Dissection of Human Tropoelastin: Solution Structure, Dynamics and Self-Assembly of the Exon 5 Peptide[+]

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Abstract: The elastic properties of elastin have essentially been discussed in terms of dominant entropic components, with questions still remaining about whether the basic mechanism is compatible with the classical theory of rubber elasticity. A better understanding of the structure–function relationships in terms of the protein's elastic properties remains an important goal in elastin science. Recently, we succeeded in the exon-by-exon synthesis of all polypeptide sequences encoded by the so-called hydrophobic exons and almost all of the cross-linking exons of human tropoelastin. Among these, the peptide encoded by exon 5 (PGGLAGAGLGA) has been extensively studied by classical spectroscopic methods, such as CD and NMR spectroscopy, and by molecular dynamics simulations. The results obtained clearly evidenced a large flexibility of the polypeptide chain, which oscillates between rather extended conformations, such as PPII, and folded ones, such as β turns. At the supramolecular level, we obtained evidence by TEM that shows that the peptide encoded by exon 5 is able to self-assemble in fibrillar structures, a result indicating that the "information" for self-assembly is also contained within a small domain of tropoelastin.

Keywords: conformation analysis · molecular dynamics · peptides · self-assembly · structure–activity relationships

Introduction

Elasticity in vertebrate tissues and organs is conferred by a polymeric, cross-linked protein, elastin. The monomeric form, tropoelastin, is a soluble protein that undergoes cross-linking and assembly into elastic fibres in the extracellular matrix. Many effectors have been suggested to play significant role in the process of elastic-fibre assembly, mainly enzymes (LOX) and other proteins like fibrillins, MAGP, or fibulin-5. Nevertheless, the details of the events that induce elastic-fibre assembly are still not known at the molecular level. Recently, some studies have evidenced the importance of self-assembly of tropoelastin for elastic-fibre formation.[1] Furthermore, recombinantly expressed polypeptides based on sequences of human elastin have been shown to contain sufficient information to self-organise into fibrillar structures and to promote the formation of lysine-derived cross-links, thereby producing a polymeric material with properties very similar to those of native elastin.[2]

Since polypeptides containing repetitive sequences of elastin were able to self-organise in fibrillar structures, the intriguing question that remains to be answered is, what is the smallest sequence/domain of tropoelastin that contains the information necessary for self-organisation?

Quite recently, we succeeded in the exon-by-exon synthesis of all polypeptide sequences encoded by the so-called hydrophobic exons and almost all of the cross-linking exons of human tropoelastin.[3] The rationale for this reductionist approach is given by the peculiar cassette-like organisation of the elastin gene, an organisation that suggested the possibility of each exon having its own structural (and possibly functional) autonomy. In all species, the elastin gene exhibits this cassette-like organisation with distinct exons coding for functionally and structurally distinct domains. The basic motif structure is a repeating unit of a cross-linking domain

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[+] Abbreviations: Boc = *tert*-butoxycarbonyl, DCC = dicyclohexylcarbodiimide, DIPEA = *N*,*N*-diisopropylethylamine, DSS = 3-(trimethylsilyl)-1-propanesulfonic acid, Fmoc = 9-fluorenylmethoxycarbonyl, HOBT = 1-hydroxy-1*H*-benzotriazole, LOX = lysil-oxidase, PPII = poly-L-proline II left-handed helix, TFA = trifluoroacetic acid, TFE = 2,2,2-trifluoroethanol.

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followed by polypeptide sequences putatively responsible for the elastic properties of the protein.[4]

Studying structural motifs based on individual exons can be useful for elastin (and most probably for other elastomeric proteins) because each exon encodes an independent and self-contained structure. This strategy will not apply to other proteins (for example, globular proteins) where individual exons do not encode autonomously folded structural modules. Furthermore, elastin has been demonstrated to be a fractal protein,[5] which means that the protein is characterised by the property of statistical self-similarity. Accordingly, even very short sequences show molecular and supramolecular features very similar to those of the whole protein.[6] This finding is most likely related to the abundance of repeating sequences of elastin, with the repetition being found at different scales along the entire sequence of the molecule.[7]

Summing up, the structural features of tropoelastin appear to be substantially different from those of classical globular proteins where the conformation of particular sequences is largely dictated by long-range interactions and, therefore, is dependent on almost the entire protein.[8] On the contrary, in the case of tropoelastin, because of its peculiar gene organisation and the abundance of repetitive sequences, one can suggest that some representative conformational building blocks exist, mostly determined by short-range interactions. This could be the case for the PPII and β-turn structures already found in elastin. The first results fully confirmed this working hypothesis, in that the elastin sequences ALGGGALG, LGAGGAG and LGAGGAGVL containing the simple dipeptide LG, were found to be structured according to the PPII conformation in aqueous solvent. On a smaller scale the same structure is adopted by the dipeptide LG, as ascertained by X-ray diffraction studies. Furthermore, the protected tripeptide Boc-VGG-OH was found to adopt a β turn with VG at the corners. It is known that many much longer sequences of elastin contain well-established β turns with XG sequences at the corners (X = V,L,A).[9] Accordingly, we were prompted to carry out an extensive and complete investigation on all of the polypeptide sequences encoded by the single exons.

Here, we report on the free peptide encoded by exon 5 (PGGLAGAGLGA, **EX5**), which has been extensively studied by classical spectroscopic methods, such as CD and NMR spectroscopy, and molecular dynamics simulations. Previous preliminary results showed the **EX5** peptide to have a high propensity to adopt short stretches of PPII structure,[3] a conformation rather ubiquitous in the hydrophobic, putatively elastomeric sequences of elastin and recently suggested to play an important role in both the elasticity of elastin and the self-assembly of extracellular matrix proteins such as elastin and lamprin.[10]

In order to study the influence of terminal charges on the adopted conformation(s), we also studied the *N*-acetylated **EX5** peptide. Furthermore, a possible interaction with **EX6**, the peptide encoded by the following exon, was checked by studying the synthetic peptide PGGLAGAGLGAGLGA (**EX5–6**), which contains an additional GLGA sequence pertaining to the N-terminal region of cross-linking **EX6**.

The results obtained clearly evidenced a large flexibility of the polypeptide chain, which oscillates between rather extended conformations, such as PPII, and folded ones, such as β turns. At the supramolecular level, we obtained evidence showing that the **EX5** peptide is able to self-assemble into fibrillar structures, as found by TEM, a result indicating that the "information" for self-assembly is also contained in a small domain of tropoelastin, as the domain encoding **EX5** is.

Results

CD spectroscopy: Figure 1 shows the CD spectra of **EX5** in water, TFE and urea solutions. The curve at 0 °C in water (Figure 1 a) shows a positive band at 215 nm and a strong

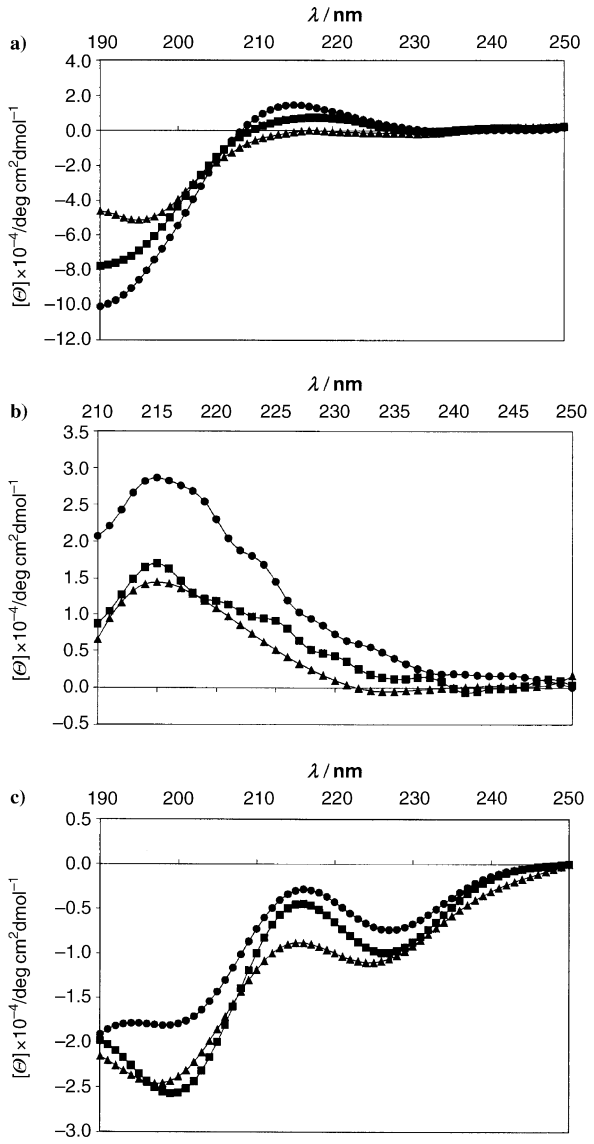

Figure 1. CD spectra of the **EX5** peptide: a) in water, recorded at different temperatures: 273 K (●), 298 K (■), 343 K (▲); b) in aqueous solution with different concentrations of urea (all recorded at 273 K): 3 M (●), 1 M (■), 0 M (▲); c) in TFE, recorded at different temperatures: 273 K (●), 298 K (■), 343 K (▲).

negative band below 195 nm, indicative of the PPII conformation.[11–13] Both the positive and negative bands decrease in magnitude at 25 °C, and at 70 °C the band at 215 nm takes values close to the zero line. This trend is typical of peptides structured in the PPII conformation as an increase in temperature increases the amount of unordered coil.[14]

In order to further confirm the presence of the PPII conformation, we registered the CD spectra of **EX5** at 0 °C in urea solutions of different concentrations (Figure 1b), because urea is known to induce stabilisation of the PPII conformation.[12] In fact, the band at 215 nm shows a urea-concentration dependence, in that it becomes more positive with increasing urea concentration.

In TFE (Figure 1c), the CD curves are characterised by two negative bands at 200 and 225 nm. The strong reduction of intensity and the slight red shifting of the band at the shorter wavelength, relative to the band that appears in the spectra measured in water, points to a significant presence of folded conformations, mainly β turns. Cooling the solution from 25 to 0 °C leads to a further reduction of the band intensity, which indicates an increased turn population.

Analogous measurements were carried out on the *N*-acetylated **EX5** peptide (Figure 2). In aqueous solution (Figure 2a), the spectra are very similar to those obtained for free **EX5** peptide, thereby demonstrating that the PPII structure does not depend on the presence of an N-terminal positive charge. In TFE (Figure 2b), a positive band appears at around 212 nm, a result strongly suggesting an increased contribution from a (most probably) type II β-turn conformation. Although the spectra are not so dissimilar from those in water, nevertheless they are not diagnostic of the PPII structure because of the opposite trend for the band at 200 nm as a function of temperature. As a matter of fact, on increasing the temperature the intensity slightly increases, a result indicating that the band is associated with the π–π* amide transition of the unordered structures and not with that of the PPII structure. NMR spectroscopy results confirmed this suggestion (see below).

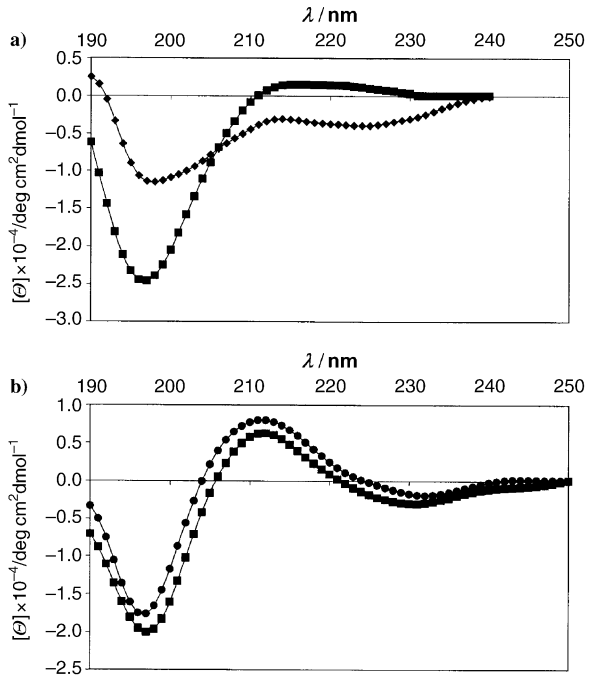

Figure 2. CD spectra of the *N*-acetylated **EX5** peptide: a) in water, recorded at different temperatures: 298 K (■), 343 K (◆); b) in TFE, recorded at different temperatures: 278 K (●), 298 K (■).

Figure 3 shows the CD spectra of the peptide PGGLAGAGLGAGLGA (**EX5–6**) under different conditions. The bands in the spectra in water and methanol (Figure 3a and b) are clearly attributable to the presence of the PPII structure, as in the case of **EX5**, and spectra comparable to those of **EX5** are also obtained in TFE. These results demonstrate that 1) no conformational changes are induced by lengthening the polypeptide chain by four residues and 2) no significant interaction occurs with the N-terminal region of the **EX6** sequence.

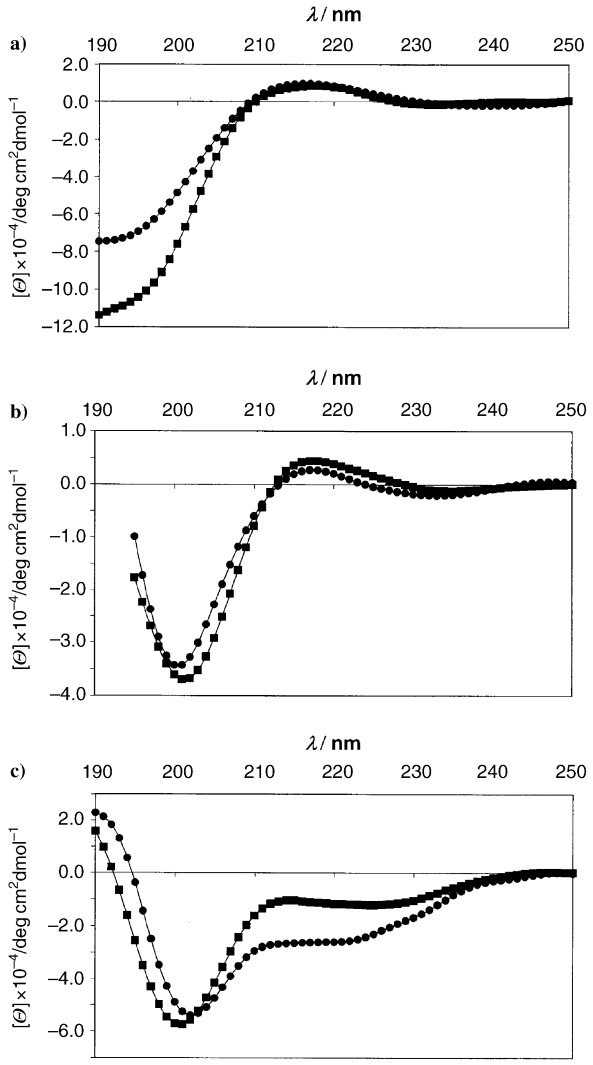

Figure 3. CD spectra of the **EX5–6** peptide: a) in water, recorded at different temperatures: 273 K (●), 298 K (■); b) in MeOH, recorded at different temperatures: 273 K (●), 298 K (■); c) in TFE, recorded at different temperatures: 273 K (●), 298 K (■).

Taken together the above results suggest that the **EX5** peptide could indeed have an autonomous folding that is essentially independent of long-range stabilising interactions (see also the Discussion section).

NMR spectroscopy: The **EX5** peptide was analysed by 2D ^{1}H NMR spectroscopy under different conditions, in aqueous solution (H_2O/D_2O (90:10)) at 298 K and 278 K, in 3 M [D_4]urea aqueous solution at 278 K and in a mixed aqueous/organic solution ([D_3]TFE/H_2O (70:30)) at 298 K and 283 K. The resonance assignments of the peptide in the different solutions were straightforwardly achieved by standard sequential assignment procedures with a combined analysis of 2D-TOCSY and 2D-ROESY spectra.[15] TOCSY spectra were used to identify spin systems, while ROESY spectra allowed the assignment of resonances to individual amino acids through sequential NOE connectivities.

While the addition of 3 M [D_4]urea into the aqueous solution induced only small changes in the chemical shifts of the **EX5** peptide resonances, the presence of 70% [D_3]TFE gave rise to dramatic changes in all of the resonance parameters of the sample. This confirmed that the conformation of the peptide in water and in TFE is significantly different (Figure 4).

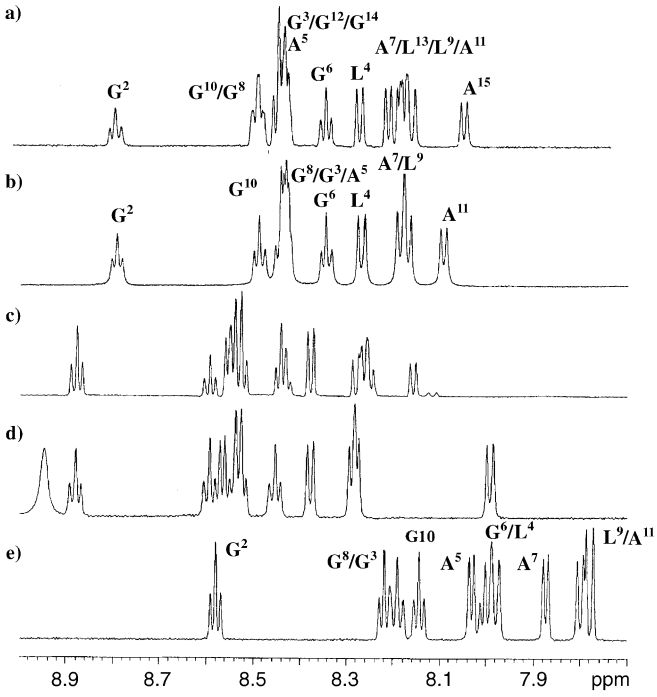

Figure 4. Amide region of ^{1}H NMR spectra: a) The **EX5–6** peptide in H_2O/D_2O (90:10) at 298 K; the **EX5** peptide recorded under different conditions: b) H_2O/D_2O (90:10) at 298 K; c) H_2O/D_2O (90:10) at 278 K; d) 3 M [D_4]urea at 278 K; e) [D_3]TFE/H_2O (70:30) at 283 K.

However, since CD spectroscopy of the **EX5** peptide in water[3] suggested the presence of a significant population of the PPII conformation that increases at lower temperatures, we tried to identify the residues mostly populating the PPII conformation by careful analysis of the NMR data. In particular, we determined the volume of some not-overlapping sequential peaks, $\alpha N(i,i+1)$ and $NN(i,i+1)$, and evaluated their ratios. As found by Fiebig et al.[17] by statistical analysis, the random-coil conformation should have an NOE ratio value ($\alpha N(i,i+1)/NN(i,i+1)$ of 1.4 for the sequential backbone cross-peaks, while for the extended conformations the higher distance of sequential amide protons increases the ratio to a value of 55.[18] Figure 5 shows the fingerprint and amide regions of the ROESY spectra of the **EX5** peptide recorded in H_2O/D_2O (90:10) at 5 °C. The regions are displayed with the same threshold, in order to highlight the difference in the intensity of the cross-peaks. This volume analysis was performed for the peptide in water at two different temperatures, as shown in Table 1.

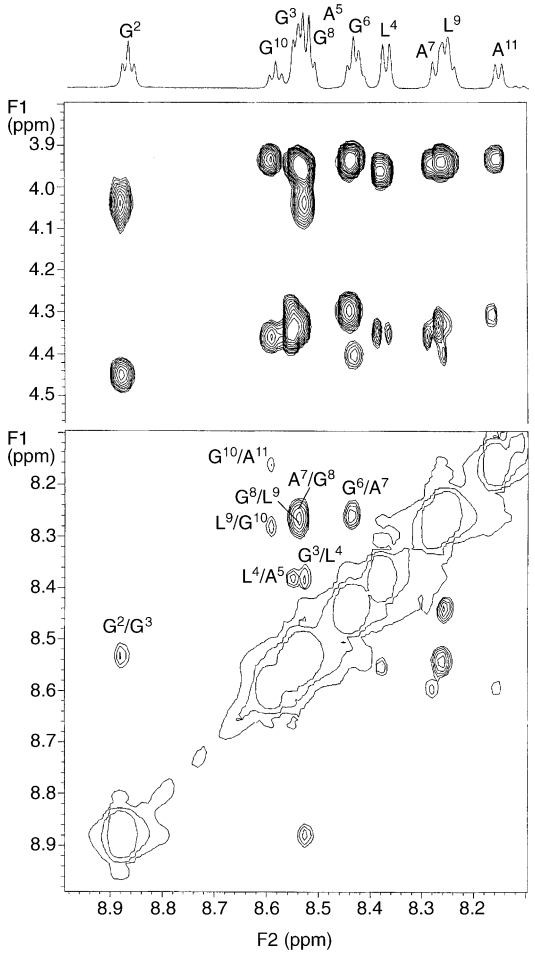

Figure 5. The fingerprint region and amide region of the ROESY spectra of the **EX5** peptide recorded in H_2O/D_2O (90:10) at 278 K. The regions are displayed with the same threshold in order to highlight the difference in the intensity of the cross-peaks.

The PPII conformation in water: The analysis of the ROESY spectra recorded at 298 K and 278 K in aqueous solution shows the complete absence of any medium range NOEs. Only intraresidue and sequential NOEs were observed. This feature is typical for highly flexible peptides where unordered conformations dominate. Also the chemical shift index (CSI) analysis points to a random-coil conformation, as the Hα chemical shift values are in the so-called random-coil range.[16]

Table 1. NOE ratio analysis results for the residues of the **EX5** peptide at 298 K and 278 K.

	T [K]	G^2	G^3	L^4	A^5	G^6	A^7	G^8	L^9	G^{10}
$\alpha N(i,i+1)$	298	–[a]	6.07	–	6.17	–	–	–	4.15	5.60
$NN(i,i+1)$	278	5.00	19.50	–	43.70	5.20	–	–	5.80	5.46

[a] Not determined because of overlapping signal.

The tabulated values for not-overlapping residues of the **EX5** peptide in water at 298 K show ratio values ranging from 4.15–6.17; all of these values are higher than the statistically determined ones for the random coil but are definitely below the value attributed to extended conformations. This finding suggests that the time spent in the PPII conformation is small, even if significant.

On decreasing the temperature to 278 K, a striking rise in the NOE ratio values was evident for some residues; this could account for the increased intensity of the band at 215 nm in the CD spectrum that was assigned to the PPII conformation (Figure 1a). Specifically, the residues with the highest propensity to PPII conformation, as determined from the NOE ratio analysis, are G^3 and A^5. Unfortunately, because of overlap of the signals it was not possible to determine the NOE ratio value of the intermediate amino acid L^4.

In order to find a rationale for the stabilising effect of urea on the **EX5** peptide, the NMR spectra of the peptide in aqueous solution containing 3 M urea were recorded and analysed. The addition of 3 M urea to the sample in aqueous solution causes a broadening of the signals and a different viscosity of the medium that affects the NOE-based experiments. The NOE ratio analysis, employed for the sample in water without urea, is possible only for well-resolved, not-overlapping peaks and thus needs high-quality spectra. The analysis of the NOESY spectra of the **EX5** peptide in aqueous solution with 3 M urea shows only a few cross-peaks in the NH–NH region corresponding to A^7/G^8 overlapping with G^8/G^9; thus, the NOE ratio analysis is unfeasible. On the other hand, it is possible to suppose that the absence of cross-peaks in the NH–NH region could be due to a distance of sequential amide protons that is higher than 4.5 Å, as tabulated for the regular PPII conformation.

Other information has been retrieved from the monodimensional spectra. On adding urea to the solution, as stated previously, some significant small changes in the chemical shift values are detected, in particular at 278 K for the residues A^5 and G^6 (Figure 4d). Interestingly, these residues are those mainly involved in the PPII conformation, as shown previously, a result suggesting that the urea molecules interact with this region.

The analysis of the **EX5–6** peptide in water shows the same chemical shift values for the residues that are common with **EX5** (Figure 4a). The NOE patterns are also similar, with no evidence of folded conformations.

β Turns in TFE: The analysis of the **EX5** peptide in TFE by NMR spectroscopy provides evidence for the presence of different β turns, that are probably rapidly interconverting. According to CD spectroscopy, the population of molecules in β-turn conformations is increased as the temperature was lowered. Indeed, although the NMR data at 298 K did not show clear evidence of stable secondary structures,[3] at 283 K typical features of β turns emerged. Apparently, there is a contradiction between the NMR spectroscopy results and the CD results at 298 K. Although the reason for this is somewhat unclear at present, a possible explanation could be the different sensitivities of the two techniques towards small conformer populations.

Temperature coefficients for some residues were low; this indicates a reduced exposition to the solvent that is usually ascribed to hydrogen bonding.[19] Furthermore, some intense sequential $\delta_{NN(i,i+1)}$ values, together with typical medium range NOE values, suggested the presence of folded conformations. In detail, A^5 and G^6 show temperature coefficients below 4 ppb K^{-1}, which suggest the presence of β turns. In addition, they show typical medium range NOE values, $\delta_{\alpha N(i,i+2)}$, between the Hα of L^4/NH of G^6 and the Hα of A^5/NH of A^7.

This NOE pattern, together with the reduced temperature coefficients, suggests the presence of rapidly interconverting (sliding) β turns involving the ^{3}GLAG^6 and ^{4}LAGA^7 sequences. In this case it is not possible from the NMR spectroscopy data to determine the types of β turn. The main reason we suggest sliding β turns is that the temperature coefficients are 3.7–3.4 ppb K^{-1}, a range that is usually considered to be the limit for hydrogen bonding (in the case of sliding β turns the time-averaged values must be noticeably higher), while stable β turns have temperature coefficients very close to 2 ppb K^{-1} (Figure 6).[20]

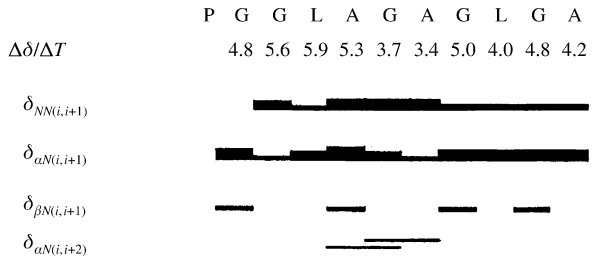

Figure 6. Summary of the NMR spectroscopy parameters of the **EX5** peptide in [D_3]TFE/H_2O (70:30) at 283 K. The summary includes the temperature coefficients ($-\Delta\delta/\Delta T$ [ppb K^{-1}]) and the sequential and medium-range NOEs. The NOE intensities are reflected by the thickness of the line.

Interestingly, for the *N*-acetylated **EX5** peptide, the determined values of the temperature coefficients for the residues G^6 and A^7 are 3.8 and 3.1, respectively (data not shown). The reduction of the value for residue A^7 in the *N*-acetylated **EX5** peptide relative to the value in the **EX5** peptide suggests that this residue is involved in a stronger hydrogen bond. Since the CD data for the *N*-acetylated peptide point to an increased presence of type II β turns (Figure 2a), we suggest that the ^{4}LAGA^7 type II β turn is more stable and therefore more populated in the *N*-acetylated **EX5** peptide.

So, the *N*-acetylation, apart from shifting the NH resonance of the G^2 residue upfield and changing the chemical shift values of the proline ring protons, does not cause great

differences in the peptide conformation. Furthermore, even if the *N*-acetylated peptide presents a PG unit able to promote a type II β turn, no evidence for such a structure is elicited from the NMR spectroscopy data. The subsequent G^3 residue probably dramatically reduces the stability of the turn. A similar behaviour was shown for the peptide encoded by exon 9 of tropoelastin.[3]

Molecular dynamics simulations: Two simulations of **EX5** in explicit water were performed at 300 and 278 K, respectively (Figure 7). All the initial conditions (velocities) were changed at the two temperatures, thereby leading to two

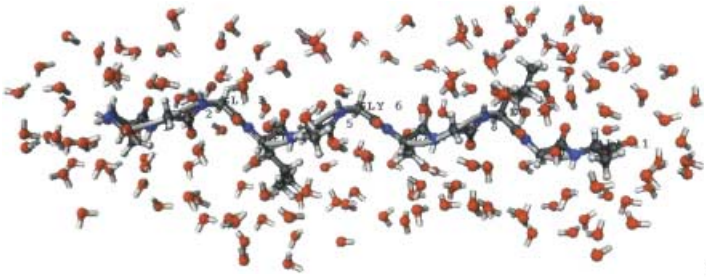

Figure 7. Ball-and-stick and backbone ribbon representations of the starting conformation used for the molecular dynamics simulation of the **EX5** peptide. The PPII structure was assigned by using the $\varphi = -75°$ and $\psi = +145°$ dihedral angles.

fully independent trajectories. The effective time of simulation was 4 ns at 300 K and 4.8 ns at 278 K. In both cases, we observed a highly flexible peptide chain, as indicated by the gyration radii reported in Figure 8, leading to a succession

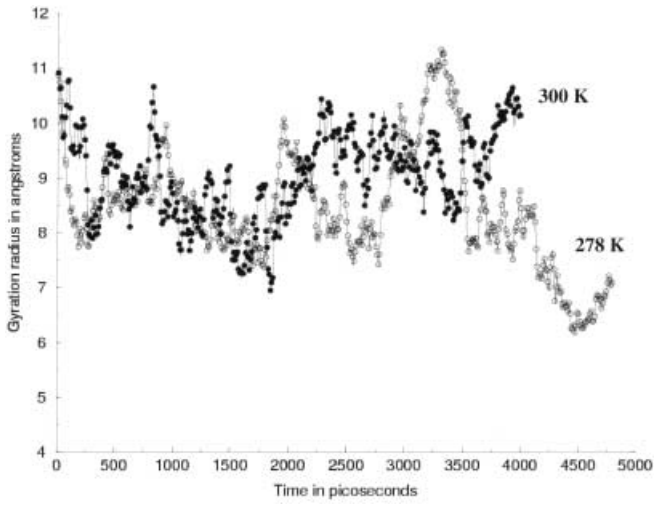

Figure 8. Gyration radius with time for the two trajectories of the **EX5** peptide at 300 K (black) and 278 K (grey).

of extended conformations and more folded ones, where extended means the conformations appearing in the northwest region of the Ramachandran plot (including the PPII structure) and folded refers to different types of β turns. Accordingly, we reduced the whole set of structures explored during the two trajectories into representative families of conformations. This clustering analysis is presented in Figure 9 for the two temperatures.

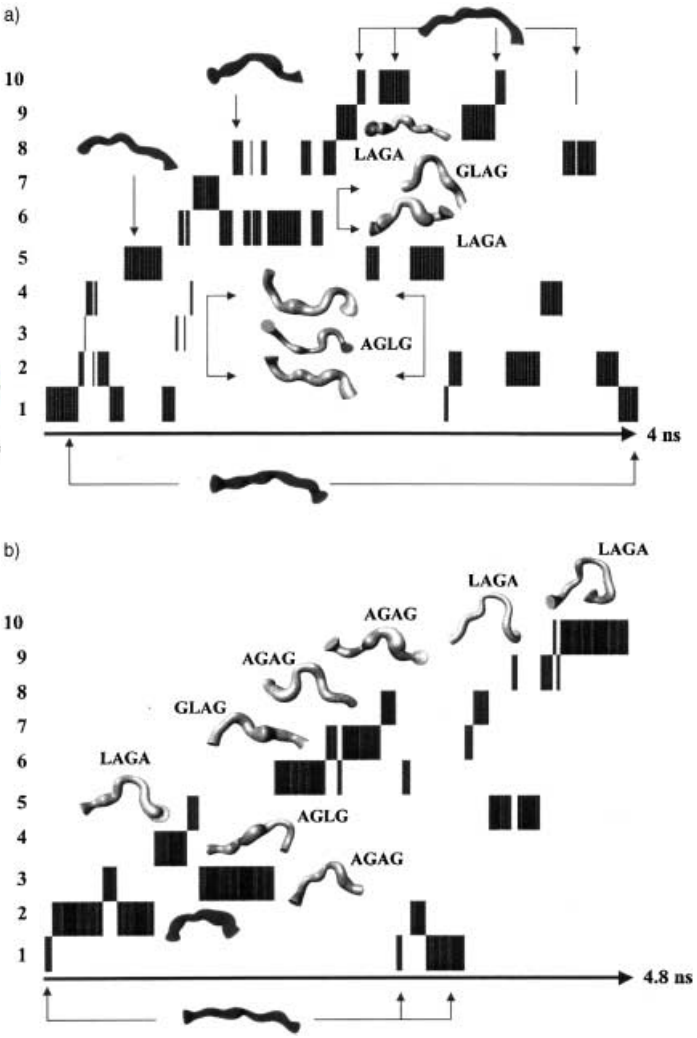

Figure 9. Time-course cluster analysis of the representative families of conformations obtained during two molecular dynamics simulations of the **EX5** peptide a) at 300 K and b) at 278 K. Each family is represented by its mean structure; the wider the ribbon is, the larger the fluctuations are at this point of the peptide.

Depending on the temperature, the behaviour of the peptide is slightly different, with extended and folded conformations continuously interchanging at 300 K and more stable folded conformations existing at 278 K. In fact, only 2 clusters are representative of quasiextended structures at 278 K, versus 4 at 300 K. However, in both trajectories we observe a return from folded (turn) structures to quasiextended ones, an observation showing an equilibrium between the extended/folded conformations in both cases. Thus, we analysed the two trajectories in terms of PPII, β turns and parallel β-sheet formation and interconversion as a function of time. The presence of PPII was confirmed in both simulations. The PPII content for each residue was extracted from the coordinates files by using dihedral canonical values ($\varphi = -75°$; $\psi = +145°$) with either a $\pm 30°$ or $\pm 22°$ deviation

(Figure 10). The 22° authorised deviation is able to distinguish between PPII and parallel β-sheet ($\varphi = -119°$; $\psi = +113°$) structures. The glycine residues are found mainly in extended conformations, where the term extended refers to the entire region of the Ramachandran plot containing the PPII, β-sheet and fully extended structures, independently of the temperature and of their position in the sequence.

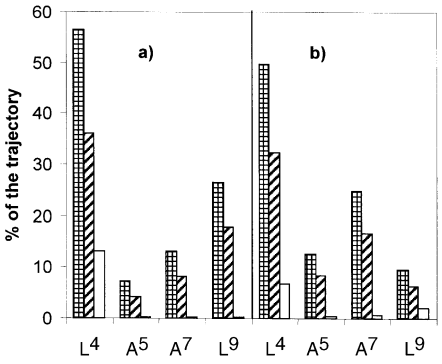

Figure 10. The PPII (⊞, ±30°; ▨, ±22°) and parallel β-sheet content (□, ±22°) as a percentage of the trajectory at a) 278 K and b) 300 K.

A deeper analysis was carried out in the case of non-glycine residues (L^4, A^5, A^7, L^9) for which characteristic free motions were observed on the Ramachandran plot, often exploring the PPII region. The Ramachandran plots of residues 2–10 at both temperatures are shown in Figure 11.

These results seem to indicate a higher propensity to form the PPII conformation for residues in the middle of the peptide, around the A^5 and A^7 residues, at 278 K. In contrast, a higher PPII propensity was observed for L^4 and L^9 at 300 K. These results, on the whole, demonstrate that both trajectories have similar characteristics and that only the distribution of PPII residues differs with changes in the temperature: when the thermal agitation is lower (at 278 K), the PPII conformation is concentrated in the middle of the peptide chain, whereas the PPII conformation is more likely to occur at the extremities of the peptide when the temperature is higher.

Identification of β turns was performed by using the classical definition of Thornton et al.,[21] with a Cα(*i*)–Cα(*i*+3) distance lower than 7 Å and with turn types defined by the simultaneous orientation of both the *i*+1 and *i*+2 residues. Once more, a similar behaviour was observed for the peptide at 300 and 278 K (Figure 12a). At 300 K, the most occurring turn span was the ^{4}LAGA^7 residues (25% of the whole trajectory) corresponding to a type IV (undefined) turn, with a clear tendency for types I and VIII. Turns are dynamic structures that can continuously interchange, and types I and VIII in this case only differ by the orientation of the *i*+2 residue. The same turn was significantly observed at 278 K (17%) but was mostly undefined (type IV). At this temperature, the ^{5}AGAG^8 (type IV) turn was the most probable (23%) and showed a trend towards a type II′ turn.

Only two hydrogen-bonded turns were observed: the ^{5}AGAG^8 at 278 K and ^{1}PGGL^4 at 300 K. Both of them are type IV β turns (Figure 12b). In any case, no significant hydrogen bonds were found as far as the stabilisation of the turns is concerned. Peptides containing several glycine residues can easily adopt both PPII and turn conformations; the high flexibility of such peptides, caused by glycine residues, also allows a very fast turn–turn exchange (by changing types and/or positions along the chain) and turn–PPII conformation exchange, all in equilibrium. Only the PPII/turns ratio and the distribution along the chain may change as a function of temperature; the general behaviour of the peptide in solution remains about the same.

Supramolecular organisation: Figure 13a shows a TEM micrograph of the **EX5** peptide where a network of filaments and of bundles of laterally aligned filaments is evident. The diameters are in the range 9.5–16.5 nm. This supramolecular organisation is also found in elastin. More generally, fibrillar assemblies are common for polypeptide chains structured in the PPII conformation and/or with β turns.[10,22] Figure 13b shows a different morphology of kinked ribbons that have previously been found for elastin-based biopolymers.[23] A detailed study, by TEM and rotary-shadowing TEM, of **EX5** and other human tropoelastin exon peptides in order to get a complete picture of their self-assembly properties will be published elsewhere.

Discussion

The experimental results (NMR and CD spectroscopy) coherently indicated the PPII structure as a significant conformer for the **EX5** peptide in aqueous solution, while in the less polar solvent, TFE, β turns were identified. In particular, in aqueous solution, NOE ratio analysis gave evidence for a stretch of PPII structure for the sequence ^{3}GLAG^6. This finding is corroborated by the results of the molecular dynamics simulation that show a quick interconversion between extended (mainly PPII) and folded (β turn) conformations.

The addition of a chaotropic agent, such as urea, which is denaturating for globular proteins because it interferes with the protein intramolecular hydrogen bonds, results in stabilisation of the PPII conformation. Also, for the **EX5** peptide, urea led to the PPII conformation being favoured (as determined by CD and NMR spectroscopy), probably because it forms hydrogen bonds with the backbone. Indeed, the PPII conformation is an extremely solvent-exposed conformation which, according to previous molecular dynamics calculations,[24] is particularly stabilised by water. The water molecules form a hydrogen-bond network with the backbone. The role of urea in additionally stabilising the PPII conformation is probably due to an even more efficient hydrogen-bond interaction formed in place of the water molecules.

The finding of significant amounts of PPII structure for this peptide is not surprising if we consider the proposal that the PPII conformation is a significant contributor to the conformational ensemble in flexible, unordered polypeptides.[11,12] In fact, a new picture of unordered conformations recently emerged from the analysis of unfolded proteins and unordered peptides that points to more restricted conforma-

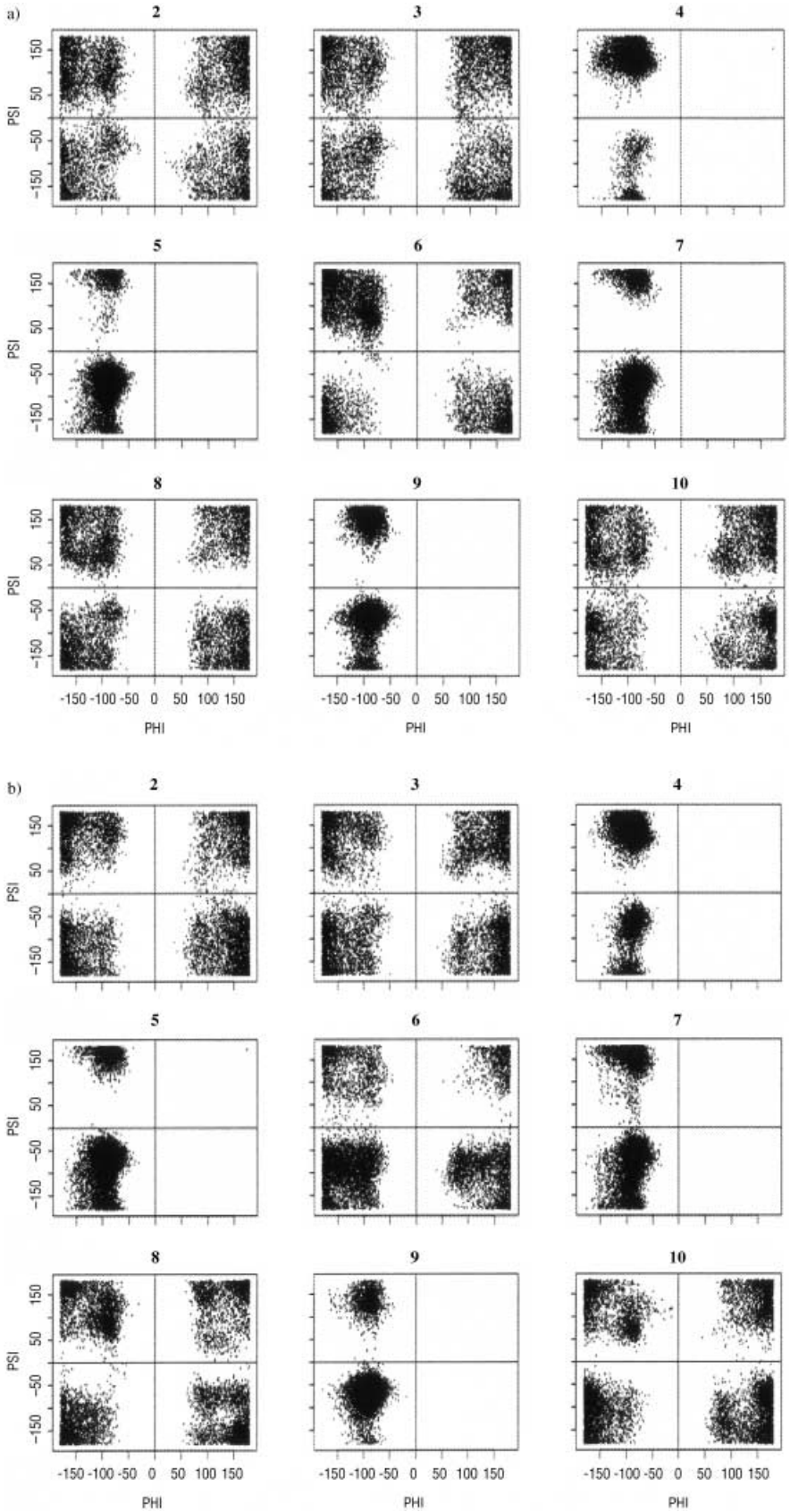

Figure 11. Ramachandran plots for residues 2–10 of the **EX5** peptide during the molecular dynamics trajectories at a) 300 K and b) 278 K.

tional sampling in the unordered state with a limited range of accessible conformations. Many spectroscopy studies (CD, VCD, and ROA spectroscopy) and molecular dynamics simulations propose that short stretches of 2–5 residues in the PPII structure are the most favoured conformation in solution for unordered and unfolded peptides. Furthermore, the unordered form is constituted not only of PPII helical stretches with three or more residues but also by conformers in which individual residues are in the PPII form, or β regions of the Ramachandran map, and rapidly interconverting. There is some evidence indicating that the PPII conformation is at the global energy minimum for peptides in water.[11]

A general caveat is that the above considerations are not valid for all sequences of short peptides. The common view that short peptides are flexible and "unordered" must be taken with caution. In fact, it has been shown that ordered conformations are present even in very short peptides such as tripeptides.[25] Accordingly, the results found for the **EX5** peptide are peculiar to that particular sequence and, per se, could be suggestive of a similar conformation when that sequence is part of the entire protein. Of course, this has to be fully demonstrated in future work by studying, where possible, two or three contiguous exon peptide sequences.

Due to the characteristics of CHARMM algorithm we used, only molecular dynamics trajectories in water were accessible. Accordingly, a direct comparison between the molecular dynamics and spectroscopy results is only possible for the aqueous solvent. Apparently, no evidence of turns was found by CD or NMR spectroscopy in the aqueous solvent, whereas the molecular dynamics simula-

300K

a)

1	2	3	4	5	6	7	8	9	10	11
P	G	G	L	A	G	A	G	L	G	A

5%
< 5%
5%
25%
< 5%
10%
10%
10%

Turn Types						
I	I'	II	II'	VIII	IV	
3			7		90	PGGL
				20	80	GGLA
2				1	97	GLAG
5				20	75	LAGA
			1		99	AGAG
5				15	80	GAGL
	1		1	8	90	AGLG
5				10	85	GLGA

278K

1	2	3	4	5	6	7	8	9	10	11
P	G	G	L	A	G	A	G	L	G	A

0%
0%
< 10%
17%
23%
10%
10%
< 10%

Turn Types						
I	I'	II	II'	VIII	IV	
						PGGL
						GGLA
2				3	95	GLAG
				1	99	LAGA
			9	1	90	AGAG
1				34	65	GAGL
			2		98	AGLG
2				13	85	GLGA

b)

	NH	O	Occurrence	% trajectory
H-BONDS	G^2	L^4	1.00	0.25
	G^3	P^1	8.00	2.00
	L^4	P^1	4.00	1.00
	L^4	G^2	5.00	1.25
	A^5	G^3	1.00	0.25
	G^6	L^4	1.00	0.25
	A^7	G^2	2.00	0.50
	A^7	A^5	21.00	5.25
	L^9	A^7	7.00	1.75
	G^{10}	G^8	1.00	0.25
	A^{11}	L^9	14.00	3.50

278K

	NH	O	Occurrence	% trajectory
H-BONDS	G^3	P^1	6.00	1.23
	G^3	A^1	2.00	0.41
	L^4	G^2	2.00	0.41
	L^4	G^{10}	1.00	0.21
	A^5	G^3	3.00	0.62
	A^7	A^5	9.00	1.85
	G^8	A^5	3.00	0.62
	G^8	G^6	5.00	1.03
	L^9	A^7	17.00	3.50
	A^{11}	L^9	10.00	2.06

Figure 12. a) Extraction of β turns during the trajectories of the **EX5** peptide at 300 K and 278 K. For each tetrapeptide the percentage of turn observed during the trajectory as well as its corresponding type(s) are reported; b) occurrence of hydrogen bonds and their percentages during the trajectories.

tions provided evidence for the presence of several turns, mainly type IV β turns. According to Wilmot and Thornton,[26] the type IV β turn is un undefined β turn that is presumably devoid of intramolecular hydrogen bonds and, therefore, is a rather labile structure in the aqueous solvent. Indeed, in TFE, evidence was found for the presence of two β turns spanning the sequences ^{3}GLAG^6 and ^{4}LAGA^7 (these are also found in MD simulations), turns that are evidently transient in water but stabilised in the organic solvent. As the polarity of the physiological microenvironment might be intermediate between water and TFE, we can suggest a conformational equilibrium for elastin that comprises the PPII, β turn and unordered conformations. This is corroborated by the presence of an isoelliptic point in the CD spectra

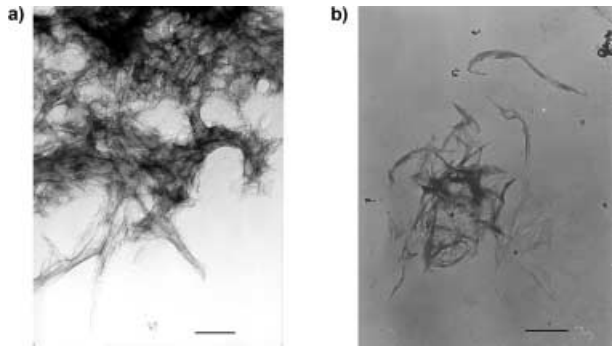

Figure 13. TEM micrographs of the **EX5** peptide. Bars represent 500 nm. The two different morphologies refer to the same sample as found on the same grid.

(Figure 1a) that is a clear indication of transconformational equilibria. Furthermore, this phenomenon has already been seen for many exon peptides of elastin.[3]

An important point to be emphasised is that, according to both NMR data and molecular dynamics simulations, the β turns are characterised by an extensive sliding, that is, they are interconverting with each other along the chain. This phenomenon, previously revealed by Tamburro and coworkers,[9,27] is considered as one of the possible sources of the entropy in the relaxed state of elastin.[28] Recently, analogously to the conclusions drawn for elastin and lamprin, an equilibrium comprising the PPII, β turn and unordered conformations has been suggested as a major structural feature for elastic segments of titin.[29]

Taken together, the results described in the present paper validate the reductionist approach that we used for understanding the complete structure–function relationships of elastin.[3] A further validation of this approach can be provided by the observation that even a short polypeptide, like the **EX5** peptide, has been found to adopt fibrillar supramolecular structures similar to those typical of elastin.

Furthermore, both experimental results and theoretical simulations indicated conformational equilibria of the extended/folded type that are the main source of entropy in the molecular model of elastin elasticity as described by Debelle and Tamburro.[28] It is to be noted that "extended" refers here to subequilibria between conformer populations belonging to the north-west region of the Ramachandran map. In its turn, "folded" means "sliding" β turn equilibria and also equilibria between different β turns.

In conclusion, as far as the **EX5** peptide can be a representative conformational building block of elastin, the results obtained in this work give an additional detailed insight into the molecular mechanism of elasticity of elastin.

Experimental Section

Peptide synthesis and purification: The unblocked peptides **EX5** and **EX5–6** were synthesised by using an automatic Applied Biosystem model 431A synthesiser. Solid-phase peptide synthesis is based on sequential addition of Fmoc-protected amino acid residues, through the C terminus of the residue, to an insoluble polymeric support. After removal of the Fmoc protecting group, the next protected amino acid is added by using the coupling-reagent combination DCC/HOBT. The coupling steps involving prolyl residues were carried out twice in order to overcome the problem of low yields that occurs because of the well-known steric hindrance of proline. The Fmoc-protected amino acids were purchased from Novabiochem (Laufelfingen, Switzerland) and from Inbios (Pozzuoli, Italy). The cleavage of peptides from the resin was achieved by using an aqueous mixture of 95% TFA. After about two hours the reaction mixture was filtered, concentrated and precipitated with diethyl ether. The *N*-acetylated **EX5** peptide was synthesised by dissolving the **EX5** peptide (24 mmmol) in acetic anhydride (5 mL) and DIPEA (1 mL). The reaction mixture was stirred for one hour, then a precipitate was obtained by adding diethyl ether. The peptides were obtained in pure form after lyophilisation and purification by semipreparative and preparative reversed-phase HPLC. A binary gradient was used and the solvents were H_2O (0.1% TFA) and CH_3CN (0.1% TFA). The purity of peptides was assessed by MALDI-TOF spectrometry.

CD spectroscopy: CD spectra of 0.1 mg mL^{-1} solutions of peptides were recorded on a Jasco J600 CD spectropolarimeter with a Haake waterbath as a temperature controller and generally by using a cell with a 1-mm optical pathlength. The usual precautions to avoid optical artifacts and to verify the absence of light scattering were taken. Usually, 16 scans were acquired in the range 190–250 nm at a temperature of 273, 289 or 343 K by taking points every 0.2 nm, with a scan rate of 100 nm min^{-1}, an integration time of 2 s and a bandwidth of 1 nm. The same parameters were adopted for CD spectra of 0.1 mg mL^{-1} solutions of peptide in 3 M and 1 M urea aqueous solutions.

NMR spectroscopy: All ^{1}H NMR spectroscopy experiments were performed on a Varian Unity INOVA 500 MHz spectrometer equipped with a 5-mm triple-resonance probe and *z*-axial gradients. The purified peptides were dissolved in H_2O/D_2O (90:10, 700 μL) or [D_3]TFE/H_2O (70:30, 700 μL), containing 0.1 mM of DSS as the internal reference standard at $\delta = 0$ ppm. 3 M [D_4]urea was added to the sample in water, for experiments with the PPII-inducing agent. Usually 1–3 mM peptide solutions were used. Under these conditions no concentration-dependent variations were observed. One-dimensional ^{1}H NMR spectra were acquired with 32 K data points and a spectral width of 6000 Hz. The residual HDO signal was suppressed by presaturation during the relaxation time. TOCSY spectra were collected by using 256 *t*1 increments and a spectral width of 6000 Hz in both dimensions.[30] Relaxation delays were set to 2.5 s and the spinlock (MLEV-17) mixing time was 80 ms. Shifted sine bell squared weighting and zero filling to 2 K × 2 K was applied before Fourier transformation. ROESY[31] data were collected in essentially the same manner as TOCSY data, with mixing times ranging from 150–250 ms. The residual HDO signal was suppressed by presaturation during the relaxation time for TOCSY experiments, while for ROESY experiments a Watergate pulse sequence was performed prior to acquisition.[32] Amide-proton temperature coefficients were measured from 1D ^{1}H NMR spectra recorded in 5 K increments from 278–318 K. Spectra were processed and analysed by VNMR Version 6.1C software (Varian, Palo Alto, CA).

Molecular dynamics simulations: Molecular dynamics simulations were performed by using CHARMM software (Version c27b3), with potential set 22, on a SUNFIRE 6800 24X supercomputer.[33] For the starting structure, the **EX5** peptide was built into its polyproline II conformation (Figure 4) by using the canonical dihedral angle values for this conformation ($\varphi = -75$; $\psi = +145$). **EX5** was then embedded in a TIP3P cubic water box, thereby warranting a density near the unity at around room temperature.[34] Periodic boundary conditions were applied during all stages of the simulation by using the IMAGE facility of the CHARMM software. Briefly, the solvent box was duplicated along all spatial directions to simulate an environment constituted by an infinite number of solvent molecules. The entire solute–solvent system was then minimised, by applying several successive minimisation cycles, firstly with the peptide atoms fixed and secondly with the solvent atoms fixed. In a final cycle, all constraints were removed. The system was then slowly heated to 300 K or 278 K, with the temperature increasing by 1 K steps every 50 fs. Equilibration was performed in two stages, either rescaling or assigning velocities, for a total time of 40 ps. The Verlet algorithm was used, and the integration step was 1 fs.[35] For the nonbonded terms, we used a protocol that has been shown to be efficient and accurate in cut-off-based simulations, with a force-switching function for electrostatics

and a potential-shifting function for van der Waals interactions, both applied between 10–12 Å. Analyses of the trajectories were performed by coupling home-made programs and the CHARMM program facility. For clustering analysis, we extracted conformations at 10 ps intervals; the corresponding snapshots were then fitted on their heavy atoms and an RMSD value was stored for each pair of conformations. A hierarchical clustering was then performed on the resulting 2D RMSD plot, by grouping iteratively the two most-related conformations and to obtain the desired number of representative families of conformations (clusters). The choice of ten conformations per cluster was done in order to give the maximum value of RMSD (for the backbone atoms) between 1.0–1.7 Å. For each cluster, the corresponding members were visualised, and the mean structure was calculated and graphed with the MolMol program.

Transmission electron microscopy measurements: The synthetic peptide was dissolved in water at a concentration of 1 mg mL^{-1}. The sample was investigated at room temperature. Specimens were prepared by putting the peptide solution (about 10 μL) on a electron microscopy copper grid (3 mm) previously covered by formvar and evaporated carbon. The grid was rinsed with water (20 drops), and then stained with a 12 mM uranyl acetate solution (10 drops, pH 3.5). The staining solution and the aqueous solvent were carefully kept at the same temperature as the sample. After air drying the specimen was observed by electron microscopy under high vacuum.Specimens were examined on a Zeiss EM 10 electron microscope at an accelerating voltage of 60 kV.

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