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Structural analysis of nonapeptides derived from elastin

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Running title.

Elastin-derived nonapeptides

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Abstract

Elastin-derived peptides are released from the extracellular matrix remodeling by numerous proteases and seem to regulate many biological processes, notably cancer progression. The canonical elastin peptide is VGVAPG which harbors the XGXXPG consensus pattern allowing interaction with the elastin receptor complex located at the surface of cells. Besides these elastokines, another class of peptides has been identified. This group of bioactive elastin peptides presents the XGXPGXGXG consensus sequence but the reason for their bioactivity remains unexplained. In order to better understand their nature and structure-function relationships, herein we searched the current databases for this nonapeptide motif and observed that the XGXPGXGXG elastin peptides define a specific group of tandemly repeated patterns. Further, we focused on four tandemly repeated human elastin nonapeptides, *i.e.* AGIPGLGVG, VGVPGLGVG, AGVPGLGVG and AGVPGFGAG. These peptides were analysed by means of optical spectroscopies and molecular dynamics. UV-circular dichroism and Raman spectra are consistent with a mixture of β -turn, β -strand and random chain secondary elements in aqueous media. Quantitative analysis of their conformations suggested that turns corresponded to half of the total population of structural elements while the remaining half was equally distributed between β -strand and unordered chains. These distributions were confirmed by molecular dynamics simulations. Altogether, our data suggest that these highly dynamic peptides harbor a type II β -turn located in their central part. We hypothesize that this structural element could explain their specific bioactivity.

Statement of Significance

Elastin fragmentation products, the so-called elastin peptides, may exhibit a bioactivity towards normal and tumor cells. This phenomenon depends on the sequence motif they harbor. While XGXXPG sequences bioactivity is explained by the presence of a type VIII β -

turn allowing interaction with the elastin receptor complex, the structural reasons for XGXPGXGXG specific activity remain unexplained. Using data mining, we show that elastin nonapeptides define a specific class of tandemly repeated features. Further, spectroscopic and numerical simulations methods suggest the presence of a type II β -turn in their conformation. This structural element could explain their bioactivity.

Introduction

Elastin is the extracellular matrix protein responsible for the structural integrity and function of tissues undergoing reversible extensibility or deformability (1). This protein is extremely stable and resistant and undergoes virtually no turnover. Nevertheless, during aging, mechanical stress and elastase activities contribute to the fragmentation of this macropolymer into elastin-derived peptides (EDP) (2).

Elastin is characterized by its elasticity and seems devoid of any other biological activity (1). In contrast, EDP have been shown to regulate numerous biological processes and are thought to be involved in several aging-related pathologies such as atherosclerosis (3, 4) and cancer (5–8). Most biologically active EDP, elastokines, possess a GXXPG consensus sequence adopting a type VIII β -turn structure (9) involved in their binding to the elastin receptor complex (10). The interaction of GXXPG-containing elastokines with the elastin receptor complex and the consequences of their interaction has been considerably documented during the last decades. For a comprehensive review on this topic, the reader is referred to (11).

Besides the classical GXXPG-containing sequences, another class of elastokines has also been reported. In 1988, Long and colleagues reported that nonapeptide sequences from elastin were chemoattractant for fibroblasts (12). In 1989, they further demonstrated that this biological activity could be extended to endothelial cells (13). Strikingly, since then, this peculiar class of EDP has been mostly ignored until 2007, when Maeda and colleagues demonstrated that these peptides could promote macrophages migration via a specific, but unknown, receptor (14). More recently, their biological activity has been further linked to lung carcinoma progression (8) and to promotion of invasion in triple-negative cancer cells via MMP-14 and MMP-2 (15).

The relatively low interest encountered by these nonapeptide EDP can be explained by the

fact that considerable advances have been made on both GXXPG-containing EDP and their receptor biology. As a consequence, few groups considered investigating on these peculiar peptides.

The nonapeptide AGVPGLGVG and other EDPs share similar structural features: random coil and β -turns conformations. However, AGVPGLGVG induces angiogenesis, tumor progression, secretion of proteases higher than other EDPs in carcinomas. Moreover, *in vitro*, it behaves like amyloid-like peptides harboring the XGGXG motif by forming cross β -sheets at the supramolecular level (16). Very recently, Brassart and colleagues have shown that the receptor mediating AGVPGLGVG biological effects, and identified as lactose-insensitive, is the ribosomal protein SA (RPSA) (17).

The aim of this work is to analyze the structure and conformational behavior of these peptides. Our structural analysis was conducted using sequence analysis, spectroscopic methods and molecular dynamics simulations.

Our results show that elastin nonapeptides define a peculiar class among peptides harboring the consensus X-G-X-P-G-X-G-X-G sequence. In elastin, these peptides are mostly tandemly repeated. Further our data show that they are engaged in a conformational equilibrium between random coil and turn conformations at the considered concentrations and temperatures. Molecular dynamics simulations suggest that the dominant conformation of these peptides is a type II β -turn. The functional significance of this conformation is discussed.

Material and Methods

Sequence analysis

Sequence retrieval. Sequences harboring the X-G-X-P-G-X-G-X-G consensus motif were searched using Python scripts on UniProtKB (Swiss-Prot and TrEMBL) database Fasta files

where spliced variants were ignored. Only exact matches were retrieved and further cleaned informatically and manually so as to ensure that a sequence belonging to a given species (identified by its unique accession number) was present only once in our results. For each sequence hit, the collected data were its accession number, the originating organism, the length of the protein and the position(s) of the nonapeptide sequence(s).

Pattern analysis. Nonapeptide exact matches were classified and distributed as a function of the taxonomic hierarchical classification lineage of the source organism of the corresponding parent protein. Hits were either individual hits, *i.e.* only one occurrence was found in the sequence, or multiple ones. In the latter case, we distinguished three possibilities. Multiple hits could be found in different regions of the protein and each occurrence was isolated from the others. Another possibility was that the repetitions could overlap, *i.e.* the end of the first occurrence (-X-G) was the beginning of the following one (X-G-). Finally, we observed that nonapeptide sequences could also be tandemly repeated, one after the other. The tree figure has been made with the Python module ETE3 (18) and the Logo sequences with WebLogo (<https://github.com/weblogo/weblogo>) (19).

Optical spectroscopy

Molecular compounds. Powder samples of amino acids were purchased from Sigma-Aldrich (Saint Quentin Fallavier, France). Lyophilized samples of nonapeptides were obtained from Genecust (Luxemburg, Luxemburg) as zwitterionic peptides in acetate salts. Their purity was at least 95% as determined by mass spectrometry.

Solution samples. Lyophilized powder of amino acids and peptides was dissolved in pure water from a Millipore filtration system (Guyancourt, France). The ionic strength of all peptide samples was increased by adding 150 mM NaCl to stock solutions. Upon dissolution, the pH was between 4 and 4.5. Raman spectra were recorded between 10°C and 80°C.

Circular dichroism (CD) spectra were obtained at room temperature between 100 and 200 μM .

CD spectroscopy. Room temperature ultraviolet-circular dichroism (UV-CD) spectra were analyzed on a JASCO J-810 spectrophotometer (Lisses, France) within the 190–300 nm spectral region. A path length of 1 mm and a spectral resolution of 0.2 nm were selected. Each spectrum corresponding to an average of five scans was recorded with a speed of 100 nm per min. The measured ellipticity for each sample, referred to as $[\phi]_{obs}$, was further normalized to obtain the so-called mean residue ellipticity, $[\phi]$, by using the expression $[\phi] = [\phi]_{obs} / ncl$, where n , c , and l are the length of the peptide, its molar concentration, and the optical path length, respectively. The normalized ellipticity was expressed in $\text{deg cm}^2 \text{dmol}^{-1}$.

Raman spectroscopy. Room temperature Stokes Raman spectra were analyzed in bulk samples at right angle on a Jobin-Yvon T64000 spectrometer (Longjumeau, France) at single spectrograph configuration, 1200 grooves/mm holographic grating and a holographic notch filter. Raman data corresponding to 1200 s acquisition time for each spectrum were collected on a liquid nitrogen cooled CCD detection system (Spectrum One, Jobin-Yvon). The effective slit width was set to 5 cm^{-1} . Solution samples were excited by the 488 nm line of an Ar^+ laser (Spectra Physics, Evry, France), with 200 mW power at the sample.

Post-record spectroscopic data treatment. Buffer subtraction and smoothing of the observed spectra was performed using the GRAMS/AI Z.00 package (Thermo Galactic, Waltham, MA, USA). Final presentation of Raman spectra was done by means of SigmaPlot package 6.10 (SPSS Inc., Chicago, IL, USA).

Secondary structure analysis. Quantification of protein secondary structures using amide I profile decomposition is a method used commonly and its intrinsic limits are readily known (20, 21). For elastin material, its overall accuracy is $\pm 5\%$ as far as the final structural estimates are concerned (22). The initial step of the reconstruction is to guess how many

underlying bands are present in the complex amide I profile to be analyzed. This is achieved using a second derivative analysis of the smoothed spectrum. For our data, 4 components were suggested. This information was provided to the curve fitting software to reconstruct the experimental spectrum using Gaussian/Lorentzian mixture profiles. The curve fitting procedure was successful when the fit was good (low chi square value), the number of components was unchanged and their width at half height were consistent. The secondary structure estimates were obtained by adding the fractional areas of amide I structural components belonging to the same secondary structure group.

Atomistic molecular dynamics simulations

Ten independent simulations of 500 ns were performed for each of the 4 human elastin nonapeptides using amber99SB-ILDN force field (23) and Gromacs 2016.5 software (24). Peptides were built in an extended conformation with PyMOL (25) and solvated with TIP3P water in a 5 nm cubic box. All studied systems were first minimized by steepest descent for 5000 steps. Then, a 1-ns simulation with the peptides under position restraints was run before the production simulations were performed. Different seeds were used for each simulation during the velocities assignments. Periodic boundary conditions were used with a 2 fs time step. The dynamics were carried out under NPT conditions (310 K and 1 bar). The temperature was maintained using the v-rescale method (26) with $\tau_T = 0.1$ ps, and an isotropic pressure was maintained using the Parrinello–Rahman barostat (27) with a compressibility of $4.5 \times 10^{-5} \text{ bar}^{-1}$ and $\tau_P = 2$ ps. Short-range non-bonded interactions were treated with a cutoff of 1.0 nm and long-range interactions were calculated with the particle mesh Ewald method (28) with a grid spacing of 0.16 nm. Bond lengths were maintained with the LINCS algorithm (29) and long range dispersion corrections for energy and pressure were applied. The 3D structures were analyzed with both PyMOL and VMD (30) softwares.

The secondary structures were computed with DSSP (31) and the different types of β -turns

have been assigned based on the $\Phi\Psi$ angles of two residues following each other as defined by Lewis *et al.* in 1973 (32) and Hutchinson *et al.* in 1994 (33). Distances between each i and $i+3$ C α have been computed and, if this distance was less than 0.7 nm and the amino acids were not part of an helix conformation, the $\Phi\Psi$ angles of residues $i+1$ and $i+2$ were considered. To assign a peculiar type of β -turn, Hutchinson and colleagues (33) considered a range of $\pm 30^\circ$ or $\pm 40^\circ$ around the standard angles with a deviation of 50° allowed for one of the angle.

The distribution of $\Phi\Psi$ angles of residues $i+1$ and $i+2$ we observed during the simulation permitted to easily identify type I, I', II, II' and VIII β -turn populations as broad plot regions (Fig. S1 in the Supporting Material). As these regions were larger than those proposed by Hutchinson and colleagues (33), we adapted the considered $\Phi\Psi$ angles ranges to encompass the ranges we observed. The main effect of this change was the reduction of unassigned turn contribution, *i.e.* type IV β -turns. The corresponding figure and $\Phi\Psi$ angle ranges for type I, I', II, II' and VIII β -turns are provided as supplemental data (Fig. S1, Table S1 in the Supporting Material). As in Hutchinson, other β -turn types are considered with a range of $\pm 40^\circ$ around the standard angles and type IV β -turns correspond to otherwise unassigned β -turns. Clustering was performed using the gromos method (34) with a cut-off of 0.1 nm on C α atoms of residues 2 to 6.

In order to assess the quality of our simulations, CD spectra have been computed from the peptide trajectories by using the SESCO software (35) with the basis set HBSS-3SC1.

Results

Sequence analysis.

The retrieval of sequences possessing the X-G-X-P-G-X-G-X-G yielded 96 293 unique sequences. These sequences (Table S2 in the Supporting Material) were classed as a function

of their taxonomy (Figure 1). Most sequences were from *Bacteria* (71 504 sequences for 75 718 occurrences with 2 129 overlaps and 297 tandem repeats) then *Eukariota* (22 919 sequences for 24 828 occurrences with 811 overlaps and 297 tandem repeats), *Archae* (1 630 sequences for 1 694 occurrences with 16 overlaps and 6 tandem repeats) and *Virus* (240 sequences for 245 occurrences with no overlaps and no tandem repeats). Strikingly, when these results are observed, it comes that elastins define a specific group. They exhibit numerous occurrences per sequences (171 for 38) with no overlap and a very high ratio of tandem repeats. This specificity of elastin nonapeptides is even more evident when the most frequent residues occurring at positions X are considered. While other group mostly favor the presence of G, A, K, P or L at these positions, elastins allow almost none of those with the AGVPGFGVG being the dominating motif. In fact, out of the 140 million sequences searched, this motif was always found in elastin sequences but one bacterial sequence.

The nonapeptide sequences found in elastin entries are reported in Table 1 where they are compared. The sequences are reported as a function of their alphabetical order. In human elastin, nonapeptides can be observed in exon domains 18, 20 or 26. Single occurrences are observed in the two first exons while they appear almost exclusively as tandem repeats in exon 26. Strikingly, the human repeats can also be observed in other species. For instance, the human elastin motif AGVPGFGVG is also present in mouse exon 26. Overall, human exon 26 (or its equivalent in other species) is a sequence domain characterized by tandem repeats of these nonapeptides. As a consequence, those are supposedly important for the biological function of the polymer and we decided to further analyze the structural behavior of the 4 human exon 26 nonapeptides, *i.e.* AGIPGLGVG (hN3), VGVPGLGVG (hN4), AGVPGLGVG (hN5) and AGVPGFGAG (hN6).

CD spectroscopy

In order to describe the structural behavior of the nonapeptides in solution, their CD spectra were recorded in different (polar vs low dielectric constant) environments. Routinely, methanol is considered as a primary step in studying the peptides conformational behavior in hydrophobic environments, such as membrane interior or hydrophobic pockets of proteins (36).

The analysis of the CD spectra in water (150 mM NaCl), in a water/methanol mixture (50/50, v/v), as well as in methanol (Fig. 2) reveal a progressive conformational change of the nonapeptides in going from water to methanol solutions.

The CD spectra recorded in water all present a double negative band shape, *i.e.* composed of a deep minimum at ~198 nm followed by a weaker and broader negative band centered at ~225 nm. It is a matter of fact that random chains are generally identified by a deep negative band at ~198 nm (37, 38). However, a negative double band, as that observed in the case of the nonapeptides (Fig. 2), is an indicator of turns. For instance, certain β -turns are known to give rise to a negative double band. It is to be mentioned that β -turns are recognized as the most frequent ones in proteins (39)(40)(41). Particularly, the CD fingerprints of four categories of β -turns, *i.e.* type-I (37), -II and -II' (42–44) and -VIII (45) β -turns are identified by a double negative band, with unequal ellipticities. It is interesting to note that amongst all the mentioned β -turns, that corresponding to type-VIII is formed by a deep minimum at ~198 nm followed by a broad shoulder at ~220 nm (45), *i.e.* strikingly similar to that observed in the case of the presently analyzed nonapeptides. However, this overwhelming resemblance between CD markers does not permit rejection of possible unordered chains, of which the CD marker can be overlapped with the negative band at ~198 nm. In the same manner, a probable overlap may occur between the CD fingerprint of β -strands, generally characterized by a unique negative band at ~215 nm, and the ~225 nm negative band from β -turns. Nevertheless, any uncertainty about the presence of PPII (polyproline II) type chains can be discarded

because of the recent CD studies highlighting the fact that the PPII fingerprint is composed of a deep negative band at ~198 nm followed by a positive one centered at ~220 nm. The presence of the latter band cannot be suspected in the CD spectra displayed in Figure 2.

In methanol (Fig. 2), the CD signals of the nonapeptides are composed of a broad positive band peaking at ~205 nm followed by a weak and broad negative band at ~225 nm. It is worth noting that the CD spectra observed in water/methanol mixture (1:1, v/v) are somehow an overlap of those observed in water and methanol. To provide a reliable assignment for the CD signal observed in methanol, we can recall that while a type-II β -turn gives rise to a positive band located between 190 and 210 nm (37), the observed negative band at ~225 nm in methanol can be ascribed to β -strands and/or to type-I' β -turn (37). Based on the observed CD spectra, no unordered chain can be expected in the presently studied nonapeptides.

Raman spectroscopy

Raman spectra of the nonapeptides were recorded in the middle wavenumber region (1800-550 cm^{-1}). They presented a low water contribution enabling an accurate analysis of the observed bands (Fig. 3A-B). Whatever the nonapeptide, the spectra were identical for the two tested concentrations, i.e. 2.5 mM and 20 mM (data not shown). Indeed, no substantial change, assignable to either a possible conformational transition or a detectable molecular aggregation, could be observed. Because the spectral features were more pronounced at 20 mM (higher Raman intensities due to higher concentration), we will limit our discussion to the Raman spectra recorded at that concentration.

The tentative assignments of the Raman spectra of the four studied nonapeptides are reported in Table 2. These assignments have been performed in keeping with the features observed in the Raman spectra of their constitutive amino-acids (Fig. S2 in the Supporting Material). The bands observed in the 1500-1320 cm^{-1} range, mainly arising from the bending modes of CH_2 and CH_3 side chain moieties, as well as those located below 1200 cm^{-1} in

amino acids spectra, were taken as references for assigning the observed peaks in the spectra of peptides.

The spectrum of hN6 is characterized by the occurrence of the six Phe characteristic Raman bands, referred to as F1 to F6 (46, 47). Being all of in-plane type and localized in the Phe phenyl ring, the wavenumbers of these six characteristic markers remained very close to those collected from the free amino acid F (Fig. 3B). The Raman spectra of hN3, hN4 and hN5 (Fig. 3A) do not present such narrow and resolved aromatic markers, but their striking spectral shape similarity is to be emphasized (Fig. 3). Two regions corresponding to amide I (1700-1640

cm⁻¹) and amide III (1300-1230 cm⁻¹) vibrations provide valuable structural information through their decomposition into band components (42–44). It is noteworthy that while amide I vibrations results from the backbone C=O bond stretch motion, more or less coupled to its adjacent N-H angular bending, amide III vibrations mainly arise from the backbone N-H angular bending. The Raman spectra obtained for the free amino acids (Fig. S2) are devoid of amide (I and III) vibrations. However, the presence of low intensity bands arising from the side chains of Val, Leu, Ile and Pro, falling within the amide III range should be stressed. These bands can be naturally superimposed to those relative to amide III vibrations in peptides. Nevertheless, because of their weakness, one cannot expect a considerable distortion in the structural analysis on the basis of amide III vibrations. In all nonapeptides, amide I and amide III vibrations are characterized by two strong, broad and incompletely resolved bands peaking at ~1685 and ~1265 cm⁻¹, respectively. To go farther in extracting the structural information from amide vibrations, the decomposition of the amide I region for four nonapeptides is displayed in Figure 4. Through a systematic investigation on the structural analysis of the peptide chains (42–44, 48, 49), in aqueous environment, we have been led to select four band components located at ~1690±5 (assignable to random chain), ~1660±5

(assignable to β -strand), $\sim 1675 \pm 5$ and $\sim 1650 \pm 5$ (both assignable to turns) to decompose the amide I region in nonapeptides. The populations corresponding to different secondary structural elements, as estimated by the normalized areas of the band components (expressed in percent) are reported in Table 3. As it can be seen between 50% to 60% of the total population is formed by turn structures, and the rest is equally distributed between β -strands and random chains.

Solution samples containing nonapeptides at 20 mM were heated up to 80°C. Little changes appeared in their Raman spectra, but slight changes could be observed in the amide III region. Figure 5 exemplifies this behavior for hN5 and hN6. The main effect is a wavenumber downshift of the spectral profile consistent with a progressive shift from turn to β -strand upon increasing temperature. Albeit the turns remain the major population of secondary structures in the 10-80° C temperature range (data not shown), they show a little tendency to be transformed into β -strands, presumably because of the weakening/breakdown of the inter-turn stabilizing hydrogen bonds occurring upon thermal annealing.

Atomistic molecular dynamics simulations

To get a more detailed representation of the structures that can be adopted by the four nonapeptides, 10 molecular dynamic simulations of 500 ns have been performed for each peptide. The adopted conformations, analyzed with DSSP (31), are presented on Figure 6A and Figure S3 in the Supporting Material. The figures show that the peptides mainly adopt random coil conformations with some residues favoring turns but no stable structure can be observed. The formation of β -turns is evidenced by distances between $C\alpha$ atoms of residues i and $i+3$ being less than 0.7 nm with residues $i+1$ and $i+2$ being non helical, which is mainly observed in the middle of the peptides between residues 3 and 6 and notably for the hN3 peptide (Fig. S4 in the Supporting Material, panel A). The type of β -turns can be identified by analyzing the $\Phi\Psi$ angles of two amino acid residues following each other as determined by

Lewis *et al.* (32) and Hutchinson *et al.* (33). Figure 6B shows that type II β -turns are formed during a third to a half of the simulation time ($52.9 \pm 4.1\%$ for hN3, $37.8 \pm 4.7\%$ for hN4, $33.9 \pm 2.8\%$ for hN5 and $33.5 \pm 3.8\%$ for hN6) for the PG residues being at positions 4 and 5. PG residues are strongly favored at $i+1$ and $i+2$ positions in experimental structure of type II β -turns and they are known to promote this type of β -turn. The population of type II β -turns is greater for the hN3 peptide than for the hN4, hN5 and hN6 peptides. Figures S4B-E show that type I, I', II, II' and VIII β -turns can also be formed in smaller amounts at other positions of the peptides and that type VIII β -turns are mainly observed at the start of the peptides (up to 3% of the simulation time). A proline at position 4 has been shown to promote the formation of this type of turn (33). Analysis of the simulation underlines that the structures are rapidly changing on the nanosecond time scale from extended to more packed conformations which mainly corresponds to type II β -turns (Fig. S4F). The population of β -turns has also been analyzed through clustering methods and the central structures of the dominant cluster population of each peptide is presented as an insert in Figure 6B. Altogether, our analysis of MD data strongly suggests that the PG motif of the nonapeptides could mainly adopt a type II β -turn conformation.

To test the agreement of these results with the measured CD spectra, CD spectra were computed from our simulated trajectories with the SESCA software (35). The results (Fig. S5 in the Supporting Material) show a very good agreement between the measured and computed spectra despite noticeable differences around ~ 220 nm. This suggests that the computed profiles might have failed to detect several experimental signals. Nevertheless, they reinforce our proposal that β -turns and notably type II β -turns are the prevailing structures adopted by the nonapeptides.

Discussion

Among elastin peptides, elastin nonapeptides have been identified initially as chemoattractants for fibroblasts (12). Now, there are growing evidences that these elastin peptides could define a peculiar class of matrikines.

Brassart and colleagues have recently shown that hN5 (*i.e.* AGVPGLGVG) plays a key role in tumor progression by promoting tumor cell blebbing and extracellular vesicle shedding following its interaction with the ribosomal protein SA (17). The structure of hN5 has been analyzed previously by CD, FT-IR and NMR spectroscopies (16). This report concluded that the structure of the peptide was a mixture of random coils and β -turns. Our data fully agree with these findings but they also expand our knowledge of the nature and structural behavior of these elastin peptides.

The occurrence of the X-G-X-P-G-X-G-X-G consensus sequence was analyzed in a large sequence data bank. Our results show that among the observed hits, the group of elastin sequences defines a specific group (Fig. 1). Further, our analysis underlines the fact that these peptides mostly occur in tandem repeats in human exon 26 (and corresponding sequences in other species). Finally, as far as human sequences are concerned, we observed that similar sequences were also present in numerous species (Table 1). The most striking observation is that hN6 is present in numerous species and is tandemly repeated five times in mouse sequence. In contrast, hN3 is only found in 3 species. These observations suggest that, besides their matrikines activity, these sequences could play a key, and yet undescribed, role in elastin biology, possibly elastin assembly. Indeed, among elastin sequences, these sequences appear much more conserved than the canonical elastin hexapeptide sequence (say VGVAPG), which occurrence is mostly limited to human exon 24 sequence. Further works are needed to test this hypothesis.

Our data (Fig. 1) show that the X-G-X-P-G-X-G-X-G consensus sequence can be found in most filum and/or species. To the best of our knowledge, sequences harboring this consensus

have caught the attention only in elastin because these elastin peptides are bioactive (8, 12–15, 17). Up to now, neither a biological activity nor a dedicated receptor have been described for the non-elastin sequences.

As evidenced by the consensus profile reported for each of them (Fig. 1), the non-elastin sequences (96 259 sequences) present a sequence pattern that is mostly dominated by the occurrence of G, A or K at X positions. These residues are not favored in elastin sequences where AGVPGFGVG is the prevailing motif. This is remarkably pronounced when non-elastin animal sequences are considered. Their consensus is GGGPGGGGG. These important sequence differences suggest that nonapeptides found in non-elastin sequences would most probably be structured and behave differently than elastin nonapeptides. Nevertheless, a comprehensive structural analysis is required to address this point.

In the present study, we focused our analysis on the nonapeptide sequences found in human exon 26 aiming at understanding their structure and structural behavior. First, this investigation was performed using optical spectroscopies. The results gathered by both CD and Raman spectra are consistent with previous reports underlining a conformational equilibrium between random, strand and turns conformations within elastin sequences (50). The Raman data underline that the conformation of these peptides is dominated by β -turns as deduced from the quantitative analysis of their amide I profiles. These conformations appear very favored as increasing the temperature up to 80°C did not drastically change the spectra and hence their structure, albeit a small increase of the β -strands content could be observed upon heating. Altogether our experimental data suggest that the structures of the nonapeptides are dominated by β -turns and that these conformations could be engaged in a conformational equilibrium with extended and random structures.

In order to have a better understanding of the structural behavior of these peptides, molecular dynamics simulations were undertaken in explicit water. Ten independent 500-ns

simulations were performed for each of the 4 nonapeptides. The results obtained are extremely consistent with our experimental data. Indeed, they show that the most common structures observed during the simulation is a type II β -turn occurring at the X-P-G-X motif. Quantitatively, this structure seems more abundant in hN3 (about 50%), than hN4, hN5 or hN6 (25-35%). This finding is in very good agreement with the conclusions of Lessing and colleagues (51) who observed that the preference for the X-Pro-Gly sequence to form a β -turn increased with the complexity and hydrophobicity of the side chain at position X (Gly < Ala < Val). In contrast to hN4, hN5 and hN6 which harbor a valyl residue before PG, hN3 possesses an isoleucyl residue at that position. Thus, the higher propensity of hN3 to adopt a β -turn when compared to the other three nonapeptides is consistent with the work of Lessing and colleagues.

Since the first report of their bioactivity on bovine fibroblasts (12), nonapeptide sequences have been tested on various cellular systems: bovine endothelial cells (13), rat macrophages (14) and human tumor cell lines (8, 9). Nevertheless, among those publications few used more than one peptide sequence, thereby forbidding the comparison of their respective effects. In the work of Maeda and colleagues, hN5 and hN6 were identified as chemoattractants for rat macrophages (14). In this work, the authors also analyzed the effect of the canonical VGVAPG sequence. Their conclusion was that the two nonapeptides could compete for receptor binding on EBP. Indeed, while VGVAPG appeared as an exclusive ligand for EBP, they underlined that hN5 and hN6 could also bind EBP and a lactose-insensitive receptor, later identified as RPSA. Their work therefore suggest that the EBP-binding motif can be harbored by both hexa- and nonapeptides while RPSA binding motif can be only adopted by nonapeptides. It has been established that VGVAPG bioactivity relies on its ability to adopt mainly a type VIII β -turn conformation (10). Our molecular dynamics simulations show that the major conformation of nonapeptides could be the type II β -turn, but

also that type VIII turns can be present. Our experimental data also suggest that both type VIII and type II β -turn can be present in the conformation of the nonapeptides. Thus, at the difference of VGVAPG, nonapeptides could be favorably structured to bind EBP and RPSA. However, it has to be emphasized that the sequences of the hexa- and nonapeptides are different. That way, binding affinity could also be supported by favorable residue side chain interactions in addition to the locally adopted conformation. Sequence differences could also contribute to the differential bioactivity exhibited by these peptides.

Altogether our data suggest that the structural signature of elastin nonapeptide is a type II β -turn. Consequently, it is reasonable to propose that this conformation could be relevant of the observed bioactivity of these peptides. This proposal is supported by the observations made by Toupance and co-workers where they measured a lesser biological effect of hN6 towards human tumor cells than hN5 (8). In our work, hN6 is predicted to present less β -turn conformations than hN5. Therefore, it seems coherent that the bioactivity of hN5 is greater than that of hN6 supporting the proposal that the turn motif is the conformation sustaining bioactivity of these peptides. If our prediction is correct, then one can anticipate that hN3 would be a peptide more bioactive than others.

Despite decades of efforts, the structure/function relationship of RPSA is still an open question (52). Notably, the interaction site of elastin nonapeptides on RPSA is unknown. In contrast, RPSA laminin binding site was described as a secondary function acquired during evolution (53). This site corresponds to the 161-180 region of the human sequence. However, the crystal structure of the protein (54) locates this binding site mostly inside the fold. Therefore, it is thought that RPSA could possess multiple sites and/or associate with other compounds to exert its functions. Consequently, our current knowledge of RPSA structure cannot explain where and how it binds elastin nonapeptides. Further structural explorations are required to address this question.

Conclusion

Our data throw new light on the nature, structure and conformational behavior of elastin nonapeptides. These peptide sequences exhibit a peculiar composition as demonstrated by our bioinformatic analysis of current databases. Further, experimental (CD and Raman spectroscopies) and theoretical data (molecular dynamics) strongly suggest that the dynamic structure of these peptides is dominated by the type II β -turn conformation. We propose that the observed bioactivity of these peptides towards stromal and tumor cells could be driven by the recognition of this structural feature by their cognate receptor, RPSA. Molecular modeling of their interaction is underway.

Authors contributions

HB and KSG performed the Raman and CD measurements and analyzed the spectra. BS conceived and ran the simulations. CJM, TJ and BN performed the sequence analyses. BS and DM funded the project and edited the manuscript. DL supervised the work and wrote the manuscript.

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References

1. Mecham, R.P. 2011. *The Extracellular Matrix: an Overview*. Berlin, Heidelberg: Springer Berlin Heidelberg.
2. Duca, L., S. Blaise, B. Romier, M. Laffargue, S. Gayral, H. El Btaouri, C. Kawecki, A. Guillot, L. Martiny, L. Debelle, and P. Maurice. 2016. Matrix ageing and vascular impacts: focus on elastin fragmentation. *Cardiovasc. Res.* 110: 298–308.
3. Maurice, P., S. Blaise, S. Gayral, L. Debelle, M. Laffargue, W. Hornebeck, and L. Duca. 2013. Elastin fragmentation and atherosclerosis progression: The elastokine concept. *Trends Cardiovasc. Med.* 23: 211–221.
4. Gayral, S., R. Garnotel, A. Castaing-Berthou, S. Blaise, A. Fougerat, E. Berge, A. Montheil, N. Malet, M.P. Wymann, P. Maurice, L. Debelle, L. Martiny, L.O. Martinez, A. Pshezhetsky, L. Duca, and M. Laffargue. 2014. Elastin-derived peptides potentiate atherosclerosis through the immune Neu1-PI3K γ pathway. *Cardiovasc. Res.* 102: 118-127.
5. Pocza, P., H. Süli-Vargha, Z. Darvas, and A. Falus. 2008. Locally generated VGVAPG and VAPG elastin-derived peptides amplify melanoma invasion via the galectin-3 receptor. *Int. J. Cancer.* 122: 1972–1980.
6. Coquerel, B., F. Poyer, F. Torossian, V. Dulong, G. Bellon, I. Dubus, A. Reber, and J.-P. Vannier. 2009. Elastin-derived peptides: Matrikines critical for glioblastoma cell aggressiveness in a 3-D system. *Glia.* 57: 1716–1726.
7. Devy, J., L. Duca, B. Cantarelli, D. Joseph-Pietras, A. Scandolera, A. Rusciani, L. Parent, J. Thevenard, S.B. Pasco, M. Tarpin, L. Martiny, and L. Debelle. 2010. Elastin-derived peptides enhance melanoma growth in vivo by upregulating the activation of Mcol-A (MMP-1) collagenase. *Br. J. Cancer.* 103: 1562–1570.
8. Toupance, S., B. Brassart, F. Rabenoelina, C. Ghoneim, L. Vallar, M. Polette, L. Debelle, and P. Birembaut. 2012. Elastin-derived peptides increase invasive capacities of lung cancer cells by post-transcriptional regulation of MMP-2 and uPA. *Clin. Exp. Metastasis.* 29: 511–522.
9. Brassart, B., P. Fuchs, E. Huet, A.J. Alix, J. Wallach, A.M. Tamburro, F. Delacoux, B. Haye, H. Emonard, W. Hornebeck, and L. Debelle. 2001. Conformational dependence of collagenase (matrix metalloproteinase-1) up-regulation by elastin peptides in cultured fibroblasts. *J. Biol. Chem.* 276: 5222–5227.
10. Blanchevoye, C., N. Floquet, A. Scandolera, S. Baud, P. Maurice, O. Bocquet, S. Blaise, C. Ghoneim, B. Cantarelli, F. Delacoux, M. Dauchez, R.G. Efremov, L. Martiny, L. Duca, and L. Debelle. 2013. Interaction between the Elastin Peptide VGVAPG and Human Elastin Binding Protein. *J. Biol. Chem.* 288: 1317–1328.
11. Scandolera, A., L. Odoul, S. Salesse, A. Guillot, S. Blaise, C. Kawecki, P. Maurice, H. El Btaouri, B. Romier-Crouzet, L. Martiny, L. Debelle, and L. Duca. 2016. The Elastin Receptor Complex: A Unique Matricellular Receptor with High Anti-tumoral Potential. *Front. Pharmacol.* 7: 32.
12. Long, M.M., V.J. King, K.U. Prasad, and D.W. Urry. 1988. Chemotaxis of fibroblasts toward nonapeptide of elastin. *Biochim. Biophys. Acta.* 968: 300–311.
13. Long, M.M., V.J. King, K.U. Prasad, B.A. Freeman, and D.W. Urry. 1989. Elastin repeat peptides as chemoattractants for bovine aortic endothelial cells. *J. Cell. Physiol.* 140: 512–518.

14. Maeda, I., N. Mizoiri, M.P.P. Briones, and K. Okamoto. 2007. Induction of macrophage migration through lactose-insensitive receptor by elastin-derived nonapeptides and their analog. *J. Pept. Sci.* 13: 263–268.
15. Salesse, S., L. Odoul, L. Chazée, C. Garbar, L. Duca, L. Martiny, R. Mahmoudi, and L. Debelle. 2018. Elastin molecular aging promotes MDA-MB-231 breast cancer cell invasiveness. *FEBS Open Bio.* 8: 1395–1404.
16. Da Silva, J., P. Lameiras, A. Beljebbar, A. Berquand, M. Villemin, L. Ramont, S. Dukic, J.-M. Nuzillard, M. Molinari, and M. Gautier. 2018. Structural characterization and in vivo pro-tumor properties of a highly conserved matrikine. *Oncotarget.* 9: 17839.
17. Brassart, B., J. Da Silva, M. Donet, E. Seurat, F. Hague, C. Terryn, F. Velard, J. Michel, H. Ouadid-Ahidouch, J.-C. Monboisse, A. Hinek, F.-X. Maquart, L. Ramont, and S. Brassart-Pasco. 2019. Tumour cell blebbing and extracellular vesicle shedding: key role of matrikines and ribosomal protein SA. *Br. J. Cancer.* 120: 453–465.
18. Huerta-Cepas, J., F. Serra, and P. Bork. 2016. ETE 3: Reconstruction, Analysis, and Visualization of Phylogenomic Data. *Mol. Biol. Evol.* 33: 1635–1638.
19. Crooks, G.E., G. Hon, J.-M. Chandonia, and S.E. Brenner. 2004. WebLogo: A Sequence Logo Generator. *Genome Res.* 14: 1188–1190.
20. Lefèvre, T., M.-E. Rousseau, and M. Pézolet. 2007. Protein Secondary Structure and Orientation in Silk as Revealed by Raman Spectromicroscopy. *Biophys. J.* 92: 2885–2895.
21. Dolui, S., A. Mondal, A. Roy, U. Pal, S. Das, A. Saha, and N.C. Maiti. 2020. Order, Disorder, and Reorder State of Lysozyme: Aggregation Mechanism by Raman Spectroscopy. *J. Phys. Chem. B.* 124: 50–60.
22. Debelle, L., A.J.P. Alix, M.-P. Jacob, J.-P. Huvenne, M. Berjot, B. Sombret, and P. Legrand. 1995. Bovine Elastin and α -Elastin Secondary Structure Determination by Optical Spectroscopies. *J. Biol. Chem.* 270: 26099–26103.
23. Lindorff-Larsen, K., S. Piana, K. Palmo, P. Maragakis, J.L. Klepeis, R.O. Dror, and D.E. Shaw. 2010. Improved side-chain torsion potentials for the Amber ff99SB protein force field. *Proteins* 78: 1950–1958.
24. Abraham, M.J., T. Murtola, R. Schulz, S. Páll, J.C. Smith, B. Hess, and E. Lindahl. 2015. GROMACS: High performance molecular simulations through multi-level parallelism from laptops to supercomputers. *SoftwareX.* 1–2: 19–25.
25. Schrodinger, L.L.C. 2010. The PyMOL molecular graphics system. Version. 1: 0.
26. Bussi, G., D. Donadio, and M. Parrinello. 2007. Canonical sampling through velocity rescaling. *J. Chem. Phys.* 126: 014101.
27. Parrinello, M., and A. Rahman. 1981. Polymorphic transitions in single crystals: A new molecular dynamics method. *J. Appl. Phys.* 52: 7182–7190.
28. Essmann, U., L. Perera, M.L. Berkowitz, T. Darden, H. Lee, and L.G. Pedersen. 1995. A smooth particle mesh Ewald method. *J. Chem. Phys.* 103: 8577–8593.
29. Hess, B., H. Bekker, H.J.C. Berendsen, and J.G.E.M. Fraaije. 1997. LINCS: A linear constraint solver for molecular simulations. *J. Comput. Chem.* 18: 1463–1472.
30. Humphrey, W., A. Dalke, and K. Schulten. 1996. VMD: visual molecular dynamics. *J. Mol.*

Graph. 14: 33–38, 27–28.

31. Kabsch, W., and C. Sander. 1983. Dictionary of protein secondary structure: Pattern recognition of hydrogen-bonded and geometrical features. *Biopolymers*. 22: 2577–2637.
32. Lewis, P.N., F.A. Momany, and H.A. Scheraga. 1973. Chain reversals in proteins. *Biochim. Biophys. Acta BBA - Protein Struct.* 303: 211–229.
33. Hutchinson, E.G., and J.M. Thornton. 1994. A revised set of potentials for β -turn formation in proteins. *Protein Sci.* 3: 2207–2216.
34. Daura, X., K. Gademann, B. Jaun, D. Seebach, W.F. van Gunsteren, and A.E. Mark. 1999. Peptide Folding: When Simulation Meets Experiment. *Angew. Chem. Int. Ed.* 38: 236–240.
35. Nagy, G., M. Igaev, N.C. Jones, S.V. Hoffmann, and H. Grubmüller. 2019. SESCO: Predicting Circular Dichroism Spectra from Protein Molecular Structures. *J. Chem. Theory Comput.* 15: 5087–5102.
36. Hernández, B., F.-Z. Boukhalfa-Heniche, O. Seksek, Y.-M. Coïc, and M. Ghomi. 2006. Secondary conformation of short lysine- and leucine-rich peptides assessed by optical spectroscopies: Effect of chain length, concentration, solvent, and time. *Biopolymers*. 81: 8–19.
37. Perczel, A., and G.D. Fasman. 1992. Quantitative analysis of cyclic β -turn models. *Protein Sci.* 1: 378–395.
38. Lopes, J.L.S., A.J. Miles, L. Whitmore, and B.A. Wallace. 2014. Distinct circular dichroism spectroscopic signatures of polyproline II and unordered secondary structures: Applications in secondary structure analyses. *Protein Sci.* 23: 1765–1772.
39. Sibanda, B.L., and J.M. Thornton. 1985. β -Hairpin families in globular proteins. *Nature*. 316: 170.
40. Gunasekaran, K., C. Ramakrishnan, and P. Balaram. 1997. Beta-hairpins in proteins revisited: lessons for de novo design. *Protein Eng. Des. Sel.* 10: 1131–1141.
41. Kaur, H., and G.P.S. Raghava. 2004. A neural network method for prediction of β -turn types in proteins using evolutionary information. *Bioinformatics*. 20: 2751–2758.
42. Hernández, B., Y.-M. Coïc, S.G. Kruglik, C. Carelli, R. Cohen, and M. Ghomi. 2012. Octreotide Used for Probing the Type-II' β -Turn CD and Raman Markers. *J. Phys. Chem. B.* 116: 9337–9345.
43. Hernández, B., Y.-M. Coïc, B. Baron, S.G. Kruglik, F. Pflüger, R. Cohen, C. Carelli, and M. Ghomi. 2014. Low concentration structural dynamics of lanreotide and somatostatin-14. *Biopolymers*. 101: 1019–1028.
44. Hernández, B., E. López-Tobar, S. Sanchez-Cortes, Y.-M. Coïc, B. Baron, A. Chenal, S.G. Kruglik, F. Pflüger, R. Cohen, and M. Ghomi. 2016. From bulk to plasmonic nanoparticle surfaces: the behavior of two potent therapeutic peptides, octreotide and pasireotide. *Phys. Chem. Chem. Phys.* 18: 24437–24450.
45. Fuchs, P.F.J., A.M.J.J. Bonvin, B. Bochicchio, A. Pepe, A.J.P. Alix, and A.M. Tamburro. 2006. Kinetics and Thermodynamics of Type VIII β -Turn Formation: A CD, NMR, and Microsecond Explicit Molecular Dynamics Study of the GDNF Tetrapeptide. *Biophys. J.* 90: 2745–2759.
46. Hernández, B., F. Pflüger, A. Adenier, S.G. Kruglik, and M. Ghomi. 2010. Vibrational Analysis of Amino Acids and Short Peptides in Hydrated Media. VIII. Amino Acids with Aromatic Side

- Chains: l-Phenylalanine, l-Tyrosine, and l-Tryptophan. *J. Phys. Chem. B.* 114: 15319–15330.
47. Hernández, B., F. Pflüger, S.G. Kruglik, and M. Ghomi. 2013. Characteristic Raman lines of phenylalanine analyzed by a multiconformational approach. *J. Raman Spectrosc.* 44: 827–833.
 48. Guiffo-Soh, G., B. Hernández, Y.-M. Coïc, F.-Z. Boukhalfa-Heniche, and M. Ghomi. 2007. Vibrational Analysis of Amino Acids and Short Peptides in Hydrated Media. II. Role of KLLL Repeats To Induce Helical Conformations in Minimalist LK-Peptides. *J. Phys. Chem. B.* 111: 12563–12572.
 49. Guiffo-Soh, G., B. Hernández, Y.-M. Coïc, F.-Z. Boukhalfa-Heniche, G. Fadda, and M. Ghomi. 2008. Vibrational Analysis of Amino Acids and Short Peptides in Hydrated Media. 3. Successive KL Repeats Induce Highly Stable β -Strands Capable of Forming Non-H-Bonded Aggregates. *J. Phys. Chem. B.* 112: 1282–1289.
 50. Debelle, L., and A.M. Tamburro. 1999. Elastin: molecular description and function. *Int. J. Biochem. Cell Biol.* 31: 261–272.
 51. Lessing, J., S. Roy, M. Reppert, M. Baer, D. Marx, T.L.C. Jansen, J. Knoester, and A. Tokmakoff. 2012. Identifying Residual Structure in Intrinsically Disordered Systems: A 2D IR Spectroscopic Study of the GVGXPGVG Peptide. *J. Am. Chem. Soc.* 134: 5032–5035.
 52. DiGiacomo, V., and D. Meruelo. 2016. Looking into laminin receptor: critical discussion regarding the non-integrin 37/67-kDa laminin receptor/RPSA protein: Looking into laminin receptor. *Biol. Rev.* 91: 288–310.
 53. Ardini, E., G. Pesole, E. Tagliabue, A. Magnifico, V. Castronovo, M.E. Sobel, M.I. Colnaghi, and S. Menard. 1998. The 67-kDa laminin receptor originated from a ribosomal protein that acquired a dual function during evolution. *Mol. Biol. Evol.* 15: 1017–1025.
 54. Jamieson, K.V., J. Wu, S.R. Hubbard, and D. Meruelo. 2008. Crystal Structure of the Human Laminin Receptor Precursor. *J. Biol. Chem.* 283: 3002–3005.

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Table 1. Nonapeptide stack of elastin Uniprot entries
Species, name of the species. Acc#, accession number of the origin sequence. #, number of the hit within the sequence. Position, position of the hit. Exon, exon number (when available). Human sequences appear in bold faces.

Species	Acc#	#	Sequence	Position	Exon
<i>Oreochromis niloticus</i>	I3JQ46	8	AGGPGSGIG	657 - 665	18
<i>Odobenus rosmarus divergens</i>	A0A2U3ZFC3	5	AGIPGFGAG	541 - 549	
<i>Canis lupus familiaris</i>	J9NW15	5	AGIPGFGVG	571 - 579	23
Homo sapiens	P15502	3	AGIPGLGVG	576 - 584	26
<i>Rhinopithecus roxellana</i>	A0A2K6NWE2	3	AGIPGLGVG	562 - 570	26
<i>Pan troglodytes</i>	A0A2I3SVF0	3	AGIPGLGVG	580 - 588	27
<i>Macaca mulatta</i>	A0A1D5R663	2	AGIPGVGPG	312 - 320	15
<i>Papio anubis</i>	A0A2I3MW49	3	AGVPGFGAG	472 - 480	16
<i>Cercocebus atys</i>	A0A2K5LP54	3	AGVPGFGAG	512 - 520	18
<i>Felis catus</i>	A0A337S1Z3	4	AGVPGFGAG	404 - 412	18
<i>Ailuropoda melanoleuca</i>	G1MA94	4	AGVPGFGAG	499 - 507	23
<i>Canis lupus familiaris</i>	J9NW15	6	AGVPGFGAG	580 - 588	23
<i>Myotis lucifugus</i>	G1QCG1	2	AGVPGFGAG	483 - 491	24
<i>Myotis lucifugus</i>	G1QCG1	3	AGVPGFGAG	492 - 500	24
<i>Myotis lucifugus</i>	G1QCG1	4	AGVPGFGAG	501 - 509	24
<i>Sus scrofa</i>	A0A287AXU0	5	AGVPGFGAG	599 - 607	25
Homo sapiens	P15502	6	AGVPGFGAG	603 - 611	26
<i>Rattus norvegicus</i>	Q99372	3	AGVPGFGAG	642 - 650	26
<i>Rattus norvegicus</i>	Q99372	4	AGVPGFGAG	658 - 666	26
<i>Mus musculus</i>	P54320	2	AGVPGFGAG	613 - 621	27
<i>Mus musculus</i>	P54320	3	AGVPGFGAG	622 - 630	27
<i>Mus musculus</i>	P54320	4	AGVPGFGAG	631 - 639	27
<i>Mus musculus</i>	P54320	5	AGVPGFGAG	640 - 648	27
<i>Mus musculus</i>	P54320	6	AGVPGFGAG	649 - 657	27
<i>Mustela putorius furo</i>	M3YXB8	4	AGVPGFGAG	608 - 616	27
<i>Pan troglodytes</i>	A0A2I3SVF0	6	AGVPGFGAG	607 - 615	27
<i>Erinaceus europaeus</i>	A0A1S3W547	3	AGVPGFGAG	584 - 592	
<i>Erinaceus europaeus</i>	A0A1S3W547	4	AGVPGFGAG	593 - 601	
<i>Enhydra lutris kenyonii</i>	A0A2Y9J5B3	4	AGVPGFGAG	615 - 623	
<i>Tursiops truncatus</i>	A0A2U4A8K1	1	AGVPGFGAG	87 - 95	
<i>Ursus maritimus</i>	A0A384CQV9	6	AGVPGFGAG	583 - 591	
<i>Mesocricetus auratus</i>	A0A1U8CCM2	2	AGVPGFGAG	643 - 651	
<i>Mesocricetus auratus</i>	A0A1U8CCM2	3	AGVPGFGAG	652 - 660	
<i>Mesocricetus auratus</i>	A0A1U8CCM2	4	AGVPGFGAG	661 - 669	
<i>Mesocricetus auratus</i>	A0A1U8CCM2	5	AGVPGFGAG	670 - 678	
<i>Dipodomys ordii</i>	A0A1S3GKU4	6	AGVPGFGAG	587 - 595	
<i>Erinaceus europaeus</i>	A0A1S3W547	5	AGVPGFGGG	602 - 610	
<i>Balaenoptera acutorostrata scammoni</i>	A0A384AI97	3	AGVPGFGPG	584 - 592	
<i>Felis catus</i>	A0A337S1Z3	2	AGVPGFGVG	386 - 394	18
<i>Felis catus</i>	A0A337S1Z3	3	AGVPGFGVG	395 - 403	18
<i>Canis lupus familiaris</i>	J9NW15	3	AGVPGFGVG	553 - 561	23
<i>Canis lupus familiaris</i>	J9NW15	4	AGVPGFGVG	562 - 570	23
<i>Saimiri boliviensis boliviensis</i>	A0A2K6UXV4	1	AGVPGFGVG	491 - 499	25
<i>Sus scrofa</i>	A0A287AXU0	3	AGVPGFGVG	581 - 589	25
<i>Sus scrofa</i>	A0A287AXU0	4	AGVPGFGVG	590 - 598	25
<i>Callithrix jacchus</i>	A0A2R8P8B8	3	AGVPGFGVG	523 - 531	26
<i>Mustela putorius furo</i>	M3YXB8	2	AGVPGFGVG	590 - 598	27
<i>Mustela putorius furo</i>	M3YXB8	3	AGVPGFGVG	599 - 607	27
<i>Odobenus rosmarus divergens</i>	A0A2U3ZFC3	4	AGVPGFGVG	532 - 540	
<i>Enhydra lutris kenyonii</i>	A0A2Y9J5B3	2	AGVPGFGVG	597 - 605	
<i>Enhydra lutris kenyonii</i>	A0A2Y9J5B3	3	AGVPGFGVG	606 - 614	
<i>Ursus maritimus</i>	A0A384CQV9	4	AGVPGFGVG	565 - 573	
<i>Ursus maritimus</i>	A0A384CQV9	5	AGVPGFGVG	574 - 582	
<i>Dipodomys ordii</i>	A0A1S3GKU4	4	AGVPGLGAG	569 - 577	
<i>Dipodomys ordii</i>	A0A1S3GKU4	5	AGVPLGAG	578 - 586	
<i>Danio rerio</i>	F8W4J7	4	AGVPLGGG	568 - 576	18
<i>Ailuropoda melanoleuca</i>	G1MA94	3	AGVPLGVG	490 - 498	23
<i>Saimiri boliviensis boliviensis</i>	A0A2K6UXV4	2	AGVPLGVG	500 - 508	25
<i>Rhinopithecus bieti</i>	A0A2K6MP70	3	AGVPLGVG	522 - 530	25
<i>Bos taurus</i>	P04985	1	AGVPLGVG	562 - 570	26
<i>Bos taurus</i>	P04985	2	AGVPLGVG	571 - 579	26
<i>Bos taurus</i>	P04985	3	AGVPLGVG	580 - 588	26
Homo sapiens	P15502	5	AGVPLGVG	594 - 602	26
<i>Rattus norvegicus</i>	Q99372	2	AGVPLGVG	633 - 641	26

Species	Acc#	#	Sequence	Position	Exon
<i>Callithrix jacchus</i>	A0A2R8P8B8	4	AGVPLGLGVG	532 - 540	26
<i>Callithrix jacchus</i>	A0A2R8P8B8	5	AGVPLGLGVG	541 - 549	26
<i>Callithrix jacchus</i>	A0A2R8P8B8	6	AGVPLGLGVG	550 - 558	26
<i>Colobus angolensis palliatus</i>	A0A2K5JW24	3	AGVPLGLGVG	548 - 556	26
<i>Bos mutus</i>	L8IPH2	1	AGVPLGLGVG	564 - 572	26
<i>Macaca nemestrina</i>	A0A2K6BHK1	4	AGVPLGLGVG	563 - 571	26
<i>Pan troglodytes</i>	A0A2I3SVF0	5	AGVPLGLGVG	598 - 606	27
<i>Ursus maritimus</i>	A0A384CQV9	3	AGVPLGLGVG	556 - 564	
<i>Ficedula albicollis</i>	U3KH66	1	AGVPGVGVG	359 - 367	15
<i>Equus caballus</i>	F7BWV3	1	FGVPGYGVG	418 - 426	17
<i>Oryzias latipes</i>	A0A3B3HSK9	1	GGAPGFGPG	383 - 391	14
<i>Tetraodon nigroviridis</i>	H3CXZ1	1	GGAPGSGPG	373 - 381	8
<i>Sus scrofa</i>	A0A287AXU0	2	GGFPGFVG	429 - 437	19
Homo sapiens	P15502	2	GGFPGFVG	401 - 409	20
<i>Pan troglodytes</i>	A0A2I3SVF0	2	GGFPGFVG	405 - 413	20
<i>Callithrix jacchus</i>	A0A2R8P8B8	2	GGFPGFVG	408 - 416	21
<i>Balaenoptera acutorostrata scammoni</i>	A0A384AI97	2	GGFPGFVG	440 - 448	
<i>Erinaceus europaeus</i>	A0A1S3W547	2	GGFPGYGAG	426 - 434	
<i>Canis lupus familiaris</i>	J9NW15	2	GGFPGYGIG	399 - 407	17
<i>Ailuropoda melanoleuca</i>	G1MA94	2	GGFPGYGIG	395 - 403	19
<i>Odobenus rosmarus divergens</i>	A0A2U3ZFC3	2	GGFPGYGIG	396 - 404	
<i>Enhydra lutris kenyoni</i>	A0A2Y9J5B3	1	GGFPGYGIG	456 - 464	
<i>Ursus maritimus</i>	A0A384CQV9	2	GGFPGYGIG	426 - 434	
<i>Felis catus</i>	A0A337S1Z3	1	GGFPGYGVG	289 - 297	14
<i>Papio anubis</i>	A0A2I3MW49	1	GGFPGYGVG	338 - 346	14
<i>Cercocebus atys</i>	A0A2K5LP54	1	GGFPGYGVG	354 - 362	15
<i>Mandrillus leucophaeus</i>	A0A2K5XQM6	2	GGFPGYGVG	284 - 292	15
<i>Rattus norvegicus</i>	Q99372	1	GGFPGYGVG	457 - 465	18
<i>Rhinopithecus bieti</i>	A0A2K6MP70	2	GGFPGYGVG	377 - 385	20
<i>Mus musculus</i>	P54320	1	GGFPGYGVG	454 - 462	21
<i>Colobus angolensis palliatus</i>	A0A2K5JW24	2	GGFPGYGVG	405 - 413	21
<i>Mustela putorius furo</i>	M3YXB8	1	GGFPGYGVG	445 - 453	21
<i>Rhinopithecus roxellana</i>	A0A2K6NWE2	2	GGFPGYGVG	405 - 413	21
<i>Macaca nemestrina</i>	A0A2K6BHK1	2	GGFPGYGVG	405 - 413	21
<i>Mesocricetus auratus</i>	A0A1U8CCM2	1	GGFPGYGVG	466 - 474	
<i>Dipodomys ordii</i>	A0A1S3GKU4	3	GGFPGYGVG	405 - 413	
<i>Canis lupus familiaris</i>	J9NW15	1	GGGPAGLGL	54 - 62	2
<i>Danio rerio</i>	F8W4J7	1	GGGPAGLGL	33 - 41	2
<i>Odobenus rosmarus divergens</i>	A0A2U3ZFC3	1	GGGPAGLGL	71 - 79	
<i>Dipodomys ordii</i>	A0A1S3GKU4	1	GGGPAGLGL	81 - 89	
<i>Oreochromis niloticus</i>	I3JQ46	1	GGPGFQGG	541 - 549	16
<i>Oreochromis niloticus</i>	I3JQ46	2	GGPGFQGG	559 - 567	16
<i>Oreochromis niloticus</i>	I3JQ46	3	GGPGFQGG	577 - 585	16
<i>Oreochromis niloticus</i>	I3JQ46	4	GGPGFQGG	589 - 597	16
<i>Oreochromis niloticus</i>	I3JQ46	9	GGPGLGLG	900 - 908	23
<i>Oreochromis niloticus</i>	I3JQ46	7	GGIPGLGYG	632 - 640	16
<i>Danio rerio</i>	F8W4J7	28	GGIPGVGYG	1190 - 1198	30
<i>Danio rerio</i>	F8W4J7	13	GGLPGGGAG	821 - 829	22
<i>Danio rerio</i>	F8W4J7	22	GGLPGGGAG	1033 - 1041	26
<i>Danio rerio</i>	F8W4J7	23	GGLPGGGAG	1041 - 1049	26
<i>Danio rerio</i>	F8W4J7	26	GGLPGGGAG	1117 - 1125	28
<i>Danio rerio</i>	F8W4J7	18	GGLPGGGIG	956 - 964	24
<i>Danio rerio</i>	F8W4J7	16	GGLPGGGLG	895 - 903	24
<i>Danio rerio</i>	F8W4J7	20	GGLPGGGLG	1005 - 1013	26
<i>Danio rerio</i>	F8W4J7	21	GGLPGGGLG	1025 - 1033	26
<i>Danio rerio</i>	F8W4J7	25	GGLPGGGLG	1090 - 1098	28
<i>Oreochromis niloticus</i>	I3JQ46	11	GGLPGGGPG	1844 - 1852	49
<i>Oreochromis niloticus</i>	I3JQ46	10	GGLPGGGTG	932 - 940	23
<i>Danio rerio</i>	F8W4J7	17	GGLPGGGVG	948 - 956	24
<i>Danio rerio</i>	F8W4J7	2	GGLPGIGAG	144 - 152	5
<i>Danio rerio</i>	F8W4J7	19	GGLPGSGIG	964 - 972	24
<i>Danio rerio</i>	F8W4J7	24	GGLPGSGIG	1049 - 1057	26
<i>Danio rerio</i>	F8W4J7	9	GGLPGSGLG	721 - 729	20
<i>Danio rerio</i>	F8W4J7	14	GGLPGSGTG	837 - 845	22
<i>Danio rerio</i>	F8W4J7	5	GGLPGSGVG	661 - 669	20
<i>Danio rerio</i>	F8W4J7	6	GGLPGSGVG	676 - 684	20
<i>Danio rerio</i>	F8W4J7	7	GGLPGSGVG	691 - 699	20
<i>Danio rerio</i>	F8W4J7	8	GGLPGSGVG	706 - 714	20

Species	Acc#	#	Sequence	Position	Exon
<i>Danio rerio</i>	F8W4J7	10	GGLPGSGVG	736 - 744	20
<i>Danio rerio</i>	F8W4J7	27	GGLPGSGVG	1180 - 1188	30
<i>Oreochromis niloticus</i>	I3JQ46	5	GGVPFGGG	613 - 621	16
<i>Myotis lucifugus</i>	G1QCG1	1	GGVPLGIG	442 - 450	22
<i>Oreochromis niloticus</i>	I3JQ46	6	GGVPGVGG	625 - 633	16
<i>Macaca nemestrina</i>	A0A2K6BHK1	3	GGVPGVGV	421 - 429	21
<i>Sus scrofa</i>	A0A287AXU0	1	PGAPGFGPG	330 - 338	17
<i>Balaenoptera acutorostrata scammoni</i>	A0A384AI97	1	PGAPGFGPG	315 - 323	
<i>Dipodomys ordii</i>	A0A1S3GKU4	2	PGFPGVGAG	303 - 311	
<i>Mandrillus leucophaeus</i>	A0A2K5XQM6	1	PGGPGFGPG	203 - 211	12
<i>Ailuropoda melanoleuca</i>	G1MA94	1	PGGPGFGPG	315 - 323	17
Homo sapiens	P15502	1	PGGPGFGPG	323 - 331	18
<i>Pan troglodytes</i>	A0A2I3SVF0	1	PGGPGFGPG	327 - 335	18
<i>Rhinopithecus bieti</i>	A0A2K6MP70	1	PGGPGFGPG	294 - 302	18
<i>Callithrix jacchus</i>	A0A2R8P8B8	1	PGGPGFGPG	339 - 347	19
<i>Colobus angolensis palliatus</i>	A0A2K5JW24	1	PGGPGFGPG	322 - 330	19
<i>Rhinopithecus roxellana</i>	A0A2K6NWE2	1	PGGPGFGPG	322 - 330	19
<i>Macaca nemestrina</i>	A0A2K6BHK1	1	PGGPGFGPG	322 - 330	19
<i>Ursus maritimus</i>	A0A384CQV9	1	PGGPGFGPG	333 - 341	
<i>Erinaceus europaeus</i>	A0A1S3W547	1	PGVPGFGRG	343 - 351	
<i>Callorhynchus milii</i>	V9KAK8	1	QGEPGLGGG	243 - 251	
<i>Danio rerio</i>	F8W4J7	3	TGLPLGIGP	463 - 471	12
<i>Danio rerio</i>	F8W4C7	1	TGRPNGRNG	137 - 145	3
<i>Macaca mulatta</i>	A0A1D5R663	1	VGAPGGGLG	302 - 310	13
<i>Danio rerio</i>	F8W4J7	11	VGLPGGGLG	792 - 800	22
<i>Danio rerio</i>	F8W4J7	15	VGLPGGGLG	875 - 883	24
<i>Balaenoptera acutorostrata scammoni</i>	A0A384AI97	5	VGVPFGGAG	602 - 610	
<i>Odobenus rosmarus divergens</i>	A0A2U3ZFC3	3	VGVPFGGAG	523 - 531	
<i>Trichechus manatus latirostris</i>	A0A2Y9RHH3	1	VGVPFGGAG	569 - 577	
<i>Heterocephalus glaber</i>	G5BG87	1	VGVPFGGAG	589 - 597	
<i>Papio anubis</i>	A0A2I3MW49	2	VGVPGLGVG	463 - 471	16
<i>Cercocebus atys</i>	A0A2K5LP54	2	VGVPGLGVG	503 - 511	18
<i>Mandrillus leucophaeus</i>	A0A2K5XQM6	3	VGVPGLGVG	423 - 431	19
<i>Macaca mulatta</i>	A0A1D5R663	3	VGVPGLGVG	535 - 543	23
<i>Macaca mulatta</i>	A0A1D5R663	4	VGVPGLGVG	544 - 552	23
Homo sapiens	P15502	4	VGVPGLGVG	585 - 593	26
<i>Bos mutus</i>	L8IPH2	2	VGVPGLGVG	573 - 581	26
<i>Bos mutus</i>	L8IPH2	3	VGVPGLGVG	582 - 590	26
<i>Macaca nemestrina</i>	A0A2K6BHK1	5	VGVPGLGVG	572 - 580	26
<i>Pan troglodytes</i>	A0A2I3SVF0	4	VGVPGLGVG	589 - 597	27
<i>Balaenoptera acutorostrata scammoni</i>	A0A384AI97	4	VGVPGLGVG	593 - 601	
<i>Tursiops truncatus</i>	A0A2U4A8K1	2	VGVPGLGVG	96 - 104	
<i>Danio rerio</i>	F8W4J7	12	YGLPGGGLG	813 - 821	22

Table 2. Tentative assignment of the Raman bands observed in the middle wavenumber region.

AGIPGLGVG hN3	VGVPGLGVG hN4	AGVPGLGVG hN5	AGVPGFGAG hN6	Tentative assignment
1700-1620 (br)	1700-1620 (br)	1700-1620 (br)	1700-1620 (br)	Amide I
			1605 (s)	F1
			1586 (m)	F2
1465 (s)	1468 (s)	1466 (s)		P, I, L, V, A
1451 (s)	1452 (s)	1452 (s)	1454 (s)	V, L, I
1421 (s)	1421 (s)	1420 (s)	1421 (s)	G, A, L, I, V, P
1391 (m)	1394 (m)	1395 (m)	1392 (m)	G, A, L, I, V, P
1343 (s)	1345 (s)	1343 (s)	1341 (s)	A, L, I, V, P
1315 (s)	1313 (s)	1317 (s)	1320 (s)	A, L, I
1300-1240 (br)	1300-1240 (br)	1300-1240 (br)	1300-1240 (br)	Amide III
			1207 (s)	F3
			1183 (m)	
1161 (m)	1175 (m)	1161 (m)	1159 (m)	V, L, I, P
1128 (s)	1127 (s)	1126 (s)	1126 (m)	V, L, I
			1101 (m)	P
			1031 (s)	F4
1031 (m)	1034 (m)	1031 (m)		G, I, P
1011 (m)	1014 (m)	1014 (m)		A, V
			1004 (vs)	F5
960 (s)	962 (s)	960 (s)		V, L, I
937 (s)	938 (s)	937 (s)	937 (br)	A, V, L, I
891 (s)	891 (s)	891 (s)		V
857 (m)	860 (m)	856 (sh)	851 (br)	L, P
845 (m)	840 (m)	843 (m)		A, V, L, I, P
			758 (br)	P
736 (br)	735 (br)	737 (br)		V, L, I, P
			621 (m)	F6

(vs) very strong; (s) strong; (m) medium; (br) broad Raman bands.

F1 to F6 refer to the six characteristic Raman bands of phenylalanine. Amide I and amide III vibrations give rise to a broad and strong Raman band peaking at ~ 1685 and 1255 cm^{-1} , respectively.

Table 3. Underlying secondary structure elements in the Amide I profile of the nonapeptides and fractional areas.

Position (assignment)	hN3	hN4	hN5	hN6
~1690 (random)	20	20	24	25
~1680 (turn)	41	40	37	38
~1660 (β -strand)	26	23	21	25
~1640 (turn)	13	17	18	12

Fractional areas				
Random	20	20	24	25
Turn	54	57	55	50
β -strand	26	23	21	25

Component positions are $\pm 5 \text{ cm}^{-1}$. During the computation, their widths were kept between 15 and 25 cm^{-1} . The figures correspond to the normalized areas of the components expressed in per cent of the whole Amide I profile. Fractional areas estimate the global secondary structures with an accuracy of $\pm 5\%$ (22).

Figure captions

Figure 1. Nonapeptide occurrences and distribution. The X-G-X-P-G-X-G-X-G consensus was found in 96 293 unique sequences amongst which 22 919 were from *Eukariota*, 71 504 from *Bacteria*, 240 from *Virus* and 1630 from *Archae*. The *Eukariota* branch distribution is dominated by Animals in which Vertebrates are predominant. For each branch of the tree, the total number of hits is reported as well as the number of observed overlapping (O) sequences and of tandem (T) repetitions. The consensus motif is also presented with the most frequent residues found at the four X positions.

Figure 2. Ultraviolet-circular dichroism (UV-CD) spectra of the four nonapeptides observed in water, water-methanol mixture (50/50), and methanol.

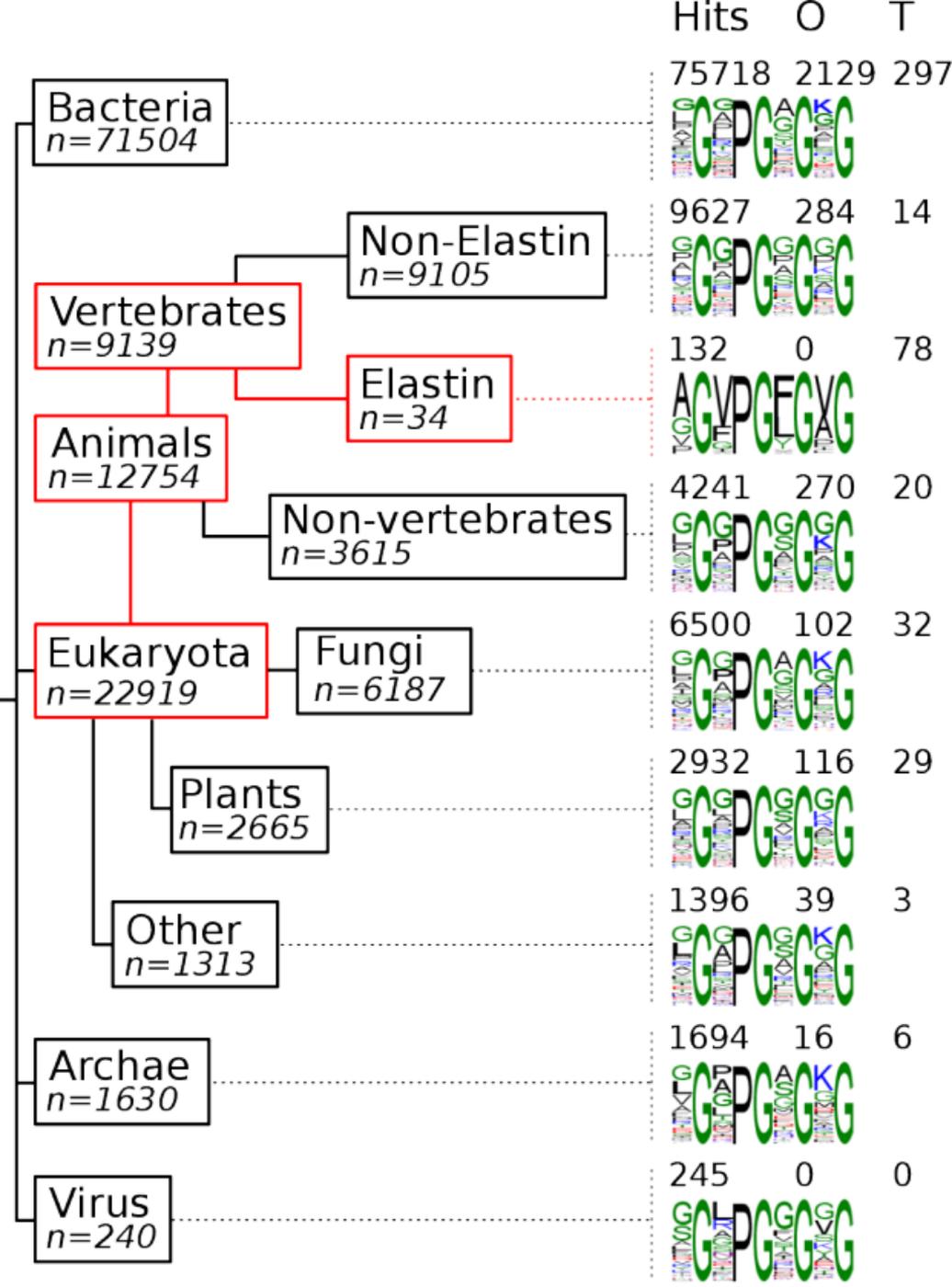
Figure 3. Room temperature Stokes Raman spectra in the middle wavenumber region for (A) hN3, hN4, hN5 and (B) hN6, compared to F. Spectra were recorded at 20 mM. See Table 2 for tentative assignments.

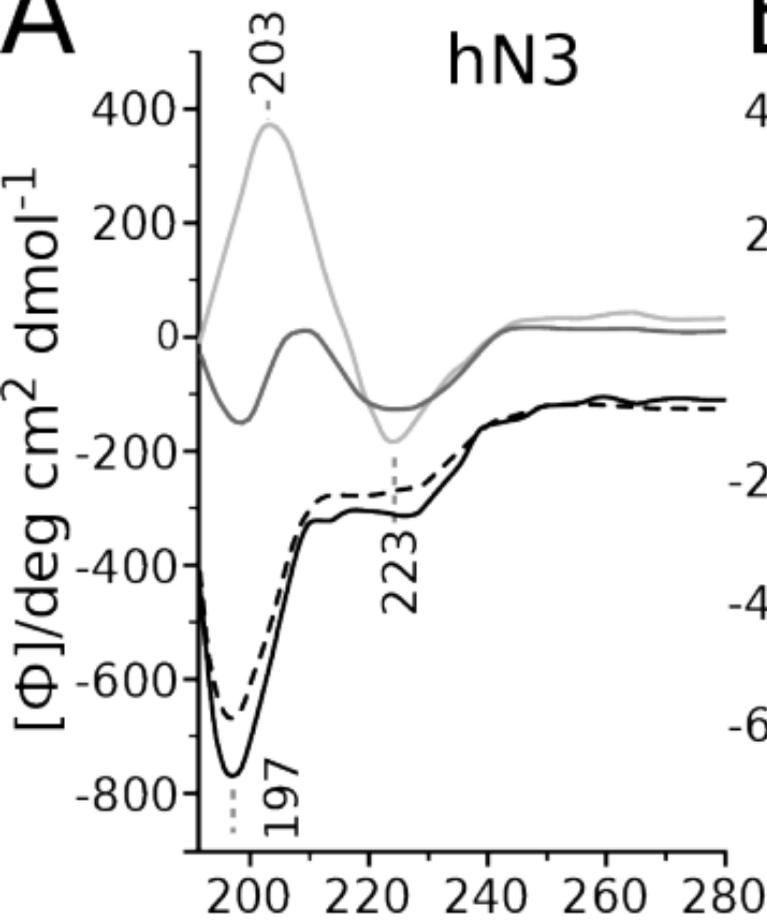
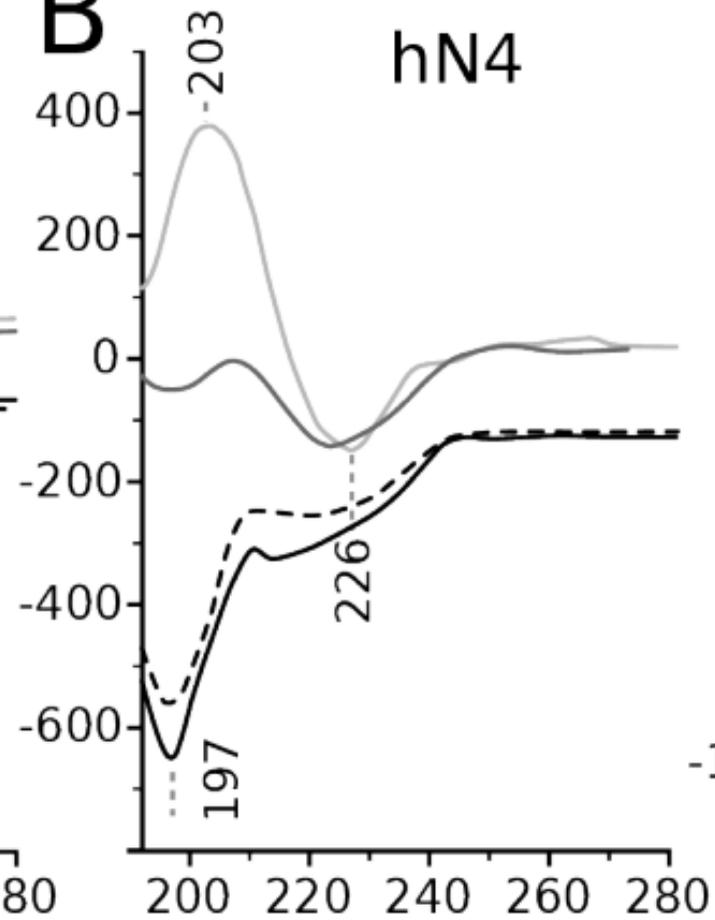
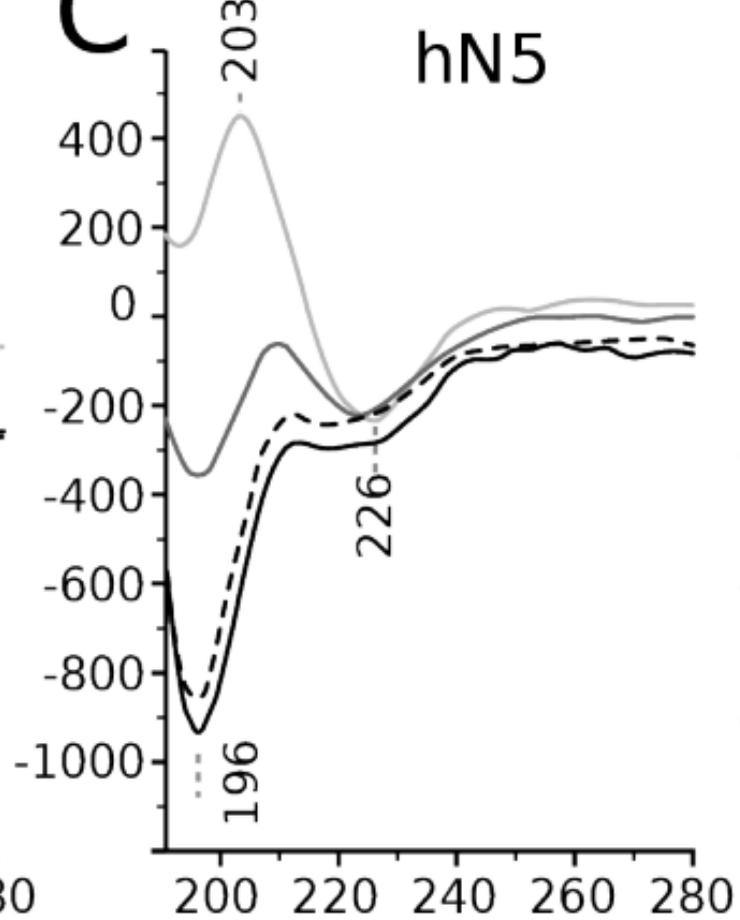
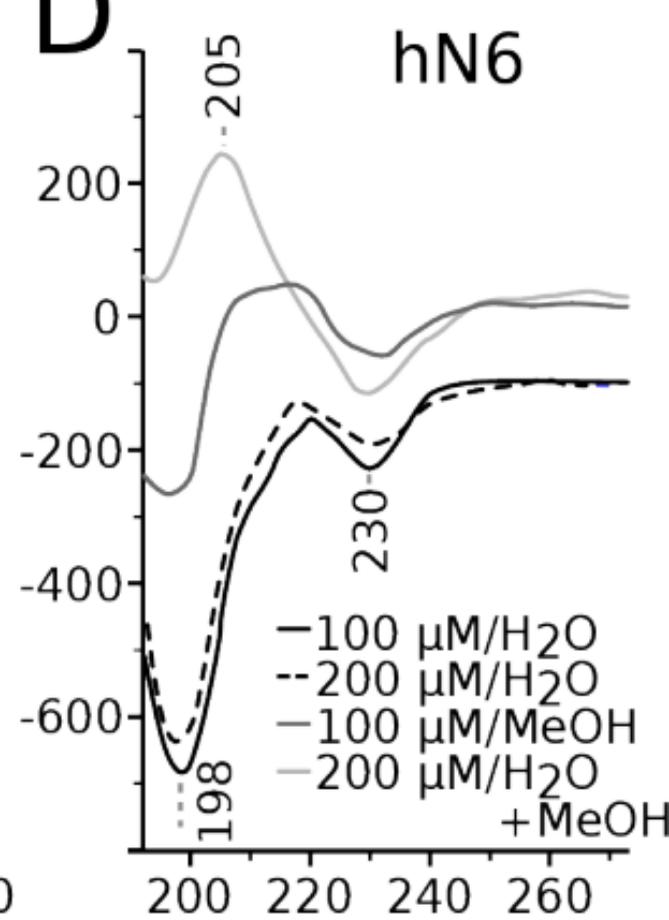
Figure 4. Band decomposition in the amide I region of the nonapeptides observed at 20 mM. Observed spectra are drawn with black lines, component bands with dark gray lines. The light gray trace corresponds to the sum of component bands (fit). Maximum band wavenumber of each used component is reported. In parentheses: (R) random; (T) turn; (S) β -strand. For the estimated populations of different secondary structural elements, see Table 3.

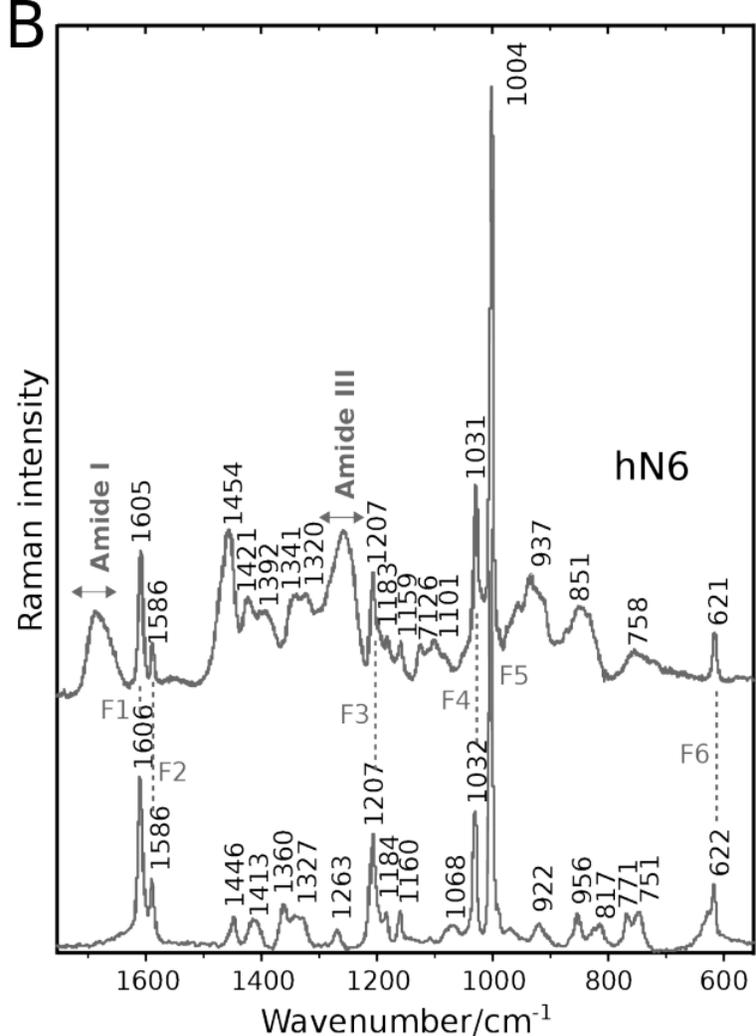
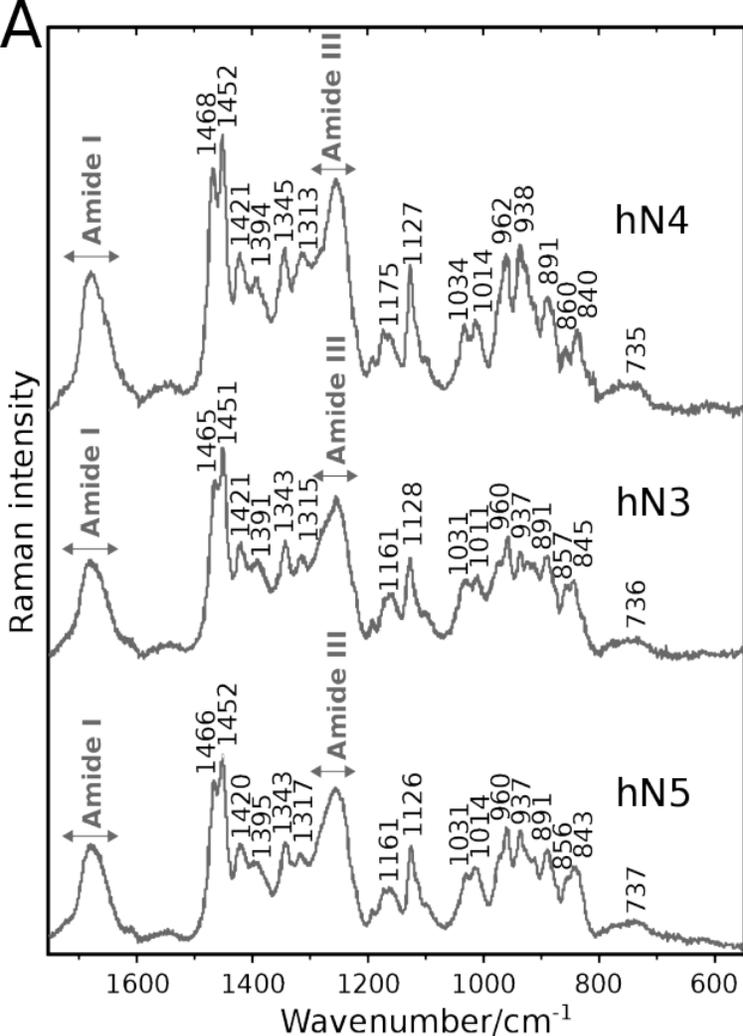
Figure 5. Effect of temperature on the amide III vibrations of nonapeptides. The spectra were recorded for a concentration of 20 mM. Gray scales are used to display the spectra obtained as a function of temperature.

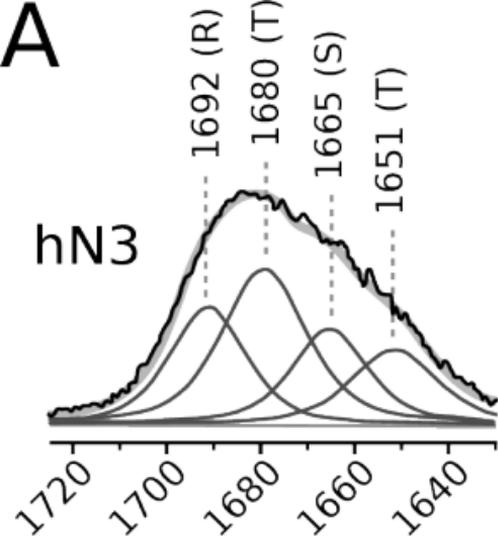
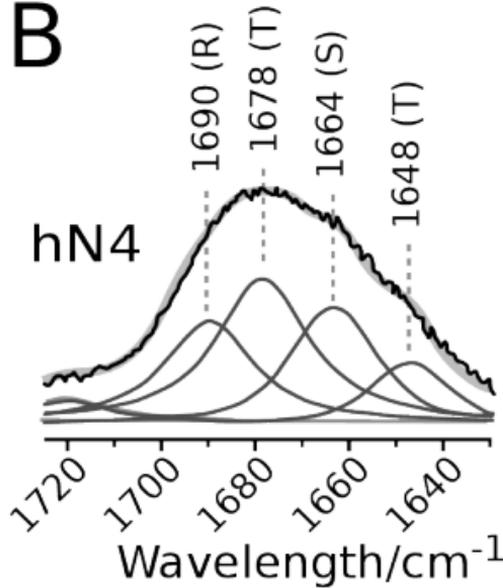
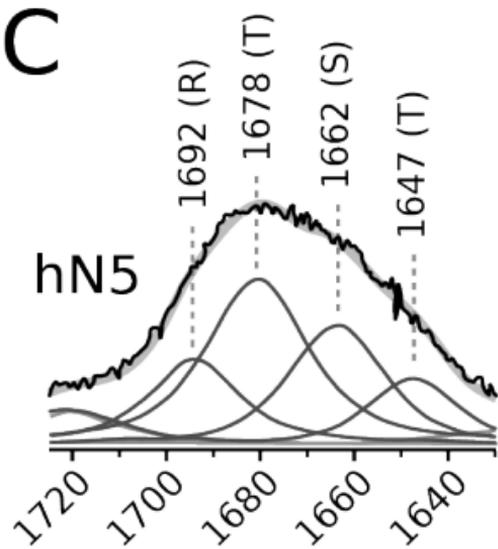
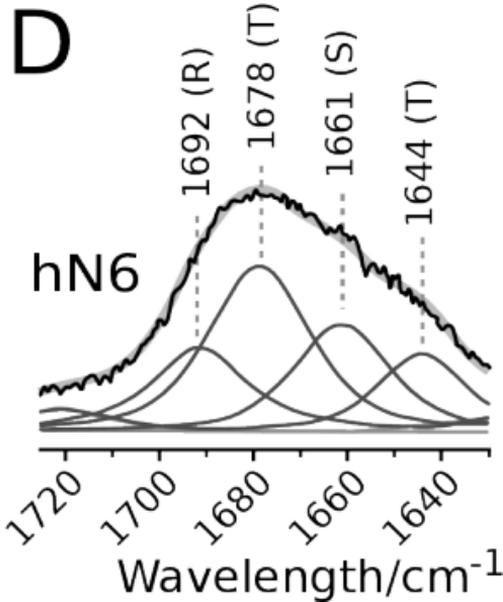
Figure 6. Conformational analysis of the molecular dynamics simulations. A, secondary structures adopted during the simulation calculated with DSSP (31). B, occurrence of β -turns types during each frame of the simulation trajectories. β -turns are computed considering the

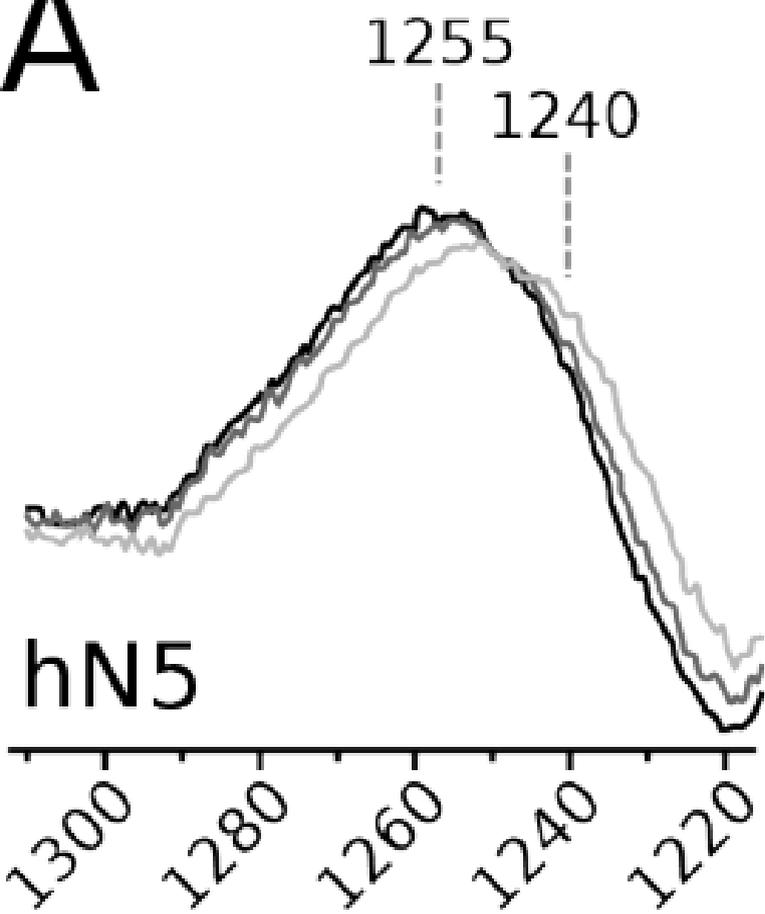
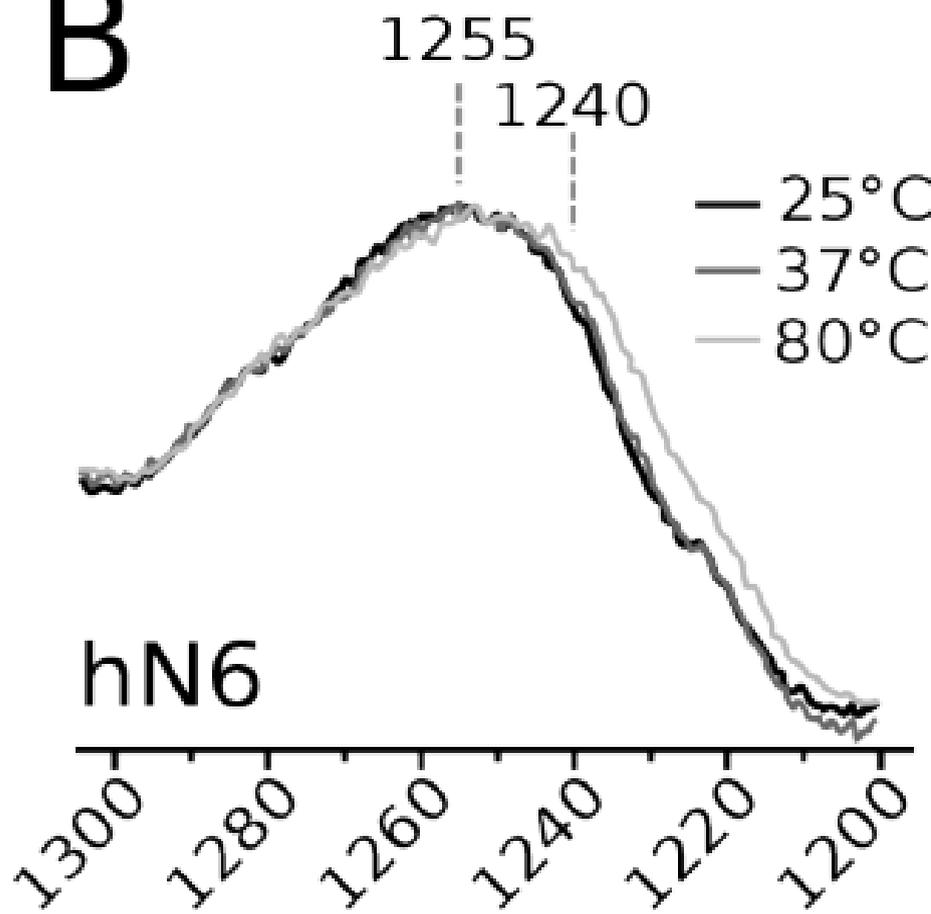
$\Phi\Psi$ angles of the PG residues at positions 4 and 5. Insert, overlay of the conformation representative of the main cluster computed for each of the four nonapeptides. Residues 3 to 6 are represented with a thicker line. Carbon atoms have the same colors as those defined for each peptide in panel B.



A**B****C****D**



A**B****C****D**

A**hN5****B****hN6**Wavelength/cm⁻¹

