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ORIGINAL ARTICLE

Proline-glutamate chimera's side chain conformation directs the type of β-hairpin structure

Jyotirmoy Maity · Ulla I. M. Gerling · Stella Vukelić · Andreas Schäfer · Beate Koksch

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Abstract Our aim was to study the impact of two proline chimeras, containing a glutamic acid side chain in *cis*- or *trans*-configuration, on secondary structure formation. We further investigated to what extent the configuration of the side chain contributes to the overall peptide conformation. We used a 10 residue peptide (IYSN**P**DGTWT) that forms a β-hairpin in water. The turn-forming proline was substituted with either a *cis*- or *trans*-proline-glutamic acid chimera, resulting in the peptides IYSN$\mathbf{P}^{cis\text{-}\mathbf{E}}$DGTWT (*P1_*$P^{cis\text{-}E}$) and IYSN$\mathbf{P}^{trans\text{-}\mathbf{E}}$DGTWT (*P1_*$P^{trans\text{-}E}$). We studied the conformation of the modified peptides by circular dichroism (CD) and NMR-spectroscopy, and SEC/static light scattering (SLS) analysis. NMR analysis reveals that the modified peptides maintain the β-hairpin conformation in aqueous solution. At 5 °C and pH 4.3, the peptide (*P1_*$P^{cis\text{-}E}$) was found to adopt two coexisting β-hairpin conformations (2:2 β-hairpin, and 3:5 β-hairpin). In contrast to that, the peptide (*P1_*$P^{trans\text{-}E}$) adopts a 2:2 β-hairpin that exists in equilibrium with a 4:4 β-hairpin conformation. The adoption of ordered β-hairpin structures for both modified peptides could be confirmed by CD spectroscopy, while SEC/SLS analysis showed a monomeric oligomerization state for all three investigated peptides. With the combination of several NMR methods, we were able to elucidate that even small alterations in the side chain conformation of the proline-glutamate chimera (*cis* or *trans*) can significantly influence the conformation of the adopted β-hairpin.

Keywords Proline chimera · β-Hairpin conformation · β-Turn · Conformational analysis · CD · NMR

Abbreviations

CD	Circular dichroism
HPLC	High performance liquid chromatography
Fmoc	Fluorenylmethoxy carbonyl
AU	Analytical ultracentrifugation
SEC	Size exclusion chromatography
MD	Molecular dynamics
DIC	Diisopropylcarbodiimide
HOBT	1-Hydroxybenzotriazole
HOAT	1-Hydroxy-7-azabenzotriazole
TFA	Trifluoroacetic acid
TIS	Triisopropylsilane
$P^{cis\text{-}E}$	*cis*-Proline glutamate chimera
$P^{trans\text{-}E}$	*trans*-proline glutamate chimera
NMR	Nuclear magnetic resonance
^{1}H NMR	Proton nuclear magnetic resonance
COSY	Correlation spectroscopy
TOCSY	Total correlation spectroscopy
NOE	Nuclear Overhauser effect
NOESY	Nuclear Overhauser effect spectroscopy
ROESY	Rotating frame nuclear Overhauser effect spectroscopy
SPPS	Solid phase peptide synthesis

J. Maity and U. I. M. Gerling contributed equally.

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J. Maity · U. I. M. Gerling · S. Vukelić · B. Koksch (✉)
Institut für Chemie und Biochemie, Freie Universität Berlin, Takustrasse 3, 14195 Berlin, Germany
e-mail: Beate.Koksch@fu-berlin.de

A. Schäfer
Gerätezentrum BioSupraMol, Freie Universität Berlin, Takustrasse 3, 14195 Berlin, Germany

Introduction

Secondary structure formation plays a crucial role in protein folding (Kim and Baldwin 1990; Dyson and Wright 1991, 1993; Munoz et al. 1997). To better understand this process, short segments of proteins have been studied to explore their ability to form α-helical (Ishikawa et al. 1996; Pagel et al. 2006; Horne et al. 2007, 2009; Price et al. 2010; Rezaei Araghi et al. 2011) or β-hairpin structures (Blanco et al. 1993, 1994; Searle et al. 1995; Ramirez-Alvarado et al. 1996; de Alba et al. 1997; Robinson 2008) in aqueous solution. Among the secondary structures that naturally occurring proteins can adopt, β-hairpin conformations are important folding motifs that play a key role in biomolecular recognition. For example, antibodies and T cell receptors contain β-hairpin motifs. Numerous short peptides that form β-hairpin structures in aqueous solution have been investigated, in the last two decades. Scaffolds of β-hairpin-like folding motifs have been investigated by mimetic design (Robinson 2008). With the aim of designing an ideal β-hairpin model the conformational properties of non-natural amino acids have been studied in β-hairpin conformations (Haque et al. 1994; Haque et al. 1996). Also non-peptidic scaffolds (Nesloney and Kelly 1996; Wu et al. 2008; Loughlin et al. 2010), and α/β peptides (Lengyel et al. 2011) have been investigated. Especially, non-natural building blocks based on proline have been incorporated into turn regions of β-hairpins (Mothes et al. 2010; Guitot et al. 2011). Due to their rigid backbone, proline residues induce turns or bends within peptide structures. Thus, prolines occur often at tight turn conformations of β-hairpins, and have been incorporated into sequences that usually do not contain this residue, to stabilize the β-turn region of the structures (Wilmot and Thornton 1988; MacArthur and Thornton 1991). Proline was also used in systematic scans to verify the internal peptide architecture of amyloid-forming peptides (Williams et al. 2004; Gerling et al. 2010). Diproline segments are useful templates to devise well-structured peptide sequences, whereas heterochiral diproline segments have been found to adopt β-turn conformations (Aubry et al. 1985; Chatterjee et al. 2008).

To combine the conformational rigidity of proline with the functionality of other natural amino acids, several substituted proline derivatives have been synthesized (Karoyan et al. 2003; Oba et al. 2009; Delaye et al. 2010; Fatas et al. 2012). In a previous study, we reported the synthesis of two 3-substituted proline glutamic acid chimeras. Starting from *trans*-4-hydroxy proline, a straightforward synthesis yielded two enantiomerically pure forms, of Fmoc protected 3-substituted *cis*- and *trans*-proline-glutamic acid, which were suitable for incorporation into peptide sequences via solid phase peptide synthesis (SPPS) (Maity et al. 2012).

Here, we present the incorporation of both proline-glutamic acid chimeras into a short β-hairpin sequence and the comprehensive study of the structural alteration that occur upon the substitution of a turn-contributing proline residue. The utilized short β-hairpin contains a proline residue as one of the four turn-contributing residues. Our aim was to study the impact of an additional side chain on the conformation of the proline ring. Furthermore, we wanted to investigate to what extent the configuration of the proline side chain (*cis* or *trans*) influences the overall conformation of the hairpin. Following the maximum probability of the four residue sequence NPDG (Asn-Pro-Asp-Gly) to form a regular tight β-turn, the natural sequences of the α-amylase inhibitor *Tendamistat* were modified and found to form a 2:2 β-hairpin type in aqueous solution (Blanco et al. 1993). Introducing the same four residue sequence into a short linear peptide derived from the N-terminal sequence of ubiquitin led to a non-native β-hairpin structure (Searle et al. 1995). Fully designed peptides that contain the NPDG-turn sequence and differ only with regard to one residue in the sequence have been found to adopt different β-hairpin structures that exist in equilibrium with one another in aqueous solution. Altered turn conformations of the studied sequences result in different H-bond patterns in the several types of β-hairpin adopted (Fig. 1) (de Alba et al. 1997). The nomenclature of β-hairpins according to the turn conformation they adopt was introduced by (Sibanda et al. 1989). The turn type is defined by the number of H-bonds that connect turn-contributing residues and differentiates residues of the turn sequence that form H-bonds with the opposing β-strand. The model peptide IYSNPDGTWT was found to adopt two types of β-hairpins which coexist in aqueous solution (de Alba et al. 1996). The predominant form (3:5 β-hairpin) contains a type I β-turn, while the less abundant conformation (4:4 β-hairpin) features a G1 β-bulge (Fig. 1). Substitution of the turn-inducing Pro residue by Ser (P5-S) does not significantly influence the conformation; the same ratio of β-hairpin conformations (3:5 β-hairpin and 4:4 β-hairpin) was observed for the Ser-containing peptide.

The Pro-containing sequence was used as a model peptide for the current conformational investigations. Pro 5 of the sequence IYSN**P**DGTWT (*P1_P*) is part of the turn moiety and was replaced by either *cis*-proline-glutamic acid chimera ($P^{cis\text{-}E}$) or *trans*-proline-glutamic acid chimera ($P^{trans\text{-}E}$). The consequences of the substitution on the turn conformation were studied with a combination of several analytical techniques. The peptides (*P1_P*, *P1_P*$^{cis\text{-}E}$, and *P1_P*$^{trans\text{-}E}$) were manually synthesized using the Fmoc strategy. The conformations of the resulting hairpins were studied with the help of one-dimensional- and two-dimensional- NMR analysis and circular dichroism (CD) spectroscopy. The oligomerization

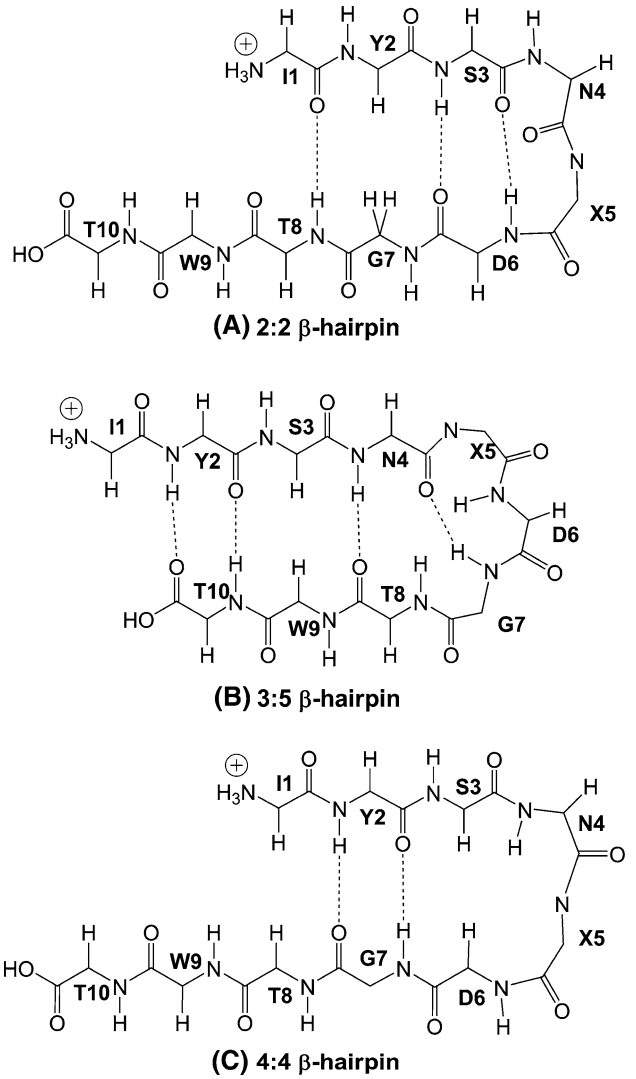

Fig. 1 Schematic representation of the backbone conformations of different β-hairpins. Position X5 represents the proline residue that was replaced with several amino acids in previous studies (de Alba et al. 1997). The *dotted lines* indicate the hydrogen bond pattern of the backbone that differs for the presented types of β-hairpins

of the hairpins was determined with a combination of size exclusion chromatography (SEC) and static light scattering (SLS) analysis. Introducing an alkyl chain at C3 of the pyrrolidine ring of proline has been found to influence the ring conformation to allow a conformation in which an intramolecular hydrogen bond between the nitrogen atom of the ring and the carboxyl group can be formed (Mezzache et al. 2003). The incorporation of electron-withdrawing residues in the pyrrolidine residue has also been found to affect the ring puckering (Milner-White et al. 1992). As described below, the incorporation of a side chain functionality at C3 of the pyrrolidine ring has a strong influence on ring conformation. The altered ring conformation, on the other hand, directly affects the type of β-hairpin formed by the peptide.

Materials and methods

Peptide synthesis and purification

Peptides were manually synthesized according to standard Fmoc strategy. A NovaSyn®TGA-resin was pre-loaded with the first amino acid Fmoc-Thr-(^{t}Bu)-OH (0.35 mmol/g, 0.05 mmol scale). C-terminal activation was carried out using 4 eq DIC/HOBt. The Fmoc-protected proline chimera and the amino acid directly succeeding it in the sequence were coupled manually using 2 eq amino acid and HOAt/DIC activation with elongated coupling times. Gly (7) was introduced carrying a DMB protecting group on the amino function to avoid aspartimide formation with the neighboring Asp (6) under basic conditions during Fmoc deprotection steps (Coin et al. 2007). Fmoc deprotection was achieved by treatment of the resin with 20 % piperidine in DMF. Peptides were cleaved from the resin with 2 mL of a solution containing triisopropylsilane (10 %, w/v), water (1 %, w/v), and trifluoroacetic acid (TFA) (89 %, w/v). To cleave the ethyl ester of the proline chimera side chain, the crude peptide was stirred in 1 M LiOH solution over night. The peptide was precipitated in cold ether, and purified with the help of preparative reversed-phase high-performance liquid chromatography (HPLC) using linear CH_3CN/H_2O gradients containing 0.1 % TFA, and identified by ESI-ToF MS using an Agilent 6210 ESI-ToF LC–MS spectrometer (Agilent Technologies Inc., Santa Clara, CA, USA).

^{1}H-NMR spectroscopy

^{1}H-NMR and two-dimensional NMR spectra were recorded in 0.6 mL of H_2O/D_2O (9:1 ratio by volume) or in pure D_2O at 275 K on a AVANCE III 700 MHz pulse spectrometer from Bruker equipped with a cryo probe. The peptide solutions of 5 mM each were adjusted to pH 4.3, with minute amounts of HCl or NaOH, or DCl or NaOD, and were not corrected for the isotope effect. One-dimensional spectra were acquired using a 90° pulse of 6.7 µs at 7 W. Phase sensitive two-dimensional correlation spectroscopy (COSY) (Aue et al. 1976), total correlation spectroscopy (TOCSY) (Rance 1987), nuclear Overhauser effect spectroscopy (NOESY) (Jeener et al. 1979; Kumar et al. 1980) and rotating frame nuclear Overhauser effect spectroscopy (ROESY) (Braunschweiler and Ernst 1983; Bothner-By et al. 1984) were recorded with standard techniques using presaturation of the water signal and the time proportional phase incrementation mode. A mixing time of 200 ms was used for NOESY spectra, whereas a spinlock pulse of 200 ms at 24 mW was used for ROESY spectra. TOCSY spectra were recorded using spinlock

pulses of 400 ms. Additional NOESY and ROESY experiments were performed for peptide samples in pure D_2O to facilitate the observation of the H_α–H_α NOE cross peaks close to the water signal. Obtained data were processed using Bruker TOPSPIIN software.

Circular dichroism spectroscopy

The lyophilized peptide was dissolved in H_2O/D_2O (9:1 ratio by volume) and the pH was adjusted to 4.3 to maintain the conditions used during the NMR-measurements. The concentration of peptide stock solutions was determined according to the tyrosine and tryptophan absorbance in 6 M guanidine hydrochloride ($\varepsilon_{Tyr;280\ nm} = 1{,}200\ mol^{-1}cm^{-1}$, $\varepsilon_{Trp;280\ nm} = 5{,}560\ mol^{-1}cm^{-1}$) using a Varian Cary 50 spectrophotometer (Varian Medical Systems, Palo Alto, CA, USA) and PMMA cuvettes (10 mm path length, 1.5 mL, Plastibrand®, VWR International GmbH, Darmstadt, Germany). Stock solutions of 1 mM concentration were prepared and subsequently diluted to give solutions with final concentrations of 100, 300, and 500 µM. CD spectra of the peptide variants were recorded using a Jasco J-810 spectropolarimeter at 5 °C. Quartz cells (0.2 mm path length for 300 and 500 µM samples, and 1.0 mm path length for 100 µM samples) were used throughout. The denaturation experiments were conducted using 1.0 mm Quartz cells. The spectra were the average of three scans, obtained by collecting data from 240 to 195 nm (240–200 nm for the denaturation experiments) at 0.5-nm intervals, 2-nm bandwidth, and 2 s response times. Spectra were background-corrected by subtracting the corresponding H_2O/D_2O spectra.

Size exclusion chromatography/static light scattering

The oligomerization state was determined by applying a combined SEC/SLS analysis. SEC analysis was performed using a WTC-015S5 column (5 µm, 150 Å, 7.8 × 300 mm, Wyatt Technology) connected to a HPLC workstation (La Chrom, VWR, Hitachi, L-2130). The separation was carried out at a velocity of 0.3 ml/min at room temperature in an aqueous buffer consisting of 10 mM sodium phosphate and 150 mM sodium chloride at pH 7.3. Elution of peptide was monitored by UV detection (VWR, Hitachi, L-2400) at 280 nm. The concentration of all injected samples was 150 µM and the sample volume was 100 µl in each case. SLS experiments were performed at room temperature using a Dawn Heleos eight light scattering photometer and an Optilab rEX refractive index detector (Wyatt Technology). Molecular weights were calculated using a dn/dc value of 0.185 mL/g. All data were analyzed with ASTRA software version 5.3.4.20 (Wyatt Technology).

Results and discussion

Peptide design

The peptide sequence IYSN**P**DGTWT (de Alba et al. 1996) had previously been described to be water soluble and non-aggregating in nature. It forms β-hairpin structures almost exclusively in aqueous solution, with the 3:5 β-hairpins predominating over the 4:4 β-hairpins (Fig. 1). Two proline chimeras containing a glutamic acid side chain in either a *cis*- or *trans*-conformation ($P^{cis\text{-}E}$ and $P^{trans\text{-}E}$) at C3 of the pyrrolidine ring (Fig. 2) had been previously synthesized in our group (Maity et al. 2012), and we aimed in this study to investigate their influence on the turn conformation of the adopted β-hairpins.

Structural characterization

^{1}H-NMR analysis

The ^{1}H-NMR spectra of the peptides (*P1_P*$^{cis\text{-}E}$ and *P1_P*$^{trans\text{-}E}$) in aqueous solution were assigned using the standard sequential assignment procedure (Wüthrich et al. 1984; Wüthrich 1986). Therefore, TOCSY was conducted, and NOESY and ROESY were used to gain information

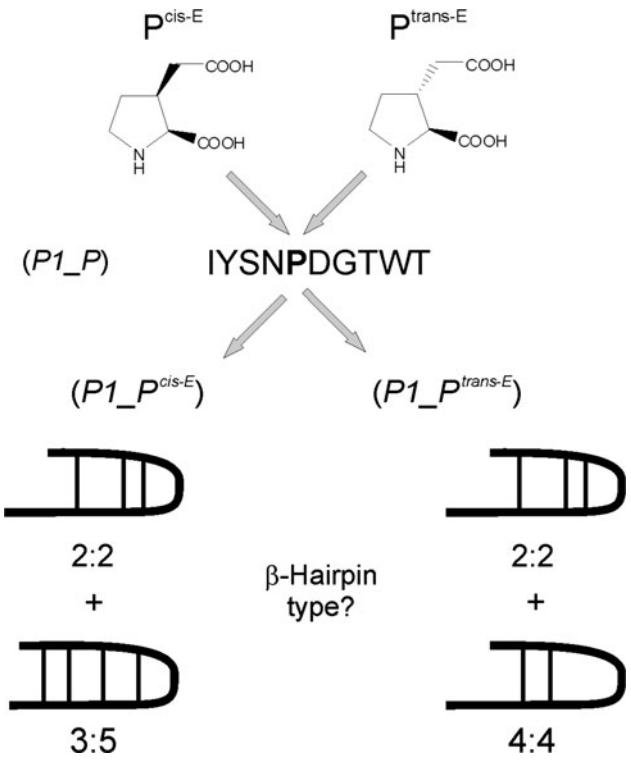

Fig. 2 Schematic illustration of the concept showing the chemical structure of the two proline chimeras and the sequence of the model peptide (*P1_P*). The proline residue 5 that was replaced is highlighted in bold

about the proximity of the protons. NMR analysis of the three peptides revealed that the substitution of Pro in the reference peptide with the two proline chimeras resulted in altered conformations of the formed β-hairpins. The stereochemistry of the glutamic acid side chain at the C3 of the proline chimera is the only distinguishing factor between the peptides (*P1_P*$^{cis\text{-}E}$) and (*P1_P*$^{trans\text{-}E}$) found to have a crucial impact on the shape of the ring conformation and thus on the type of β-hairpin that is formed.

Table 1 Chemical shift assignments of peptide (*P1_P*$^{cis\text{-}E}$)

	NH	H_α	H_β	Other
Ile 1		3.80	1.91	$C_\gamma H$ 1.42, 1.14; $C_{\gamma'}H_3$ 0.93; $C_\delta H_3$ 0.86
Tyr 2	8.50	4.65	2.89, 2.78	$C_\delta H$ 7.07; $C_\varepsilon H$ 6.78
Ser 3	8.18	4.26	3.62, 3.62	
Asn 4	8.44	4.72	2.81, 2.57	$N_\delta H_2$ 7.62, 7.00
Pro$^{cis\text{-}E}$ 5		4.44	2.79	$C_\gamma H$ 2.15, 1.72; $C_{\gamma'}H$ 2.44, 2.37; $C_\delta H$ 3.87, 3.66
Asp 6	8.78	4.59	2.98, 2.91	
Gly 7	7.98	3.85, 3.81		
Thr 8	7.96	4.28	4.11	$C_\gamma H_3$ 1.08
Trp 9	8.35	4.78	3.30, 3.20	$N_{\varepsilon 1}H$ 10.09; $C_{\delta 1}H$ 7.19; $C_{\varepsilon 3}H$ 7.57; $C_{\zeta 3}H$ 7.09; $C_{\eta 2}H$ 7.17; $C_{\zeta 2}H$ 7.44
Thr 10	7.99	4.24	4.20	$C_\gamma H_3$ 1.06

Table 2 Chemical shift assignments of peptide (*P1_P*$^{trans\text{-}E}$)

	NH	H_α	H_β	Other
Ile 1		3.82	1.93	$C_\gamma H$ 1.43, 1.14; $C_{\gamma'}H_3$ 0.94; $C_\delta H_3$ 0.86
Tyr 2	8.52	4.63	2.89, 2.89	$C_\delta H$ 7.07; $C_\varepsilon H$ 6.78
Ser 3	8.16	4.30	3.57, 3.57	
Asn 4	8.49	4.79	2.79, 2.64	$N_\delta H_2$ 7.64, 7.02
Pro$^{trans\text{-}E}$ 5		4.03	2.62	$C_\gamma H$ 2.23, 1.78; $C_{\gamma'}H$ 2.60, 2.48; $C_\delta H$ 3.96, 3.73
Asp 6	8.76	4.61	2.98, 2.92	
Gly 7	8.21	3.93, 3.88		
Thr 8	7.97	4.31	4.14	$C_\gamma H_3$ 1.10
Trp 9	8.40	4.77	3.31, 3.20	$N_{\varepsilon 1}H$ 10.11; $C_{\delta 1}H$ 7.21; $C_{\varepsilon 3}H$ 7.55; $C_{\zeta 3}H$ 7.09; $C_{\eta 2}H$ 7.19; $C_{\zeta 2}H$ 7.44
Thr 10	8.06	4.29	4.22	$C_\gamma H_3$ 1.08

The peak corresponding to $C_\delta H_3$ of the model peptide (*P1_P*) has been used as the reference peak in the ^{1}H-NMR spectra for the peptides containing the proline chimera. The ^{1}H-NMR spectra of the peptides (*P1_P*$^{cis\text{-}E}$ and *P1_P*$^{trans\text{-}E}$) show peaks arising from the *cis*/*trans* equilibrium of the proline imide bond due to the presence of the proline chimera moiety in these peptides (Kang and Young Choi 2004; Thunecke et al. 1996; Sugawara et al. 2001; Ivanova et al. 2010). We assume that the *trans* form of the peptides is the predominating species due to the similar conformation of the X-proline chimera bond with the X-proline bond in the peptides (Zimmerman and Scheraga 1976). The chemical shifts of the proton resonances for the peptides *P1_P*$^{cis\text{-}E}$ and *P1_P*$^{trans\text{-}E}$ in aqueous solution at 5 °C and pH 4.3 are given in Tables 1 and 2, respectively.

The conformational shift of the C_α protons (H_α) can be used to gain insight into the secondary structures of proteins (Wishart et al. 1991; Wüthrich 1986). The deviation of chemical shift [$(\Delta\delta H_\alpha) = \delta H_\alpha$ (observed) − δH_α (random coil)] of the H_α proton from the random coil values is negative in turn or helical regions and positive in β-strand regions. This type of analysis was conducted for all residues of the peptides containing the proline chimeras. Except for the proline chimera moieties, all residues of the peptides followed the same trend in $\Delta\delta H_\alpha$ values. In the case of proline chimera residues, we found that the conformational shift for H_α is positive for the P$^{cis\text{-}E}$ moiety, and negative for the P$^{trans\text{-}E}$ moiety (Fig. 3). Since a negative $\Delta\delta H_\alpha$ value is more suitable for residues at turn regions of β-hairpins, we infer that the negative $\Delta\delta H_\alpha$ value of the P$^{trans\text{-}E}$ residue at the turn position could be linked to the pre-organization of the peptide, which promotes folding into a β-hairpin structure. Although both proline chimera moieties have been found to be suitable for a β-turn position of the hairpin forming peptides, this result indicates that the P$^{trans\text{-}E}$ moiety is presumably better accommodated in the β-turn position than the P$^{cis\text{-}E}$ moiety.

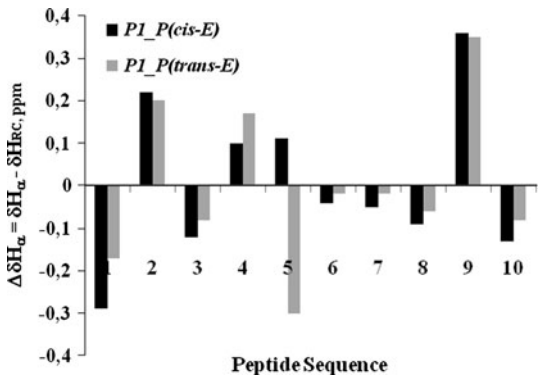

Fig. 3 Histograms of the conformational shift values of the H_α ($\Delta\delta H_\alpha$) relative to random coil values in the peptides *P1_P*$^{cis\text{-}E}$ and *P1_P*$^{trans\text{-}E}$ at pH 4.3 and 5 °C in aqueous solution

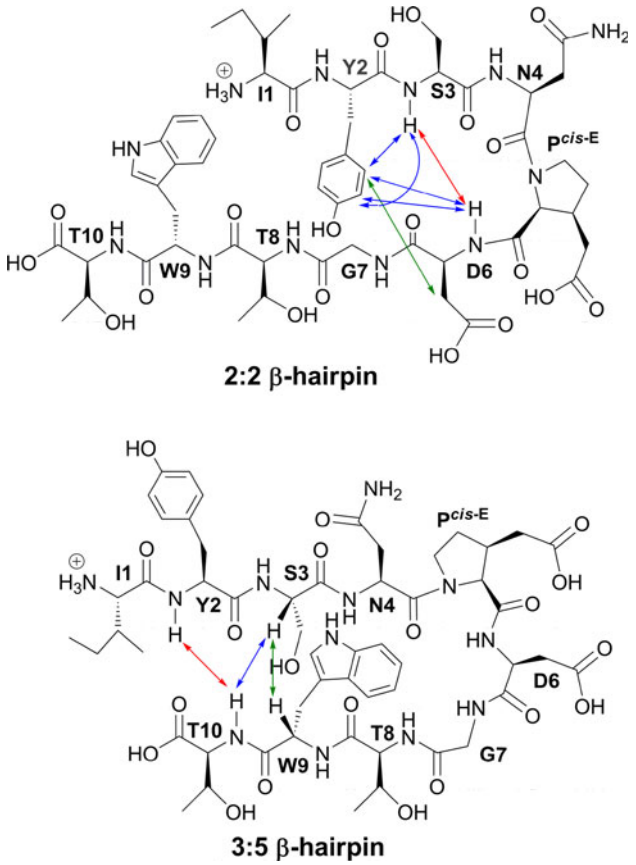

Fig. 4 Cross strand NOEs observed for $P^{cis\text{-}E}$ at 5 °C in aqueous solutions at pH 4.3. NH–NH interactions are highlighted in *red*; HN–CH interactions are indicated by *blue arrows* and CH–CH proximities are given in *green* (color figure online)

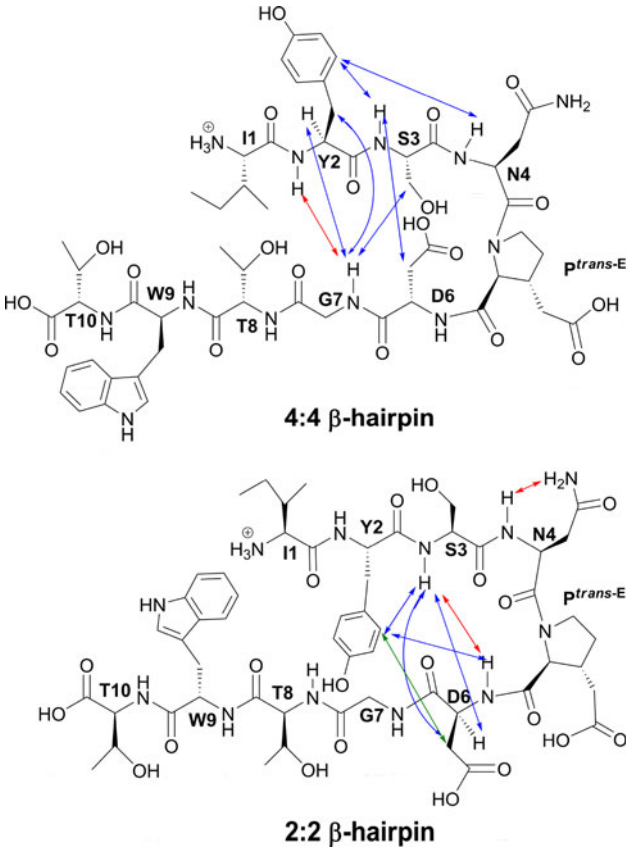

Fig. 5 Cross strand NOEs observed for $P^{trans\text{-}E}$ at 5 °C in aqueous solutions at pH 4.3. NH–NH interactions are highlighted in *red*; HN–CH interactions are indicated by *blue arrows* and CH–CH proximities are given in *green* (color figure online)

Several NMR parameters have been used to identify the β-hairpin structures adopted by $P1\_P^{cis\text{-}E}$ and $P1\_P^{trans\text{-}E}$. The observed NOE patterns of both peptides give information about sequentially non-adjacent residues that are in close proximity, such as interacting residues of opposing β-strands. ROESY spectra were obtained under the same experimental conditions that were used for the NOESY spectra (pH 4.3 and 5 °C) to obtain identical NOE patterns (*Supporting information*). The cross-strand NOEs (NH–NH, NH–H_α, H_α–H_α and involved side chains) that were observed for $P1\_P^{cis\text{-}E}$ and $P1\_P^{trans\text{-}E}$ are illustrated in Figs. 4, 5, respectively.

In the peptide $P1\_P^{cis\text{-}E}$, long range NOEs relating the backbone protons, NH(Y2)–NH(T10), H_α(S3)–NH(T10) and H_α(S3)–H_α(W9) have been found, indicating the formation of a 3:5 β-hairpin. For this type of β-hairpin, the residues (Y2–S3 and W9–T10) face towards each other. In addition to the backbone NOEs, strong NH–NH interactions of S3 and D6 were observed, which indicate the formation of a second conformation, a 2:2 β-hairpin. However, the expected H_α(Y2)–H_α(G7) interaction was not observed. The described interactions reveal that the peptide ($P1\_P^{cis\text{-}E}$) adopts two different types of β-hairpin in aqueous solution, which are in equilibrium with each other. Additional long range NOEs involving side chain protons were also found for peptide $P1\_P^{cis\text{-}E}$ (Fig. 4).

Peptide ($P1\_P^{trans\text{-}E}$) shows long range NOEs between NH(S3) and NH(D6), indicating the formation of a 2:2 β-hairpin. Furthermore, NH–NH NOEs of Y2 and G7 and H_α(Y2)–NH(G7) interactions were observed, which imply the formation of a 4:4 β-hairpin in which the residues Y2

Table 3 $^3J_{NHC\alpha H}$ values for peptides ($P1\_P^{cis\text{-}E}$), and ($P1\_P^{trans\text{-}E}$)

Residue	$P1\_P^{cis\text{-}E}$ $^3J_{NHC\alpha H}$ (Hz)	$P1\_P^{trans\text{-}E}$ $^3J_{NHC\alpha H}$ (Hz)
Tyr 2	7.8	7.3
Ser 3	7.8	7.8
Asn 4	6.9	6.4
Asp 6	6.4	6.4
Gly 7	4.3	5.6
Thr 8	7.8	8.2
Trp 9	7.3	7.4
Thr 10	8.6	8.6

and S3 face towards the residues D6 and G7 (Fig. 5). Thus, the existence of two different β-hairpin structures was also observed for this peptide. However, the 4:4 β-hairpin formed by *P1_P*$^{trans\text{-}E}$ is not exactly the same type that the reference peptide (*P1_P*) adopts. In the case of *P1_P*, the distal strand residues are S3 and T8 and the four turn participating residues are N4, P5, D6 and G7 (de Alba et al. 1996). For *P1_P*$^{trans\text{-}E}$, however, the distal strand residues are Y2 and G7 and the four residues forming the turn are S3, N4, P5 and D6. Side chain protons were also found to be involved in cross-correlation NOEs (Fig. 5). The reference peptide *P1_P* exists in equilibrium between a 3:5 β-hairpin and a 4:4 β-hairpin. *P1_P*$^{cis\text{-}E}$ adopts a 2:2 β-hairpin coexisting with a 3:5 β-hairpin, while *P1_P*$^{trans\text{-}E}$ occurs as a mixture of 2:2 β-hairpin and 4:4 β-hairpin.

Vicinal coupling constant values ($^3J_{NHC\alpha H}$) were calculated to verify β-sheet formation by the peptides (Table 3). Values larger than 7 Hz are characteristic for a β-strand structure.

Almost all residues show values larger than 7 Hz, indicating their existence in β-strand conformations. However, the residues Asn 4, Asp 6, and Gly 7 have

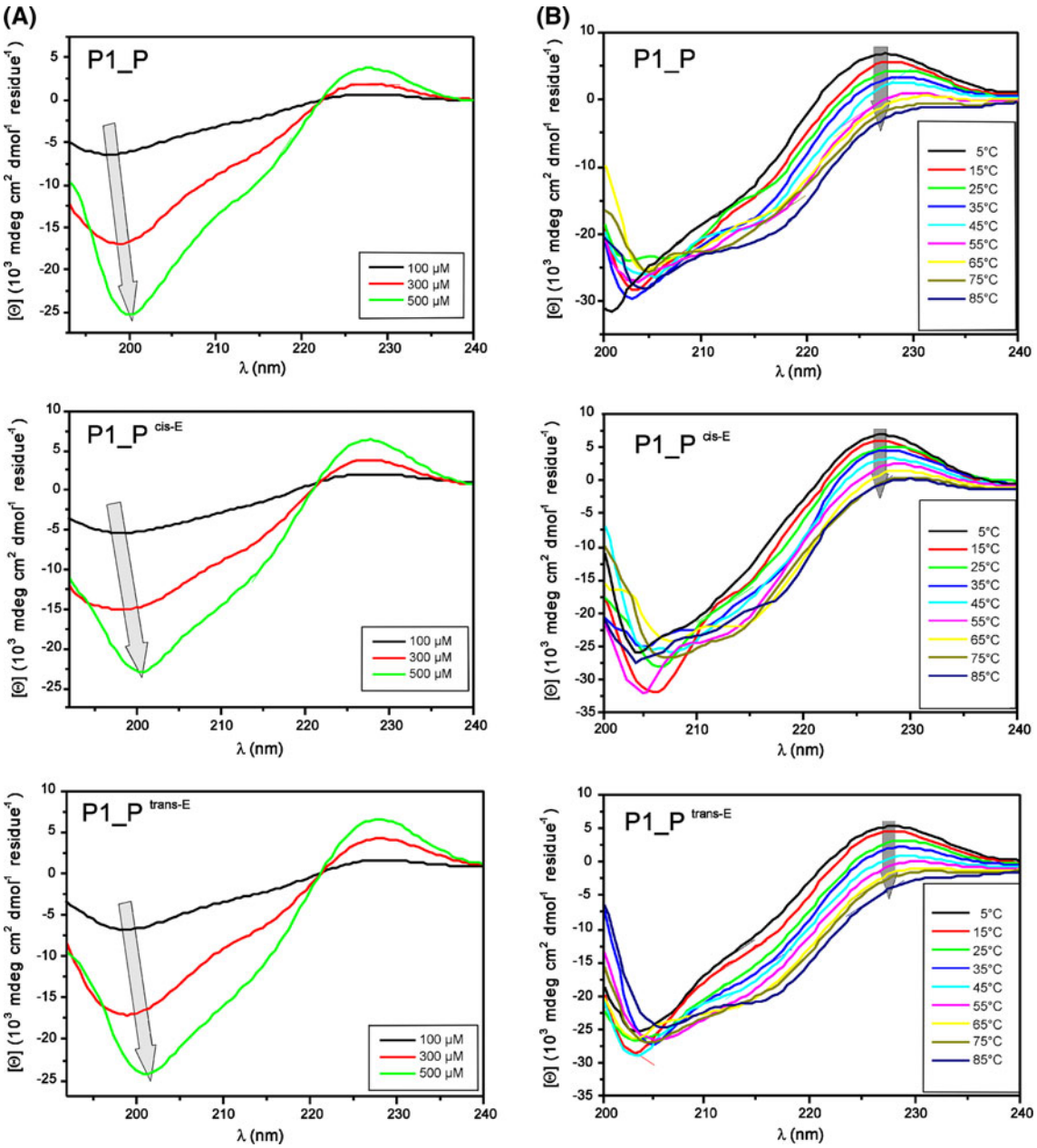

Fig. 6 CD spectra of the *P1_P* peptide variants at pH 4.3. **a** Spectra of different sample concentrations at 5 °C; **b** spectra of 300 μM samples at increasing temperatures

Table 4 Theoretical and experimental determined molecular weights of the peptide variants

Peptide	Theoretical monomer mass (Da)	SEC/SLS determined mass (Da)
P1_P	1,153	1,104 ± 145
P1_P$^{cis\text{-}E}$	1,211	1,188 ± 195
P1_P$^{trans\text{-}E}$	1,211	1,166 ± 130

smaller coupling constants because they are present within the turn structure. For residues near the C- and N-terminus of the peptide, larger $^{3}J_{\mathrm{NHC}\alpha\mathrm{H}}$ values were found, indicating that these residues also adopt β-strand structures.

Circular dichroism

In addition to the NMR analysis, the conformations of the three peptides were investigated by CD spectroscopy. Due to the short sequence of only ten residues, no characteristic β-sheet structures containing a minimum at around 216 nm have been observed. However, some characteristic signatures for secondary structures were observed. With regard to the assumed β-hairpin conformations, elucidated during NMR analysis, only three residues are expected to form β-strands. Thus, the spectra resemble more a random coil structure, although a small minimum at around 216 nm was observed. With increasing peptide concentration, the minimum at 198 nm, being characteristic for random coil structures, is slightly shifted towards 201 nm (Fig. 6a). This indicates a higher amount of order in the structure with increasing peptide concentration. Also, the maxima between 220 and 230 nm observed in all spectra indicate that the aromatic residues (Trp, Tyr) participate in ordered structures (Fernández-Escamilla et al. 2006; Lakshminarayanan et al. 2009). This assumption is further supported by the systematic decrease in this maximum with increasing temperature (Fig. 6b). The obtained results, which are in good agreement with previously published CD spectra (de Alba et al. 1996; Fernández-Escamilla et al. 2006), show that all three peptides adopt similar structures in aqueous solution, and indications for β-hairpin conformation were found in the CD spectra. However, a distinction between the different β-hairpin types that were found with NMR cannot be made with CD spectroscopy alone.

Size exclusion/static light scattering

The oligomerization state of the peptides (*P1_P*), (*P1_P*$^{cis\text{-}E}$), and (*P1_P*$^{trans\text{-}E}$) was investigated with SEC/SLS analysis to test for higher oligomers or aggregates. The obtained results confirm a monomeric oligomerization state for all peptides, which is in good agreement with the results of NMR and CD measurements. Single peaks were found for all three peptides with the help of SEC, and the corresponding molecular weights determined with SLS were comparable to the theoretical monomer masses (Table 4; Fig. 7). The reported values and spectra are the mean of three individual measurements. The results confirm that

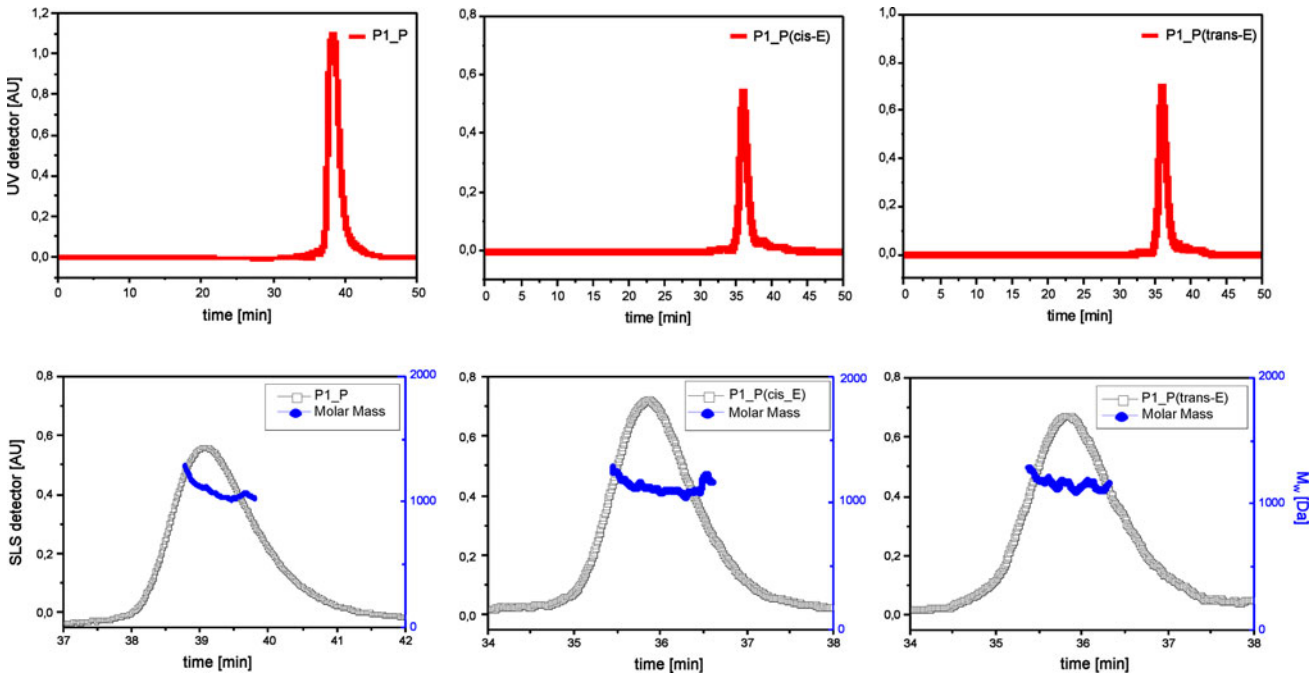

Fig. 7 SEC/SLS chromatograms of peptides (*P1_P*), (*P1_P*$^{cis\text{-}E}$), and (*P1_P*$^{trans\text{-}E}$). Single peaks were detected with UV at 280 nm (*red*). Corresponding rayleigh ratio (*black*) and molar mass distribution (*blue*) indicate monomeric species for all peptide variants (color figure online)

single peptide strands form the observed β-hairpin structures and that the interactions found in the NMR-experiments are of intramolecular nature rather than occurring between two individual hairpins.

Conclusion

The conformational analysis of short β-hairpins containing $P^{cis\text{-}E}$ and $P^{trans\text{-}E}$ in the turn region clearly demonstrates the importance of the proline ring conformation for the formation of β-hairpins. Even small alterations in the sensitive turn region can have large consequences for the whole peptide conformation as the turn type dictates the type of β-hairpin that is formed. The reference peptide (*P1_P*) contains L-proline at position 5, and forms a 3:5 β-hairpin that coexists with a 4:4 β-hairpin in aqueous solution. Substitution of their turn-contributing Pro residue with $P^{cis\text{-}E}$ results in the formation of a 2:2 β-hairpin and a 3:5 β-hairpin. The incorporation of $P^{trans\text{-}E}$ yields an equilibrium between a 2:2 β-hairpin and a 4:4 β-hairpin. These results clearly show the importance of the ring puckering of proline as a key factor in determining the type of β-hairpin that will be formed by the peptide. With two proline chimeras that differ only at a single chiral center, we were able to alter the β-hairpin structures of the corresponding peptides. Our findings show that such proline chimeras can be used to fine-tune the conformation of β-hairpin structures and are therefore likely to become interesting tools for applications in medicinal chemistry and materials sciences.

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Conflict of interest The authors declare that they have no conflict of interest.

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