SUMO-2 C48A were found to be similar (SUMO-2 $T_m = 70.7 \pm 2.4$ °C, Fig. 4B).

The impact of the Cys→Ala modification was more pronounced for SUMO-3 with a decrease of the protein α -helical content of ~2% ($15.1 \pm 0.21\%$ ($n = 8$) for SUMO-3 vs $13.3 \pm 0.29\%$ ($n = 10$) for SUMO-3 C47A). The T_m measured for SUMO-3 C47A (65.5 ± 0.7 °C) is 4.4 °C lower than those found for the wild type protein SUMO-3 (69.9 ± 1.5 °C). Therefore and contrary to SUMO-2, the effect of the Cys→Ala modification on SUMO-3 is significant.

The internal Cys (Cys48 for SUMO-2, Cys47 for SUMO-3) is located in the α -helix of the SUMO-2/3 core domain with the Cys side-chain pointing toward the interior of the hydrophobic core. Some hydrophobic residues surrounding the Cys side-chain are highlighted in Fig. 1B. The case of Phe60 (SUMO-2) which is within 5 Å of Cys48 is particularly interesting to discuss in the light of a recent report by Bhattacharya and Ainarapu.⁶⁶ The authors have proposed

that Phe60 plays a central role in the reduction of the tertiary structure and mechanical stiffness of SUMO-2, as measured by CD and single-molecule force spectroscopy (SMFS) respectively, which is observed when SUMO-2 is heated from 5 °C to 45 °C. Obviously, the thermal denaturation of SUMO-2 is complex and involves some intermediates between the native and unfolded states. Therefore, the modification of the hydrophobic core of SUMO-2 and 3 by desulfurization of the central Cys residue can potentially alter their structure, stability and/or the distribution of intermediates along the protein energy landscape. Interestingly, the data presented in Fig. 4 show that although wild-type SUMO-2 and SUMO-3 proteins adopt the same secondary structure, they respond differently to the Cys→Ala modification. SUMO-2 and 3 differ only by some amino acid residues in their tail (Figure 1A). In other words, SUMO-2 and SUMO-3 share the same core domain and the close environment of the central Cys is the same for both proteins. A likely explanation for the observed difference is the existence of a cross-talk between the tail and the SUMO-2/3 core domain which is perceived by CD when the latter is perturbed by desulfurization. In order to further investigate this point, we analyzed the synthetic SUMO-2/3 core and SUMO-2/3 core C48A proteins by CD (Figure 5). The data show that the SUMO-2/3 core domain is also sensitive to the Cys→Ala modification, with a loss of α -helical content upon Cys desulfurization which is similar to what is observed for SUMO-3. Taken together, these experiments strongly support the existence of stabilizing interactions between the N-terminal tail of SUMO-2 and the core domain that are absent or much less pronounced in SUMO-3. Therefore, the Cys→Ala modification is an interesting tool to uncover weak interactions occurring between the tail and the core domain of SUMO proteins.

Figure 4. CD analysis of synthetic SUMO-2, SUMO-2 C48A, SUMO-3 and SUMO-3 C47A proteins. A) Analysis of SUMO-2 (n = 10) and SUMO-2 C48A (n = 12) at 25 °C. B) Thermal denaturation of SUMO-2 (n = 5) and SUMO-2 C48A (n = 7) ($[\theta]_{MRW}$ at 222 nm). C) Analysis of SUMO-3 (n = 8) and SUMO-3 C47A (n = 10) at 25 °C. D) Thermal denaturation of SUMO-3 (n = 9) and SUMO-2 C47A (n = 5) ($[\theta]_{MRW}$ at 222 nm). Conditions: 10 mM phosphate buffer pH 7.2. The samples were carefully degassed during 30 min with argon and immediately transferred under argon into the CD cuvette. Protein concentration for thermal denaturations : SUMO-2 12.9-28.6 μ M, SUMO-2 C48A 12.0-32.5 μ M, SUMO-3 12.5-40.4 μ M, SUMO-3 C47A 13.5-34.7 μ M. Protein concentration for CD analysis at 25 °C: SUMO-2 13.8-25.3 μ M, SUMO-2 C48A 9.1-31.6 μ M, SUMO-3 14.4-24.0 μ M, SUMO-3 C47A 25.1-34.7 μ M. The data correspond to the mean and standard deviation.

Figure 5. CD analysis of synthetic SUMO-2/3 core and SUMO-2/3 core C48A proteins. Conditions: 10 mM phosphate buffer pH 7.2. The samples were carefully degassed during 30 min with argon and immediately transferred under argon into the CD cuvette. Protein concentration: SUMO-2/3 core 21.2 μ M, SUMO-2/3 core C48A 22.4 μ M.

Effect of the Cys→Ala modification on SUMO-2/3 conjugation assays

Having characterized the effect of the Cys→Ala modification on the secondary structure and thermal stability of SUMO-2 and SUMO-3 proteins, we next performed a classical SUMO biochemical conjugation assay to see if the modification influences the enzyme-catalyzed formation of the isopeptidic bond using RanGAP as the target protein (see Supporting Information, Figure S30). We used for this purpose reagents from a commercially available conjugation kit. The different SUMO analogues (i.e. the recombinant proteins from the kit r₂-SUMO-2/3, synthetic wild-type SUMO-2/3 and synthetic desulfurized SUMO-2(3) C48A (C47A) were efficiently transferred with no apparent difference in the amount of RanGAP-SUMO conjugate formed whether SUMO-2 or SUMO-3 were desulfurized or not. Unexpectedly, isolated desulfurized proteins SUMO-2 C48A and SUMO-3 C47A were poorly recognized by the polyclonal anti-SUMO-2/3 antibody provided with the kit.

Since the enzymatic conjugation assay described above could be biased by the differential recognition of the SUMO analogues by the anti-SUMO-2/3 antibody, we performed the FRET-based quantitative bioconjugation assay described in Fig. 6.^{68,69} In this assay, the cyan fluorescent protein (CFP)-labelled RanGAPtail target is conjugated enzymatically to the yellow fluorescent protein (YFP)-labelled SUMO-3 protein. The CFP protein absorbs light at 430 nm

and emits a fluorescence signal at 485 nm. When CFP and YFP moieties are in close proximity (*e.g.* when RanGAPtail is SUMOylated), the energy transfer from CFP to YFP results in an emission signal at 527 nm. Therefore, the intensity ratio of the emission at 527 nm and the emission at 485 nm is representative of the amount of RanGAPtail SUMOylated. The assay enables the monitoring of the conjugation reaction in real-time and in parallel. With this assay, we analyzed a commercially available and recombinant SUMO-3 protein and its chemically desulfurized analogue (Fig. 7A) or synthetic SUMO-3 and SUMO-3 C47A proteins (Fig. 7B). The different analogues were used as competitors of the YFP-labelled SUMO-3 substrate. The capacity of the synthetic and recombinant SUMO-3 proteins to compete for the YFP-SUMO-3 substrate is the same. The data also show that the Cys→Ala modification has no significant effect on the capacity of SUMO-3 to be conjugated to RanGAPtail, either with the recombinant protein or the synthetic one at different competitor/ YFP-labelled SUMO-3 ratios.

Figure 6. Principle of the FRET-based enzymatic conjugation assay using RanGAPtail as target.

Figure 7. FRET-based enzymatic conjugation assay using RanGAPtail as target.^{68,69} A) Competition with a commercially available and recombinant SUMO-3 protein and its chemically desulfurized analogue. B) Competition with synthetic SUMO-3 and SUMO-3 C47A proteins. Conditions: 15 nM E1, 20 nM E2, 300 nM YFP-SUMO-3, 300 nM CFP-RanGAPtail and competitors (75 nM-600 nM) were incubated at 37°C for 10 min in conjugation medium (110 mM KOAc, 20 mM HEPES pH 7.3, 2 mM Mg(OAc)₂, 1 mM EGTA, supplemented with 1 µg/mL of each leupeptin, pepstatin and aprotinin, 1 mM DTT, 0.05% Tween®-20 and 0.2

mg/mL ovalbumin). In vitro SUMOylation reaction was started upon addition of 1 mM ATP. Every 20 s, the ratio $\frac{E527}{E485}$ was determined upon excitation at 430 nm.

Effect of the Cys→Ala modification on the SUMO-2 and 3 deconjugation reaction

We next examined the effect of Cys→Ala modification on the cleavage of synthetic SUMO-2 and 3 conjugates by SENP1 and SENP2 enzymes (Scheme 6). The enzymatic cleavage yields peptide **10** and the SUMO-2 or SUMO-3 domains. The rate of the deconjugation reaction could be easily monitored by LC-MS (Fig. 8).

Scheme 6. SENP1-catalyzed deconjugation of SUMO-2 conjugates **8,9a** and SUMO-3 conjugates **8,9b**

The data presented in Fig. 8 show that SUMO-2 and SUMO-2 C48A conjugates **8,9a** are cleaved at the same rate, whatever SENP enzyme is used. Therefore, SUMO-2 domain is recognized by the SENP1 and SENP2 enzymes whenever it features an internal Cys or not. In contrast, SUMO-3 conjugate **8b** is cleaved by both enzymes significantly faster than the C47A analog **9b**. In this case, the Cys→Ala modification alters the functionality of SUMO-3 domain. These results are in full accordance with the CD analyses, which showed that of the two proteins SUMO-2 and SUMO-3, SUMO-3 is the more sensitive to the Cys→Ala modification.

Figure 8. Deconjugation of SUMO conjugates **8,9a** and **8,9b** by SENP1 and SENP2 enzymes. A) SENP1-catalyzed deconjugation. B) SENP2-catalyzed deconjugation. Conditions: 50 mM HEPES buffer pH 8.0, 100 mM NaCl, 10 mM DTT, 10% glycerol, 25 °C, conjugate 0.5 mg/mL, final volume 100 μ L, SENP1 5.0 ng per cleavage reaction, SENP2 0.4 units per cleavage reaction. The deconjugation reaction was performed under nitrogen atmosphere, quenched with 10% aqueous acetic acid and analyzed by UPLC-MS. The experiments were performed in triplicate. The data correspond to the mean and standard deviation.

CONCLUSIONS

In conclusion, we have successfully prepared a series of SUMO-2 and SUMO-3 proteins that enabled investigating the impact of the SUMO-2/3 domain desulfurization on the secondary structure and thermal stability of SUMO-2 and 3 proteins, the rate of SUMO-2 and 3 conjugation to RanGAP substrates and on the deconjugation of SUMO-2 and 3 conjugates by

SEN1 and SEN2 enzymes. Contrary to SUMO-2, the fold of SUMO-3 is significantly affected by the Cys→Ala modification. Likewise, the rate of cleavage of SUMO-3 conjugates by SEN1 and SEN2 is reduced when the internal Cys residue is desulfurized into Ala. The CD analysis of the wild-type and desulfurized SUMO-2, SUMO-3 and SUMO-2/3 core proteins show the existence of a stabilizing interaction between the tail and the core domain in SUMO-2 which is less apparent for SUMO-3. Taken together, our results show that mutating the internal Cys residue in SUMO-3 must be considered with caution. They also reveal subtle differences between SUMO-2 and 3 that cannot be considered as being the same although they sequences are highly similar. Therefore, we show here that SUMO-2 and SUMO-3 are distinct proteins and should not be considered as redundant.

NOTES

- i) The composition in amino acids of some commercially available recombinant SUMO-2/3 proteins was found to differ significantly from the canonical sequences of SUMO-2/3 shown in Fig. 1A. Therefore, each purchased recombinant product was trypsinized and analyzed by MALDI-TOF mass spectrometry to determine its sequence independently of the information provided by the manufacturer (see Supporting Information).
- ii) The recombinant SUMO-2 and SUMO-3 products used for SDS-PAGE and CD analyses, i.e. r₁-SUMO-2 and r₁-SUMO-3, correspond to the canonical sequences for SUMO-2/3 shown in Fig. 1A, see Supplementary Information.
- iii) For SUMO-2/3 core protein, we use the SUMO-2 numbering of amino acid residues. The Cys residue is therefore at position 48.

ACKNOWLEDGEMENTS

We thank the Ministère de l'Enseignement Supérieur, de la Recherche et de l'Innovation for financial support (PhD fellowship to Jennifer Bouchenna). We thank Jean-Jacques Lacapère and Florine Cavelier for useful discussions. We thank Frauke Melchior for giving us access to the FRET SUMO conjugation assay.

Supporting Information Available

The Supporting Information is available free of charge on the ACS Publications website at DOI: #####.

Experimental procedures and characterization data for all peptides and proteins (LC-MS, MALDI-TOF, CD and proteomic analyses) (PDF).

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