Published in final edited form as:

J Chem Inf Model. 2017 June 26; 57(6): 1330–1341. doi:10.1021/acs.jcim.7b00150.

Structural Characterization of Histatin 5–Spermidine Conjugates: A Combined Experimental and Theoretical Study

Stephanie Jephthah[†], João Henriques[†], Carolina Cragnell[†], Sumant Puri[‡], Mira Edgerton[§], and Marie Skepö^{†,*}

[†]Division of Theoretical Chemistry, Lund University, Post Office Box 124, S–221 00 Lund, Sweden

[‡]Pediatrics and Community Oral Health Sciences, Temple University, Philadelphia, Pennsylvania 19147, United States

[§]Department of Oral Biology, University at Buffalo, Buffalo, New York 14214–3092, United States

Abstract

Histatin 5 (Hst5) is a naturally occurring antimicrobial peptide that acts as the first line of defense against oral candidiasis. It has been shown that conjugation of the active Hst5 fragment, Hst5_{4–15}, and the polyamine spermidine (Spd) improves the candidacidal effect. Knowledge about the structure of these conjugates is, however, very limited. Thus, the aim of this study was to characterize the structural properties of the Hst5_{4–15}–Spd conjugates by performing atomistic molecular dynamics simulations in combination with small-angle X-ray scattering. It was shown that the Hst5_{4–15}–Spd conjugates adopt extended and slightly rigid random coil conformations without any secondary structure in aqueous solution. It is hypothesized that the increased fungal killing potential of Hst5_{4–15}–Spd, in comparison with the Spd–Hst5_{4–15} conjugate, is due to the more extended conformations of the former, which cause the bonded Spd molecule to be more accessible for recognition by polyamine transporters in the cell.

Graphical Abstract

*Corresponding Author: marie.skepo@teokem.lu.se. Phone: +46 462 223 366.

ORCID

Stephanie Jephthah: 0000-0003-4287-506X

Carolina Cragnell: 0000-0001-6751-0644

Sumant Puri: 0000-0002-5509-007X

Mira Edgerton: 0000-0002-9680-5908

Marie Skepö: 0000-0002-8639-9993

Notes

The authors declare no competing financial interest.

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jcim.7b00150.

Information regarding the charge parametrization, time series of the radius of gyration and end-to-end distances and the respective density estimates (per simulation replicate), results for supplementary simulations performed with 140 mM salt, and additional experimental SAXS scattering intensities at different conjugate concentrations as well as the form factors on a log–log scale (PDF)

INTRODUCTION

Invasive fungal infections are known problems for critically ill patients, and the occurrence of these type of infections has been increasing over the years.¹ The most frequently isolated yeast species causing such infections are the *Candida* spp., mainly *Candida albicans*, but other non-*albicans* species have also been identified as the cause of fungal infections.^{1–3} Many effective antifungal agents are available for the treatment of invasive candidiasis, such as polyenes, azoles, and echinocandins.^{1,4} However, despite the access to these drugs, failures in treatment of invasive candidiasis due to drug-resistant *Candida* species still occur.^{1,4,5} There are also other problems related to the drugs, such as frequent side effects, inconvenient drug administration, and unfavorable interactions with other drugs.^{1,4} Thus, the need to develop new therapeutic agents is imperative.⁵

Naturally occurring antimicrobial peptides have been proposed as possible therapeutic agents against fungal infections.^{5,6} One example of such a peptide is histatin 5 (Hst5), which is a histidine-rich intrinsically disordered protein that can be found in human parotid and submandibular–sublingual saliva.^{7–9} Hst5 has various properties that contribute to oral health, and one of the most important is its antifungal action. Because of its effectiveness against fungal infections, especially against the blastospore and the germinated form of *C. albicans*,¹⁰ several studies have focused on investigating the possibility of using enhanced Hst5 variants in therapeutic contexts.^{5,11–13}

A study by Rothstein et al.¹² showed that the candidacidal activity of the 12 amino acid Hst5 fragment Hst5_{4–15} is equivalent to that of the full-length protein. Later, Kumar et al.¹³ observed that Hst5 utilizes polyamine transporters for intracellular uptake and transport in fungal cells. It was also shown that Hst5 operates like an analogue of the polyamine spermidine (Spd), which uses the same means of transportation into fungal cells. Tati et al.⁵ suggested that the uptake and activity of Hst5 into fungal cells might be increased by conjugation with Spd. Two different Hst5_{4–15}–Spd conjugates were studied, consisting of the active Hst5_{4–15} fragment with a Spd molecule conjugated either to the N-terminus (NSpd) or C-terminus (CSpd) of the fragment. It was observed that the conjugates have a greater candidacidal effect against *C. albicans* and *C. glabrata* and are more resistant to protease degradation compared with the unaltered full-length Hst5.⁵

Although their fungal killing potential has been established, very little is known about the structure of the Hst5_{4–15}–Spd conjugates. In fact, until now no direct measurements or simulations have been carried out in order to assess it. Thus, the aim of this study was to

characterize the Hst5₄₋₁₅-Spd conjugates by comparing the results of atomistic molecular dynamics (MD) simulations with small-angle X-ray scattering (SAXS) data.

MATERIALS AND METHODS

Chemicals

The Hst5₄₋₁₅-Spd conjugate (CSpd) and the Hst5₄₋₁₅ fragment were obtained from Genemed Synthesis Inc. (San Antonio, TX, USA) as white to off-white powders with 97.46% and 96.18% purity, respectively. The counterion was trifluoroacetate (TFA). A 20 mM Tris (Saveen Werner AB, >99.9% purity, CAS Registry no. 72-2-25) buffer was prepared in Milli-Q water and acidified to pH 7 with HCl. The ionic strength of the buffer solution was adjusted with NaCl (Scharlau, >99.5% purity, CAS Registry no. 7647-14-5). The buffer was filtered through a 0.2 μ m hydrophilic polypropylene membrane (Pall Corporation) before any peptide was added. After the peptide was dissolved, a concentration cell (Vivaspin 20, 2 kDa molecular weight cutoff (MWCO), product no. VS02H92, Sartorius Stedim Biotech GmbH, Göttingen, Germany) was used to remove low-molecular-weight impurities. The samples were rinsed with buffer corresponding to at least 10 times the sample volume by centrifugations at 3000 rpm and 18 °C. The samples were also dialyzed (Slide-A-Lyzer MINI, 2 kDa MWCO, product no. 69580, Thermo Scientific, United States) for 12 h to ensure exact background for the SAXS measurements. The studied peptide concentrations ranged from 0.8 to 5.12 mg mL⁻¹ in order to overlap the overall physiological protein concentration range in saliva (1–3.5 mg mL⁻¹). The ionic strength of the samples was set to 140 mM to exclude any electrostatic repulsion effects and to facilitate comparison with simulations, where the protein does not interact with its periodic images.

Small-Angle X-ray Scattering Measurements

SAXS measurements were performed using beamline BM29 at the European Synchrotron Radiation Facility (ESRF) in Grenoble, France. The incident-beam wavelength was 0.99 Å. The distance between the sample and the PILATUS 1 M detector was 2867 mm, giving a scattering vector range of 0.036–4.95 nm⁻¹. The scattering vector is defined as $q = 4\pi \sin \theta / \lambda$, where 2θ is the scattering angle. For each sample and pure solvent, several successive 1 s frames (typically 10–25) were recorded and analyzed. Special attention was paid to radiation damage by comparing the successive frames prior to further processing of the data. The background (pure solvent) was subtracted from the corresponding sample solutions. All measurements were performed at 20 °C. $I(0)$ was converted to an absolute scale by measuring the scattering of water. Before SAXS measurements, the samples were further centrifuged with an ultracentrifuge (TLA55 rotor) at 13 000 rpm for at least 30 min in order to remove unspecific aggregates. Protein concentrations were measured after preparation and again immediately before the SAXS measurements using a Nanodrop spectrophotometer ($\epsilon = 1490 \text{ M}^{-1} \text{ cm}^{-1}$, $\lambda = 280 \text{ nm}$).

The scattering of a macromolecule in solution depends on the contrast (“scattering power” relative to the solvent), given by the square of the difference between the scattering length densities of the solute and solution. The scattering length density differences (determined from the electron densities) of Hst5₄₋₁₅ and Spd are 3.099×10^{10} and $0.564 \times 10^{10} \text{ cm}^{-2}$

respectively, in pure water. Furthermore, the scattering length density differences were calculated with MULCh¹⁴ to be $3.013 \times 10^{10} \text{ cm}^{-2}$ for Hst5₄₋₁₅ and $1.030 \times 10^{10} \text{ cm}^{-2}$ for Spd in 20 mM Tris and 140 mM NaCl. From these numbers we assume that the SAXS spectra give information about the peptide part of the conjugate only, and not the bonded Spd. The above values could, for example, be compared with the scattering length density difference for a 10-monomer poly(ethylene glycol) molecule (i.e., $\sim 1.5 \times 10^{10} \text{ cm}^{-2}$), which is known to give very low contrast. Experimental SAXS spectra of the Hst5₄₋₁₅ fragment and the conjugate were thus compared (see Figure 1). The origin of the small deviation between the scattering curves in the high-*q* region is most probably due to the background subtraction. For both Hst5₄₋₁₅ and the Hst5₄₋₁₅-Spd conjugate, the apparent molecular mass, determined from $I(q=0)$, was found to be within a 10% deviation from the molecular mass calculated from the amino acid sequence.

Circular Dichroism

Hst5 (60 μM) and Hst5₄₋₁₅-Spd conjugates (60 μM) were dissolved in 95% trifluoroethanol (TFE) (Sigma-Aldrich, St. Louis, MO, USA) and analyzed in a fluorimeter cell (Starna Cells, Inc., Atascadero, CA, USA) with a 1 cm path length at 25 °C. Far-UV spectra (190–280 nm) were collected with a circular dichroism (CD) spectrophotometer (model 815, Jasco, Easton, MD, USA) and a thermostatic water bath. The data were processed using Jasco software and analyzed using Prism 5.0.

Nano LC-MS/MS

Mass spectrometry was employed to ensure the purity of the Hst5₄₋₁₅-Spd samples. The experiments were performed with an EasyLC nanoflow HPLC interfaced with a nanoEasy spray ion source (Proxeon Biosystems, Odense, Denmark) connected to a Fusion Orbitrap mass spectrometer (Thermo Fisher Scientific). The sample was loaded on a 2 cm PepMap column (75 μm inner diameter packed with 3 μm resin), and the chromatographic separation was performed at 35 °C on a 25 cm (75 μm inner diameter) EASY-Spray column packed with 2 μm of resin (Proxeon Biosystems). The nanoHPLC was operated at 300 nL/min with a gradient of 5–22% solvent B (0.1% (v/v) formic acid in acetonitrile) in solvent A (0.1% (v/v) formic acid in water) over 20 min, 22–32% over 2 min, and then an increase to 98% B over 2 min. An MS scan (*m/z* 350–1500) was recorded in the Orbitrap mass analyzer, which was set at a resolution of 60 000 at *m/z* 400, 1×10 automatic gain control target, and a maximum ion injection time of 500 ms. The mass spectrometric conditions were as follows: spray voltage, 1.9 kV; no sheath or auxiliary gas flow; S-lens, 60%; ion transfer tube temperature, 275 °C. The molecular mass was determined to be 1862.084 Da (theoretical weight 1863.0 Da), and very few ions were detected apart from the conjugate, indicating a purity of >98% (Figures 2 and 3).

COMPUTATIONAL SECTION

Models

Hst5₄₋₁₅ (Ala₁-Lys-Arg-His₄-His-Gly-Tyr-Lys₈-Arg-Lys-Phe-His₁₂) and its Spd conjugates (see Table 1) were built with PyMOL.¹⁵ The initial structures were assumed to be linear to avoid subsequent biasing of the conformational sampling. All four possible isomers

were built and simulated. The nomenclature (as shown in the first column of Table 1) is XSpdY, where X is either N or C, depending on whether it is an N- or C-terminal conjugate, and Y is either S or L (which stand for short side and long side, respectively), depending on the extremity by which Spd is bonded.

Charge Parametrization

Spd and succinic acid geometry optimizations (HF/6-31G*) were performed using the Gaussian 09 program.¹⁶ The same program, method, and basis set were also used to derive the electrostatic potentials from which the restrained electrostatic potential (RESP) charges (AmberTools 14 suite¹⁷) were calculated. A two-stage RESP fit was executed for each model shown in Table 2. In the first step, the charges of all noncapping atoms were allowed to vary. The charges of the capping residues, i.e., acetyl (ACE) or methylamine (NME), were taken from Cornell et al.¹⁸ Hydrogen charge symmetry was enforced for Spd amine groups with more than one hydrogen. In the second step, only the charges of methylene bridge groups were allowed to vary. Charge symmetry was enforced for the respective hydrogen atom charges. All of the other charges were fixed to the output of the first step. The final charges (presented in Table S1) were then used to build the new residue topology entries in the AMBER ff99SB-ILDN force field.¹⁹ The remaining bonded and nonbonded parameters were taken from the aforementioned force field, using the appropriate “atom type” for each atom in the models presented in Table 2.

Molecular Dynamics Simulations

All of the molecular dynamics simulations were performed with the GROMACS package (version 4.6.7)^{20–23} using the AMBER ff99SB-ILDN force field¹⁹ and the TIP4P-D water model²⁴ in the isobaric–isothermal ensemble. All of the simulation boxes were constructed as rhombic dodecahedrons with periodic boundary conditions and a minimum distance of 1 nm between the solute and the box. All of the systems were neutralized with Cl[−] ions (five for Hst5_{4–15}, six for the different N-terminal Hst5_{4–15}–Spd conjugates, and eight for their C-terminal counterparts). Simulations with an additional 140 mM NaCl were also performed for the Hst5_{4–15} fragment and the CSpdS conjugate in order to mimic experimental conditions and to ensure that the addition of salt to the simulations had no effect on the estimation of the structural properties studied in this work (see Figure S3 and Table S2). The equations of motion were numerically integrated using the Verlet leapfrog algorithm with a time step of 2 fs. The nonbonded interactions were treated with a Verlet list cutoff scheme in order to make use of the program’s native GPU acceleration. The short-range interactions were calculated using a nonbonded pair list with a single cutoff of 1 nm, updated every 100 fs. Long-range dispersion corrections were applied to the system’s energy and pressure. Particle mesh Ewald²⁵ was used to handle the long-range electrostatic interactions with cubic interpolation and a grid spacing of 0.16 nm. The solvent and solute were separately coupled to temperature baths at 300 K with the velocity rescaling thermostat²⁶ and a relaxation time of 0.1 ps. A Parrinello–Rahman pressure coupling²⁷ was used at 1 bar with a relaxation time of 2 ps and an isothermal compressibility of $4.5 \times 10^{-5} \text{ bar}^{-1}$. All of the bond lengths were constrained using the LINCS algorithm.²⁸

The minimization procedure was performed using the steepest-descent algorithm²⁹ without setting a maximum number of steps in order to achieve convergence within the available machine precision. Initiations were performed in a two-step scheme of 500 ps and 1 ns using position restraints of 1000 kJ mol⁻¹ nm⁻² on all protein heavy atoms. The first step was performed under the canonical ensemble to stabilize the temperature, and the second step was performed under the isobaric–isothermal ensemble.

Each conjugate system was simulated for a total of 5 μ s (five replicates of 1 μ s each). The protein fragment alone (i.e., Hst5_{4–15}) was simulated for twice as long (10 replicates of 1 μ s each) because of slower convergence. Coordinates and energies were saved every 10 ps. Residue charges were set to be representative of those at pH 7, with histidines being modeled in the deprotonated form.

The MD trajectories were used in their entirety for all of the analyses because of the inherent difficulty in defining initial equilibration times for such highly dynamic systems.

Analyses

As noticed before by our group when we studied Hst5, the simulation time needed to equilibrate properties such as the radius of gyration (R_g) and the end-to-end distance (R_{ee}) around their average values is extremely short,^{30,31} so short in fact that it is negligible given the very long simulation times of the replicates of each system (see Figure S1). Figure S2 shows the individual distributions of the radius of gyration and end-to-end distance per replicate for each simulated system. It is clearly visible that satisfactory convergence was achieved for all of the conjugates. However, some replicates of the system consisting of the protein fragment alone appeared to be “stuck” in metastable, compact conformations at times. Hence the doubled number of simulations performed for this system.

Information about all of the other analyses performed in this paper can be found in the previous studies by Henriques et al.³⁰ and Henriques and Skepö.³¹

Figures

All of the graphs were created using GNUPLOT.³² Protein snapshot figures were rendered using the PyMOL ray tracer. Two-dimensional chemical structure representations were drawn with Maestro.³³

RESULTS AND DISCUSSION

General Introduction

Figure 1a in the paper by Tati et al.⁵ shows the killing percentage as a function of the peptide concentration for *C. albicans*. It is shown that the conjugates have a higher killing efficiency than Hst5, with a difference of almost a factor of 2 at the highest evaluated peptide concentration. Only at lower concentrations is there a deviance between the N- and C-terminal conjugates, where the latter have the higher fungicidal activity.

Figure 4 in this paper shows a schematic picture of the primary structures of the peptides with respect to both length and charge. As can be seen, Hst5 has a longer sequence and

contains two negatively charged residues (excluding the C-terminal carboxyl group), whereas the conjugates contain solely positively charged and neutral amino acids. Hence, the average charges per amino acid are $0.21e$ for Hst5 and $0.27e$ and $0.40e$ for the N- and C-terminal conjugates respectively, i.e., the conjugates have a higher charge than the native peptide.

As explained in the Computational Section, Spd can be attached at either the N- or the C-terminus, and at each end there are two alternatives, either short or long (see Table 1). Since it is not possible to elucidate whether the molecular structures of the conjugates from the supplier are long or short, comparisons of simulation results with experiments are done using a mixture of the two. In the second part of this paper, a computational analysis of the conjugates will be performed, where the individual molecular structures will be taken into consideration.

Histatin 5 versus the Conjugates

To be able to compare the SAXS measurements with the molecular dynamics simulations, samples with a low peptide concentration and an ionic strength of 140 mM were used. Under these conditions, the peptide conformations and locations are uncorrelated, and the solution scattering effectively gives the form factor. Four CSpd concentrations were measured to ensure concentration independence of the radius of gyration and the scattering function (see Figure S4). A CSpd concentration of 2.4 mg mL^{-1} was used as the form factor to give the appropriate signal-to-noise ratio. The Guinier approximation, restricted to $qR_g < 0.8$, and the pair distance distribution function $P(r)$ (GNOM³⁴) gave consistent values of $I(q=0)$ (0.00193 and 0.00196 cm^{-1} , respectively) and R_g (0.92 ± 0.05 and $0.94 \pm 0.009 \text{ nm}$, respectively). The apparent molecular mass, which provides an indication of monodispersity, was determined from the specific volume of CSpd ($v_p = 0.694\text{--}0.715 \text{ cm}^3 \text{ g}^{-1}$; obtained using Sednterp³⁵) and $I(q=0)$ to be within the range $1.747\text{--}2.066 \text{ kDa}$. This is in good agreement with the molecular mass calculated from the amino acid sequence (1.863 kDa), indicating that CSpd is indeed monomeric.

Figure 5 shows comparisons of the form factors, Kratky plots, and distance distribution functions, $P(r)$, for the experimental data for Hst5 and CSpd (upper panel), the experimental CSpd data and the simulated CSpd and NSpd results (middle panel), and the experimental CSpd data and the simulated results for the Hst5_{4–15} fragments in NSpd and CSpd (bottom panel). The Hst5 form factor used in this figure can be found in the previous study by Cragnell et al.⁷ The last two panels show results for both SAXS and molecular dynamics simulations, whereas the first is for experiments solely. First of all, it is clearly shown that the simulations and the experiments are in reasonable agreement for the Hst5_{4–15} fragment part of NSpd and CSpd with the SAXS scattering of the conjugate (bottom panel), i.e., the applied force fields, the water models, and the parametrization of Spd are reliable and give accurate results. The assumption stated in Materials and Methods that the SAXS curve gives information mainly about the peptide part of the conjugate also seems to be valid. Taking the whole conjugate (with the Spd part included) into account when calculating the scattering function and $P(r)$ gives rise to too-large average dimensions. From the distance distribution functions it can be seen that, as expected, Hst5 is more extended than the fragments ($D_{\text{max}} =$

5 nm vs 3 nm) independent of whether Spd is attached to the N-terminus or the C-terminus. The Kratky plot indicates that Hst5 attains a random coil conformation, which is expected since Hst5 has been shown to behave as such.^{36,37} On the other hand, as a result of their small size in combination with the higher charge density, the fragments become slightly stiffer. Hence, from a conformational point of view, the conjugates display similar trends, differing from that of Hst5, as shown in Figure 5 (upper panel). The radius of gyration of the conjugates obtained from SAXS is approximately 0.94 nm (as obtained using the pair distance distribution function $P(r)$ ³⁴), whereas the average radius of gyration obtained from the simulations ranges from 0.97 to 1.01 nm.

Figure 6 shows the secondary structure predictions obtained from the molecular dynamics simulations as well as the CD spectra for Hst5 and the conjugates. It should be noted that the CD measurements were performed in TFE. From the experimental point of view, it is well-known that Hst5 does not possess any secondary structure in aqueous solutions, which has been shown both using NMR and CD.^{36,37} This is in line with the MD predictions given in Figure 6a, although from a secondary structure analysis at the atomistic level it becomes clear that some part of the amino acid sequence possesses helical content, which can be attributed to the sequence of His-Ser-His at positions 19–21. Despite this, the amino acid sequence consists mainly of turns and bends. On the other hand, when Hst5 is dissolved in an organic solvent such as TFE, its secondary structure content is increased, adopting an α -helical structure, as shown in the CD spectra. The apparent bands at 208 and 222 nm are indicative of an α -helical structure. This is in line with previous studies of Hst5 in TFE.^{36,38} In regard to the conjugates, which lack the His-Ser-His sequence, MD simulations indicate only turns and bends for CSpd. The α -helical content for NSpd is negligible. In TFE, the conjugates still do not form any secondary structure, as shown by the absence of spectral features in the respective CD spectra (Figure 6b). Thus, the conjugates are completely disordered in both water and TFE, and it is plausible that the His-Ser-His sequence contributes to forming the α -helical structure. The helix-forming sequence in Hst5 is also a part of a Zn-binding motif (HEXXH motif).³⁹ A loss of this α -helical secondary structure upon binding of Fe and Zn has been reported.⁴⁰

Computational Analysis of the Conjugates

Table 3 presents the simple and normalized average values of the radius of gyration (R_g), the end-to-end distance (R_{ee}), and the persistence length (L_p) for each simulated system. Two different sets of numbers are reported for the different conjugates. This is the case because we are interested in studying the conjugates both in part (i.e., the Hst5_{4–15} fragment) and as a unit (i.e., including Spd). Figure 7 displays the distributions from which these values were computed, and Figure 8 depicts the representative structure of each simulated system. From an analysis of the differences between the conjugate isomers (i.e., short vs long), it is noticeable that each pair of isomers shows very consistent results regardless of the end by which the Spd molecule is attached. Despite its asymmetry, attaching Spd at either end shifts the position of the central secondary amine by only one bond (recall Table 1).

From an experimental point of view, it is more interesting to analyze the differences depending on whether Spd is attached to the N- or C-terminus of the peptide. The N-

terminal versions of the Hst5₄₋₁₅-Spd conjugate (NSpd) contain a short succinic acid linker (needed for covalent bonding of Spd and the GGG linker), which necessarily increases their maximum extension and degrees of freedom. Despite their longer contour length, the N-terminal conjugates appear to adopt more compact conformations than their C-terminal counterparts, as is visible from the normalized average radii of gyration and end-to-end distances presented in Table 3. These findings are also reflected by their average persistence lengths, which are significantly shorter than those for the C-terminal variants, indicating that the former are more flexible. One possible explanation for these observations is that the CSpd conjugates have a higher linear charge density (cf., net charge of +8e vs +6e) and shorter contour length, which necessarily results in increased intramolecular electrostatic repulsion in comparison with the NSpd counterparts.

One could hypothesize that there might be a correlation between the results from the microbiological studies performed by Tati et al.⁵ and the less flexible and more elongated average structure of the CSpd conjugates, since CSpd has experimentally been shown to have slightly superior killing activity compared with its N-terminal counterpart. It seems plausible that a more elongated and rigid structure should better expose Spd to the solvent, facilitating its recognition by the polyamine transporters.

As expected, the system consisting of the Hst5₄₋₁₅ fragment alone presents a smaller radius of gyration and end-to-end distance than the different conjugates. However, when normalized, these values clearly indicate that for its size, this system adopts more expanded conformations than its conjugate counterparts. Interestingly, this peptide is also significantly more extended than its parent protein, Hst5, for which the normalized $\langle R_g \rangle$ and $\langle R_{ee} \rangle$ are 0.56 ± 0.09 and 0.35 ± 0.14 nm, respectively, under the same simulation conditions, force field, and water model.³¹ The latter is likely due to the stronger intramolecular electrostatic repulsion arising from the higher net charge per residue of the peptide compared with full-length Hst5.

When the focus is on the fragment part only, the most pronounced differences between the simulations of the different conjugates and Hst5₄₋₁₅ alone become almost negligible, as if the fragment portion of the conjugates is oblivious to the other constituents that make up the whole conjugate molecule, behaving just as it would by itself in solution. This is particularly well illustrated by the significant overlap between the black and red curves in Figure 7, and one interesting question is whether conjugation with Spd affects the conformational space of Hst5₄₋₁₅ at all. For that purpose, a principal component analysis (PCA) of the peptide backbone for all five systems was performed, as devised by Campos and Baptista.⁴¹ In general, we found that the first two principal components (PCs) comprise the majority of the variance in each system. Furthermore, visual inspection and root-mean-square calculations (not shown) prove that corresponding minima in the free energy landscapes enclose closely related conformations and that different minima relate to distinct conformational classes, whose dissimilarity increases with their distance in the landscape. This finding validates the analysis in itself and shows that higher dimensionality (i.e., a higher number of PCs) is not necessary for this specific purpose. Figure 9 shows that all of the systems sample very similar low-energy conformational regions, which means that on average, regardless of whether there is conjugation with Spd or not, the 12 amino acid active Hst5 segment adopts

similar conformations. Hence, conjugation with Spd does not noticeably affect the conformational space of Hst5₄₋₁₅. This is also visible from the aligned representative structures in Figure 10. It should be noted, though, that while most of the sampled conformations occupy the upper right quadrant of each plot, some simulations venture all the way down to the lower left quadrant, on which compact, β -hairpin-like structures are aggregated (see Figure 11).

The computational results illustrated in Figures 7–10 are in good agreement with the experimental SAXS results (see Figure 1), as neither computations nor experiments show any significant conformational change in the Hst5₄₋₁₅ fragment when it is conjugated to Spd compared to the fragment alone.

CONCLUSION

The first part of this work investigated the conformational and structural properties of the Hst5₄₋₁₅–Spd conjugates in comparison to Hst5. Reasonable agreement between experimental and simulated SAXS results were shown, suggesting that the applied force field, water model, and Spd parametrization used for the simulations are reliable. It is known from both SAXS measurements and simulations that Hst5 adopts a random coil conformation in aqueous solution.^{7,30,31} The Hst5₄₋₁₅ fragment part of the conjugates was shown to behave similarly to Hst5, displaying slightly more rigid conformations, which is reasonable in view of the combination of shorter contour lengths and higher charge density. The remaining simulated SAXS curves were more or less identical for the two conjugates. When the secondary structure as obtained from the simulations was studied, it was observed that the conjugates do not possess any significant secondary structure in aqueous solution. This is in line with the previous results reported for Hst5, although for the latter there are some indications of helical content for the His-Ser-His (19–21) sequence. The CD spectra, however, showed that the conjugates seemed to retain their lack of secondary structure in the organic solvent. It is hypothesized that this might be due to the absence of the His-Ser-His sequence in the conjugates.

In the second part of this work, the different types of conjugates were compared on the basis of simulation results solely. It was observed that despite having a longer contour length, the NSpd conjugate still adopts a more compact conformation compared with the CSpd conjugate. This effect could arise for two reasons: (1) the intramolecular electrostatic repulsion is higher for CSpd, as it has a higher charge density, and/or (2) the succinic acid linker in NSpd gives rise to more flexibility in the molecule, which might allow the Spd to fold back toward the Hst5₄₋₁₅ fragment, hence reducing the radius of gyration of the entire conjugate. The simulations also showed that conjugation with Spd does not appear to affect the conformational ensemble of the Hst5₄₋₁₅ fragment. This was also observed in the SAXS measurements from a comparison of the form factors, assuming that the Spd part did not contribute to the scattering.

We previously observed that structural changes induced by introduction of Spd improved fungicidal abilities of both Hst5 conjugates, which were attributable to resistance of the conjugates to salivary proteases and to enhanced uptake into fungal cells. We hypothesize

that the Hst5_{4–15}–Spd conjugate has better activity compared with the Spd–Hst5_{4–15} conjugate since it assumes a structure that better conceals residues that are enzymatic cleavage sites and protects them from degradation while exposing those residues involved in cell surface and *C. albicans* Dur3 transporter binding. Indeed, although both conjugates remain unstructured, these results show differences in the rigidity and compactness of the two conjugates. The more rigid random coil conformation of Hst5_{4–15}–Spd may result in residues that are stabilized to allow better occlusion, translocation, or release of the substrate to the inner side of the Dur transporter channel.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We acknowledge financial support from OMM Linnaeus Center, the Swedish Research Council, the Swedish Foundation for Laryngectomees, and the U.S. National Institutes of Health (Grant NIH R01DE010641 to M.E.). The simulations were performed on resources provided by the Swedish National Infrastructure for Computing (SNIC) at the Center for Scientific and Technical Computing at Lund University (LUNARC). To finalize, we thank the European Synchrotron Radiation Facility (Grenoble, France) for providing beamtime and Dr. Petra Pernot for assistance in using beamline BM29.

References

1. Enoch DA, Ludlam HA, Brown NM. Invasive Fungal Infections: A Review of Epidemiology and Management Options. *J Med Microbiol.* 2006; 55:809–818. [PubMed: 16772406]
2. Eggimann P, Garbino J, Pittet D. Epidemiology of Candida Species Infections in Critically Ill Non-immunosuppressed Patients. *Lancet Infect Dis.* 2003; 3:685–702. [PubMed: 14592598]
3. Pappas PG, Rex JH, Lee J, Hamill RJ, Larsen RA, Powderly W, Kauffman CA, Hyslop N, Mangino JE, Chapman S, et al. A Prospective Observational Study of Candidemia: Epidemiology, Therapy, and Influences on Mortality in Hospitalized Adult and Pediatric Patients. *Clin Infect Dis.* 2003; 37:634–643. [PubMed: 12942393]
4. Eggimann P, Garbino J, Pittet D. Management of Candida Species Infections in Critically Ill Patients. *Lancet Infect Dis.* 2003; 3:772–785. [PubMed: 14652203]
5. Tati S, Li R, Puri S, Kumar R, Davidow P, Edgerton M. Histatin 5-Spermidine Conjugates Have Enhanced Fungicidal Activity and Efficacy as a Topical Therapeutic for Oral Candidiasis. *Antimicrob Agents Chemother.* 2014; 58:756–766. [PubMed: 24247141]
6. Danesi R, Senesi S, Wout JWvt, Dissel JTV, Lupetti A, Nibbering PH. Antimicrobial Peptides: Therapeutic Potential for the Treatment of Candida Infections. *Expert Opin Invest Drugs.* 2002; 11:309–318.
7. Cragnell C, Durand D, Cabane B, Skepö M. Coarse-grained Modelling of the Intrinsically Disordered Protein Histatin 5 in Solution: Monte Carlo Simulations in Combination with SAXS. *Proteins: Struct, Funct Genet.* 2016; 84:777–791. [PubMed: 26914439]
8. Oppenheim FG, Xu T, McMillian FM, Levitz SM, Diamond RD, Offner GD, Troxler RF. Histatins, a Novel Family of Histidine-rich Proteins in Human Parotid Secretion. Isolation, Characterization, Primary Structure, and Fungistatic Effects on *Candida albicans*. *J Biol Chem.* 1988; 263:7472–7477. [PubMed: 3286634]
9. Raj PA, Edgerton M, Levine MJ. Salivary Histatin 5: Dependence of Sequence, Chain Length, and Helical Conformation for Candidacidal Activity. *J Biol Chem.* 1990; 265:3898–3905. [PubMed: 2406266]
10. Tsai H, Raj PA, Bobek LA. Candidacidal Activity of Recombinant Human Salivary Histatin-5 and Variants. *Infect Immun.* 1996; 64:5000–5007. [PubMed: 8945538]

11. Tsai H, Bobek L. Human Salivary Histatins: Promising Anti-Fungal Therapeutic Agents. *Crit Rev Oral Biol Med.* 1998; 9:480–497. [PubMed: 9825223]
12. Rothstein DM, Spacciopoli P, Tran LT, Xu T, Roberts FD, Dalla Serra M, Buxton DK, Oppenheim FG, Friden P. Anticandida Activity is Retained in P-113, a 12-Amino-acid Fragment of Histatin 5. *Antimicrob Agents Chemother.* 2001; 45:1367–1373. [PubMed: 11302797]
13. Kumar R, Chadha S, Saraswat D, Bajwa JS, Li RA, Conti HR, Edgerton M. Histatin 5 Uptake by *Candida albicans* Utilizes Polyamine Transporters Dur3 and Dur31 Proteins. *J Biol Chem.* 2011; 286:43748–43758. [PubMed: 22033918]
14. Whitten AE, Cai S, Trehwella J. *MULCh*: Modules for the Analysis of Small-angle Neutron Contrast Variation Data from Biomolecular Assemblies. *J Appl Crystallogr.* 2008; 41:222–226.
15. The PyMOL Molecular Graphics System, version 1.2r1. Schrödinger, LLC; New York: 2009.
16. Frisch, MJ., Trucks, GW., Schlegel, HB., Scuseria, GE., Robb, MA., Cheeseman, JR., Scalmani, G., Barone, V., Mennucci, B., Petersson, GA., Nakatsuji, H., Caricato, M., Li, X., Hratchian, HP., Izmaylov, AF., Bloino, J., Zheng, G., Sonnenberg, JL., Hada, M., Ehara, M., Toyota, K., Fukuda, R., Hasegawa, J., Ishida, M., Nakajima, T., Honda, Y., Kitao, O., Nakai, H., Vreven, T., Montgomery, JA., Jr, Peralta, JE., Ogliaro, F., Bearpark, M., Heyd, JJ., Brothers, E., Kudin, KN., Staroverov, VN., Kobayashi, R., Normand, J., Raghavachari, K., Rendell, A., Burant, JC., Iyengar, SS., Tomasi, J., Cossi, M., Rega, N., Millam, JM., Klene, M., Knox, JE., Cross, JB., Bakken, V., Adamo, C., Jaramillo, J., Gomperts, R., Stratmann, RE., Yazyev, O., Austin, AJ., Cammi, R., Pomelli, C., Ochterski, JW., Martin, RL., Morokuma, K., Zakrzewski, VG., Voth, GA., Salvador, P., Dannenberg, JJ., Dapprich, S., Daniels, AD., Farkas, Ö., Foresman, JB., Ortiz, JV., Cioslowski, J., Fox, DJ. Gaussian 09, revision A.02. Gaussian, Inc; Wallingford, CT: 2009.
17. Case, DA., Babin, V., Berryman, J., Betz, R., Cai, Q., Cerutti, D., Cheatham, TE., III, Darden, T., Duke, RE., Gohlke, H., Götz, AW., Gusarov, S., Homeyer, N., Janowski, P., Kaus, J., Kolossváry, I., Kovalenko, A., Lee, T., LeGrand, S., Luchko, T., Luo, R., Madej, B., Merz, KM., Paesani, F., Roe, DR., Roitberg, A., Sagui, C., Salomon-Ferrer, R., Seabra, G., Simmerling, C., Smith, W., Swails, J., Walker, RC., Wang, J., Wolf, RM., Wu, X., Kollman, PA. Amber 14. University of California; San Francisco: 2014.
18. Cornell WD, Cieplak P, Bayly CI, Gould IR, Merz KM, Ferguson DM, Spellmeyer DC, Fox T, Caldwell JW, Kollman PA. A Second Generation Force Field for the Simulation of Proteins, Nucleic Acids, and Organic Molecules. *J Am Chem Soc.* 1995; 117:5179–5197.
19. Lindorff-Larsen K, Piana S, Palmo K, Maragakis P, Klepeis JL, Dror RO, Shaw DE. Improved Side-chain Torsion Potentials for the Amber ff99SB Protein Force Field. *Proteins: Struct, Funct Bioinf.* 2010; 78:1950–1958.
20. Berendsen H, van der Spoel D, van Drunen R. GROMACS: A Message-passing Parallel Molecular Dynamics Implementation. *Comput Phys Commun.* 1995; 91:43–56.
21. Lindahl E, Hess B, van der Spoel D. GROMACS 3.0: a Package for Molecular Simulation and Trajectory Analysis. *J Mol Model.* 2001; 7:306–317.
22. van der Spoel D, Lindahl E, Hess B, Groenhof G, Mark A, Berendsen H. GROMACS: Fast, Flexible, and Free. *J Comput Chem.* 2005; 26:1701–1718. [PubMed: 16211538]
23. Hess B, Kutzner C, van der Spoel D, Lindahl E. GROMACS 4: Algorithms for Highly Efficient, Load-balanced, and Scalable Molecular Simulation. *J Chem Theory Comput.* 2008; 4:435–447. [PubMed: 26620784]
24. Piana S, Donchev AG, Robustelli P, Shaw DE. Water Dispersion Interactions Strongly Influence Simulated Structural Properties of Disordered Protein States. *J Phys Chem B.* 2015; 119:5113–5123. [PubMed: 25764013]
25. Darden T, York D, Pedersen L. Particle Mesh Ewald: An Nlog(N) Method for Ewald Sums in Large Systems. *J Chem Phys.* 1993; 98:10089.
26. Bussi G, Donadio D, Parrinello M. Canonical Sampling Through Velocity Rescaling. *J Chem Phys.* 2007; 126:014101. [PubMed: 17212484]
27. Parrinello M, Rahman A. Polymorphic Transitions in Single Crystals: A New Molecular Dynamics Method. *J Appl Phys (Melville, NY, U S).* 1981; 52:7182.
28. Hess B, Bekker H, Berendsen H, Fraaije J. LINCS: a Linear Constraint Solver for Molecular Simulations. *J Comput Chem.* 1997; 18:1463–1472.

29. Fletcher R, Powell M. A Rapidly Convergent Descent Method for Minimization. *Comput J.* 1963; 6:163–168.
30. Henriques J, Cragnell C, Skepö M. Molecular Dynamics Simulations of Intrinsically Disordered Proteins: Force Field Evaluation and Comparison with Experiment. *J Chem Theory Comput.* 2015; 11:3420–3431. [PubMed: 26575776]
31. Henriques J, Skepö M. Molecular Dynamics Simulations of Intrinsically Disordered Proteins: on the Accuracy of the TIP4P-D Water Model and the Representativeness of Protein Disorder Models. *J Chem Theory Comput.* 2016; 12:3407–3415. [PubMed: 27243806]
32. Williams, T., Kelley, C., et al. GNUPLOT: An Interactive Plotting Program, version 5.0, patch level 3. 2016.
33. Schrödinger Release 2015-4: Maestro. Schrödinger, LLC; New York: 2015.
34. Svergun DI. Determination of the Regularization Parameter in Indirect-transform Methods Using Perceptual Criteria. *J Appl Crystallogr.* 1992; 25:495–503.
35. Hurton, T., Wright, A., Deubler, G., Bashir, B. SEDNTERP.
36. Brewer D, Hunter H, Lajoie G. NMR Studies of the Antimicrobial Salivary Peptides Histatin 3 and Histatin 5 in Aqueous and Nonaqueous Solutions. *Biochem Cell Biol.* 1998; 76:247–256. [PubMed: 9923693]
37. Raj PA, Marcus E, Sukumaran DK. Structure of Human Salivary Histatin 5 in Aqueous and Nonaqueous Solutions. *Biopolymers.* 1998; 45:51–67. [PubMed: 9433185]
38. Melino S, Rufini S, Sette M, Morero R, Grottesi A, Paci M, Petruzzelli R. Zn²⁺ Ions Selectively Induce Antimicrobial Salivary Peptide Histatin-5 to Fuse Negatively Charged Vesicles. Identification and Characterization of a Zinc-binding Motif Present in the Functional Domain. *Biochemistry.* 1999; 38:9626–9633. [PubMed: 10423240]
39. Grogan J, McKnight CJ, Troxler RF, Oppenheim FG. Zinc and Copper Bind to Unique Sites of Histatin 5. *FEBS Lett.* 2001; 491:76–80. [PubMed: 11226423]
40. Puri S, Li R, Ruszaj D, Tati S, Edgerton M. Iron Binding Modulates Candidacidal Properties of Salivary Histatin 5. *J Dent Res.* 2015; 94:201–208. [PubMed: 25365968]
41. Campos SR, Baptista AM. Conformational Analysis in a Multidimensional Energy Landscape: Study of an Arginylglutamate Repeat. *J Phys Chem B.* 2009; 113:15989–16001. [PubMed: 19778072]

Figure 1. Comparison of the scattering intensities of the Hst5_{4-15} peptide and the $\text{Hst5}_{4-15}\text{-Spd}$ conjugate. The concentrations were 1.50 and 1.29 mg/mL for conjugate and peptide sample, respectively.

Figure 2. Mass determination of Hst5₄₋₁₅-Spd using a MALDI Orbitrap instrument. The y axis displays the relative intensity and the x axis the mass range.

Figure 3. Mass determination of Hst₅₄₋₁₅-Spd using a MALDI Orbitrap instrument. The inset shows the high-resolution separation (as shown in Figure 2), which enables the accurate mass determination of the different isotopes. The monoisotopic peak is indicated by the arrow. The *y* axis displays the relative intensity and the *x* axis the mass range.

Figure 4.

Schematic picture of the charge distributions for Hst5 (top row) and the conjugates with Spd attached either to the C-terminus (middle row) or the N-terminus (bottom row). The color code for the charges (in units of e) is as follows: blue, +1; pink, -1; gray, 0; green, +2. The total net charges are +5, +8, and +6, respectively. SPD stands for spermidine, and SAC stands for succinic acid.

Figure 5.

Figure sets depicting (left to right) the form factor, the Kratky plot, and $P(r)$ determined by SAXS measurements and simulations. The upper row (a–c) shows a comparison between the experimental data for Hst5 and the Hst5₄₋₁₅-Spd conjugate. The middle row (d–f) compares the experimental conjugate results to the simulated results for the N- and C-terminal conjugates. The bottom row (g–i) compares the experimental conjugate results to the simulated results for the Hst5₄₋₁₅ part of the conjugates only. All of the Hst5 data used in this figure can be found in previous studies by Cragnell et al.⁷

Figure 6.

(a, c, d) Stacked secondary structure histograms for the simulated Hst5 and the peptide part of the conjugates. All of the histograms sum up to 100%, but the coil percentages are not shown for visual simplicity. The shaded areas indicate the conjoint amino acid sequence that can be found in all the peptides of this study. (b) CD spectra for Hst5 and the two conjugates in trifluoroethanol.

Figure 7.

Density estimates of the radius of gyration (upper row) and the end-to-end distance (lower row) for all of the simulated conjugates. The blue lines show the distributions for the complete conjugates, whereas the red lines show the distributions for the peptide part of the conjugates only (excluding the triple-glycine linkers). The result for each conjugate is compared to that for the simulated Hst54-15 fragment, which is shown in black.

Figure 8.
Aligned representative structures of the simulated systems.

Figure 9.

Free energy landscapes for the Hst5₄₋₁₅ part of each simulated system using the first two principal components. Contour lines are drawn for energies between 0 and 5 RT with 1 RT increments. Triangles mark the centers of the lowest-energy minima, and dots represent other local minima. Plots in the same row have axes of equal size.

Figure 10.

Same as Figure 8, but here only the Hst5₄₋₁₅ section of the conjugates was considered for the calculation of the representative structures.

Figure 11. Representative structure of a local free energy minimum from the bottom left quadrants of Figure 9. Hydrogen bonds within the main chain are represented as dashed yellow lines.

Table 1

Hst5₄₋₁₅-Spd Conjugate Models

NSpdS	Spermidine (short side) + succinic acid + R1 *	
NSpdL	Spermidine (long side) + succinic acid + R1 *	
CSpdS	R2 ⁺ + spermidine (short side)	
CSpdL	R2 ⁺ + spermidine (long side)	

* R1 = GGG linker + AKRHHGYKRKFH

⁺R2 = AKRHHGYKRKFH + GGG linker

Table 2

Models Used for the Charge Derivation of Spd and Succinic Acid

ACE capped spermidine (short side)
ACE capped spermidine (long side)
NME capped succinic acid (both sides)

Table 3

Averages and Standard Deviations of the Radius of Gyration (R_g), the End-to-End Distance (R_{ee}), and the Persistence Length (L_p) for Each Simulated System; The Upper Values in Each Row Represent the Complete Conjugate, Whereas the Lower Values Represent the Fragment Part of the Conjugate Only

	Hst5 ₄₋₁₅	NSpdS	NSpdL	CSpdS	CSpdL
$\langle R_g \rangle \pm \sigma$ (nm) ^a	0.97 ± 0.11	1.23 ± 0.17	1.25 ± 0.17	1.22 ± 0.12	1.20 ± 0.14
		0.97 ± 0.11	0.97 ± 0.12	1.01 ± 0.09	1.00 ± 0.10
$\langle R_g^b \rangle \pm \sigma^b$	0.76 ± 0.09	0.64 ± 0.09	0.64 ± 0.08	0.70 ± 0.07	0.69 ± 0.08
		0.71 ± 0.08	0.71 ± 0.08	0.75 ± 0.07	0.74 ± 0.08
$\langle R_{ee} \rangle \pm \sigma$ (nm)	2.49 ± 0.65	3.36 ± 1.01	3.47 ± 0.97	3.69 ± 0.79	3.62 ± 0.83
		2.40 ± 0.69	2.47 ± 0.64	2.72 ± 0.51	2.67 ± 0.54
$\langle R_{ee}^c \rangle \pm \sigma^c$	0.64 ± 0.17	0.50 ± 0.15	0.51 ± 0.14	0.59 ± 0.13	0.58 ± 0.13
		0.60 ± 0.17	0.62 ± 0.16	0.69 ± 0.13	0.68 ± 0.14
$\langle L_p \rangle \pm \sigma$ (bonds)	7.05 ± 3.91	5.68 ± 2.87	5.72 ± 2.94	7.18 ± 3.71	7.15 ± 3.79
		7.02 ± 3.84	7.01 ± 3.91	7.99 ± 3.99	7.93 ± 4.09

^a Experimental conjugate $R_g = 0.94 \pm 0.009$ nm (the latter is the error and not the standard deviation).

^b Normalized by the maximum radius of gyration.

^c Normalized by the approximate contour length.