Research Article

Journal of PeptideScience

Received: 19 November 2009 Revised: 5 August 2010 Accepted: 6 August 2010 Published online in Wiley Online Library: 27 September 2010

(wileyonlinelibrary.com) DOI 10.1002/psc.1296

Tetrad selectivity in polarity-driven switch peptides: the best turn is not always the best nucleation site

Katja Gehenn* and Jennifer Reed

In some naturally occurring protein sequences, an abrupt, concerted refolding from β-sheet to helical conformation occurs when the polarity of the surrounding medium drops below a critical level. This switch-like behaviour was first observed on the HIV-1 envelope glycoprotein gp120, where it plays a crucial role in the efficient binding of gp120 to the T-cell receptor CD4. Previous work had shown that an *N*-terminal amino acid tetrad LPCR and a Trp located 5–20 residues downstream to the tetrad are common motifs in polarity-driven switch peptides. The LPCR tetrad governs the folding of the subsequent residues and acts as a helix initiation site, whereas the Trp is responsible for the cooperative character of the structural change due to multiple, simultaneous interactions of its quadrupole moment with several amino acid residues within the sequences. Here we identify and characterize new families of switch peptides that use different, turn-probable tetrads (LPST and VPSR) as helix initiation sites at the *N*-terminus. We have also been able to demonstrate that some tetrads with extremely high turn probability do not serve as helix initiation sites. Comparison of these with LPCR and the newly discovered tetrads LPST and VPSR has allowed a more comprehensive description of the physico-chemical properties of helix-inducing tetrads. The deeper understanding of the intrinsic properties of switch sequences allows the design of artificial polarity-driven switches, applicable in engineering of, e.g. controllable binding sites in artificial proteins. **Copyright © 2010 European Peptide Society and John Wiley & Sons, Ltd.**

Keywords: switch peptides; peptide folding; helix-inducing tetrads; conformational rigidity; CD-spectroscopy

Introduction

Switch peptides are short sequences that are able to alter their conformation very abruptly and radically in response to a decreasing polarity of the surrounding environment. Typically there is a sharp transition from β-type fold into helical structures when the polarity drops below a particular critical point [1].

This special behaviour was first observed in a 15-residue minidomain derived from the glycoprotein gp120 of HIV-1 (sequence LPCRIKQFINMWQEV), involved in the binding process of HIV-1 to the CD4-receptor of its host cells, the T-lymphocytes [1]. It has been shown that the switch behaviour of this peptide is essential for a proper binding of gp120 to CD4-receptor [2].

In addition to gp120, several naturally occurring switch peptides had been identified and characterized [3–4]. They all had the *N*-terminal amino acid tetrad LPCR in common, serving as a helix initiation site. Deletion of the LPCR tetrad from the original HIV-peptide results in a complete loss of switch activity [4], indicating that the tetrad is an absolutely necessary element for steering the following residues to helical conformation. The tetrad has a high potential for forming β-reverse turns, according to the Chou–Fasman algorithm [5]. NMR measurements in the corresponding hexapeptide (TLPCRI) in isolation revealed the existence of a γ-inverse turn [6]. At the same time, preferential φ angles between the Leu2 and Cys 4 displayed values typical for a 3_{10}-helix. It is thus well suited to form the seed for a series of overlapping turns that – triggered by a particular level of polarity – force the subsequent residues into a conformation that closely resembles a 3_{10}-helix [6]. In fact, the existence of a 3_{10}-helix could be verified for the 15mer in both, NMR measurements and MD simulations [4,7]. Another important characteristic of switch sequences belonging to the LPCR family is a Trp residue 5–20 amino acids downstream from the tetrad. The strong quadrupole/dipole moment of its aromatic ring system interacts with several amino acids within the sequence simultaneously [4,8]. This network of interactions is the reason for the abrupt, cooperative refolding of all switch peptides; the β-structure remains stable until the tendency to adopt the helical form – forced by the LPCR tetrad – becomes stronger than the energy of the quadrupole moment of the tryptophan residue, weakened by the sinking polarity.

The amino acid residues between the tetrad and the tryptophan show both a high and a nearly equal probability of forming helical and β-sheet structures, which is to be expected, as the peptide adopts both structures during the refolding process.

Another crucial parameter for switch sequences is a high side chain interaction index (SCII). The SCII is a mathematical algorithm that takes all positive, advantageous side chain interactions within a peptide into account [3]. Conformational switches are characterized by an SCII value of $\geq$0.5 and the SCII also has an extremely good predictive value for the assessment of local structural stability of peptides [3,9].

TFE is the ideal solvent for folding studies of this kind because of its structure-inducing and structure-stabilising properties, mimicking protein–protein or protein–membrane interactions. The

* Correspondence to: Katja Gehenn, Tallinn Technical University, Institute of Gene Technology, Department of Genomics and Proteomics, Akadeemia tee 15, 12618 Tallinn, Estonia. E-mail: Katja.Gehenn@gmail.com

Biomolecular Interactions, German Cancer Research Center, Im Neuenheimer Feld, D-69120 Heidelberg, Germany

Copyright © 2010 European Peptide Society and John Wiley & Sons, Ltd.

structure-inducing properties of TFE arise from the hydrophobicity of its trifluoromethyl group, which is able to displace the water molecules around the peptide backbone, resulting in a positive effect concerning the total free energy of all interactions [10]. Additionally, its basicity supports the formation of intra-molecular C=O···H–N hydrogen bonds [11] and, thus, helical structures. Usually, titration series of peptides with TFE display a rather linear and gradual rise of helical structures. Switch peptides, however, show a very sharp and abrupt refolding to helical structures at a certain point of polarity, at the cost of the β-type fold.

The aim of the current work was the identification of new families of naturally occurring switch peptides, searching for other tetrads that are able to serve as helix initiation sites for switch peptides. We have identified the tetrads LPST and VPSR, which are now revealed to act as helix initiation sites. Additionally, we have characterized their structural and physico-chemical properties that are responsible for creation of a polarity-driven nucleation site for helix formation.

Material and Methods

Data Bank Searches

In order to search for appropriate peptides for the CD spectroscopic studies, the locally developed program Heidelberg unix sequence analysis resources (HUSAR) was used and SwissProt was chosen as the data bank.

Selection of Peptides

The peptides found were tested for their ability to fulfil the requirements so far known for a polarity-driven switch: the presence of a tetrad at the *N*-terminus with a high probability for forming reverse turns, a Trp residue 5–20 residues downstream to the tetrad, a high and a nearly equal probability for forming helical and β-sheet conformation for the residues between tetrad and tryptophan and a high SCII value.

The calculations for P_α, P_β and P_t were based on the Chou–Fasman algorithm ('Conformation parameters for secondary structure' table) [5]. The SCII algorithm is based on the '20 × 20 matrix of the contact propensities of the side chains in proteins' [12].

The calculations of P_α (probability for helical structures) and P_β (probability for forming β-sheets) were carried out according to the following scheme:

$$(P^1{}_{\alpha/\beta} + P^2{}_{\alpha/\beta} + P^3{}_{\alpha/\beta} + P^4{}_{\alpha/\beta} + P^5{}_{\alpha/\beta})/5 = x_1$$
$$(P^2{}_{\alpha/\beta} + P^3{}_{\alpha/\beta} + P^4{}_{\alpha/\beta} + P^5{}_{\alpha/\beta} + P^6{}_{\alpha/\beta})/5 = x_2 \ldots\ldots\ldots\ldots$$
$$(P^{n-4}{}_{\alpha/\beta} + P^{n-3}{}_{\alpha/\beta} + P^{n-2}{}_{\alpha/\beta} + P^{n-1}{}_{\alpha/\beta} + P^{n}{}_{\alpha/\beta})/5 = x_n$$

according to the values given in the tables 'Conformation parameters for secondary structure' [5], where n = total number of amino acids between the *N*-terminal tetrad and the tryptophan residue.

The total probability of forming helical structures and β-sheets respectively was then:

$$P_{\alpha/\beta\ \mathrm{tot}} = x_1 + x_2 + \cdots + x_n/n$$

where P_α should have a value $\geq$1.03 and $P_\beta \geq 1.05$ (high probability) [13].

Calculation for the probability of reverse turns, P_t, was also performed according to the Chou–Fasman algorithm:

$$P_t = f_1 \times f_2 \times f_3 \times f_4$$

where f_1, f_2, f_3, and f_4 are bend frequencies in the four positions of the β-turn [5].

$P_t > 0.75 \times 10^{-4}$ indicated a turn [5].

The SCII was calculated as follows:

$$\mathrm{SCII} = \frac{1}{n}\left[\left(\frac{\Sigma i_f}{\Sigma i_t}\right)_{a_1} + \left(\frac{\Sigma i_f}{\Sigma i_t}\right)_{a_2} + \cdots + \left(\frac{\Sigma i_f}{\Sigma i_t}\right)_{a_n}\right]$$

where Σ_{if} = total sum of unique advantageous contacts propensities available for amino acid x within the sequence, Σ_{it} = the total sum of advantageous contact propensities available for amino acid x among the other 19 amino acids, $a = 1/2/\ldots/n$th amino acid of the peptide and n = total number of amino acids within the peptide.

For every amino acid in the peptide, all numbers of the remaining residues of the peptide were added together, provided that the value was $\geq$1.0 (= advantageous). Afterwards, this sum was divided by the total sum of possible interactions of the particular amino acids. Thus, for every amino acid of the peptide, one got particular values. These were added again and then divided by the total number of amino acids within the peptide. An SCII value greater than 0.5 was desired, as it has been shown to be an important parameter for polarity-triggered switch peptides [3].

Peptide Synthesis

All peptides used in this work were synthesized by Dr R. Pipkorn (German Cancer Research Center, Service Unit 'Central Peptide Synthesis') using the SPPS method. Activation of the carboxyl group took place by the use of HBTU (*O*-benzotriazole-*N*,*N*,*N*′,*N*′-tetramethyl-uronium-hexafluoro-phosphate). The α-amino group was blocked with Fmoc (fluorenyl-9-methyloxycarbonyl). Peptides were screened for homogeneity by HPLC and MALDI mass spectrometry. Purity of all peptides was above 90%.

CD Measurements

The CD measurements of the peptides were performed by a J-710 spectropolarimeter (Jasco). The apparatus was calibrated with a solution of 0.05% β-androsterone in dioxane.

All measurements were carried out in far-UV range (190–240 nm). The temperature was held constant at 20 °C. Peptides were dissolved to a concentration of 100 µg/ml in TFE, increasing steps from 0 to 80% (90%) and in 10 mM tris-HCl, pH 7.5 as aqueous buffer. As noted before, TFE is the ideal solvent for folding studies of this kind, mimicking protein–protein or protein–membrane interactions due to its apolarity. Additionally, TFE has the advantage that it can be mixed with water easily.

For all spectroscopic examinations, quartz cuvettes were used with a path length of 0.1 cm. CD spectra were recorded using the following parameters: band width 1.0 nm, response 4 s, sensitivity 10 mdeg, data pitch 0.1 nm, scanning speed 5 nm/min, accumulation 4 or 8. All spectra are the average of four repeated measurements in order to improve the signal-to-noise ratio. Baseline was subtracted from every spectrum. The CD spectra were evaluated using the program PEPFIT [14].

Copyright © 2010 European Peptide Society and John Wiley & Sons, Ltd.

Table 1. Probabilities of forming a helix (P_α), β-sheet (P_β), reverse turn (P_t tetrad) and the SCII values of the shown peptides

#	Peptide	P_α	P_β	P_t tetrad	SCII
1	VB16_COWPX (NPGIYILVGTQMRKDMWTTL)	1.08	1.12	4.8×10^{-4}	0.66
2	NPTKIKQFINMWQEV	1.04	1.08	3×10^{-4}	0.53
3	NPCRQAQDVSMWIVG	0.98	1.07	4.8×10^{-4}	0.59
4	A1A1_BUFMA (LPSTLLGIRVAWEDR)	1.08	1.2	1.8×10^{-4}	0.58
5	RPOB_AGRT5 (VPSRMNVGQILETHLAWACA)	1.09	1.08	2×10^{-4}	0.69
6	LPSTVASMIGHWVLM	1.07	1.11	1.8×10^{-4}	0.61
7	VPSRINAQLSLWQMG	1.12	1.14	2×10^{-4}	0.57

NMR Spectroscopic Measurements

Peptide samples were dissolved in 0.5 ml 10 mM potassium phosphate buffer, pH 4.5 H_2O/D_2O (9 : 1) to a final concentration of 5 mM.

Measurements with the peptide TNPCRI were performed in the working group of Prof. Dr Paul Rösch at the Department for Biopolymers at Bayreuth University. Samples were measured at 283 K overnight with a 400-MHz NMR apparatus from Bruker. The spectra were evaluated under assistance of Dr Kristian Schweimer with the programs *NDee, Sparky* and *MestreC*. Structures were calculated with X-PLOR.

The peptide GVPSRM was measured in the group of Dr Michael Sattler at the European Molecular Biology Laboratory (EMBL) in Heidelberg. Measurements were performed with a 600-MHz NMR apparatus from Bruker at a temperature of 283 K. Data were evaluated with *NMRview* and calculation was made with *Aria 1.2*.

ROESY mixing times were 200 ms. Compensations were not used as the spinlock was generated by continuous irradiation. As the calculations were performed in vacuum and electrostatic interactions were not taken into account, the solvent did not play a role in structure calculations. Pseudo atoms were not used. In case of several similar protons (e.g. CH_2 and CH_3), there was an addition over all distances. The cross peaks for the *cis* form of the proline were so weak that, although they were included in the constraints used for structure determination, the program did not consider any solutions that included the *cis* form.

Results

A Productive Blind Alley – the Tetrad NPGI

The first criterion used on the search for new natural helix initiation sites was a high probability of forming reverse turns, which in case of the LPCR tetrad had a value of 1.8×10^{-4}.

The tetrad NPGI has a very high probability for reverse turns ($P_t = 4.8 \times 10^{-4}$) and seemed to serve as a good candidate for a tetrad. The data bank searches for this tetrad plus a tryptophan residue 5–20 amino acids downstream to it uncovered several sequences that also fulfilled all other described criteria (see Table 1). These were as follows: ABB1_MOUSE, Amyloid β a4 precursor protein-binding family from *Mus musculus* (NPGIKCFAVRSLGWVEM), HMGD_DROME, High mobility group protein from *Drosophila melanogaster* (NPGISIGDVAKKLGEMWNNL) and VB16_COWPX [1], Interleukin-1 binding protein precursor from cow pock virus (NPGIYILVGTQMRKDMWTTL).

Figure 1(A) and (B) shows the results of CD spectroscopic studies on the peptide VB16_COWPX as an example.

As can be seen from both, the CD spectrum and the secondary structure plot, no switch-like behaviour could be observed for the VB16_COWPX peptide. Although there is a transition from β-sheet to the helical structure, this transition occurs linearly over a wide range of polarities and not abruptly over a narrow range of polarity in a cooperative manner – as is typical for polarity-triggered switch peptides.

Surprisingly, none of the NPGI sequences postulated on the basis of the data bank search and calculations showed the expected behaviour despite fulfilling all the parameters known so far. This situation, together with the fact that earlier measurements with the tetrad *NPTK* peptides (unpublished data) also revealed no switch characteristics, led to the conclusion that the high probability of building reverse turns, while it may be necessary, is evidently not a sufficient criterion for tetrads with respect to the switch characteristics of their associated sequences. It could also be concluded that the *N*-terminal amino acid Asn might be responsible for the loss of the switch-like properties.

In order to test the latter suggestion, we designed a number of new peptides with Asn substitutions in the tetrad. The classical tetrad LPCR was replaced by either the entire new tetrad NPGI or NPTK, or only the first Leu in **L**PCR was substituted with Asn (**N**PCR).

Tetrad/Single Amino Acid Substitutions

When substituting tetrads, we controlled that the SCII continued to yield a favourable value. For this reason, only particular combinations were possible. These were as follows: **NPTK**IKQFINMWQEV [2] and **NPGI**AFGDVQLKWSKE.

The original sequence of the first peptide stems from the CD4-binding region of gp120 of HIV-1 described earlier, but with NPTK replacing the native LPCR tetrad. The second peptide is derived from a sequence within pyruvate-dehydrogenase [lipoamide]-phosphatase 2 from *Rattus norvegicus*.

The CD spectra and the secondary structure plots demonstrate that the tetrad substitution is responsible for a complete loss of switch behaviour. In both cases, even the transition from sheet to helix of the native sequence was now absent. Figure 2(A) and (B) shows the CD spectrum and the secondary structure plot of the peptide NPTKIKQFINMWQEV as an example.

For the amino acid substitution of Leu to Asn at the *N*-terminus of an established switch peptide, it was equally necessary to keep the SCII value above 0.5. Two peptides were chosen: **NPCR**QAQDVSMWIVG [3] and **NPCR**IKQFINMWQEV.

The first peptide comes from a designed, fully artificial switch peptide [3] and the second from the CD4-binding region of gp120.

From the CD spectroscopic results, it could be seen that even in the case of a single replacement of the *N*-terminal Leu for Asn

 Copyright © 2010 European Peptide Society and John Wiley & Sons, Ltd.

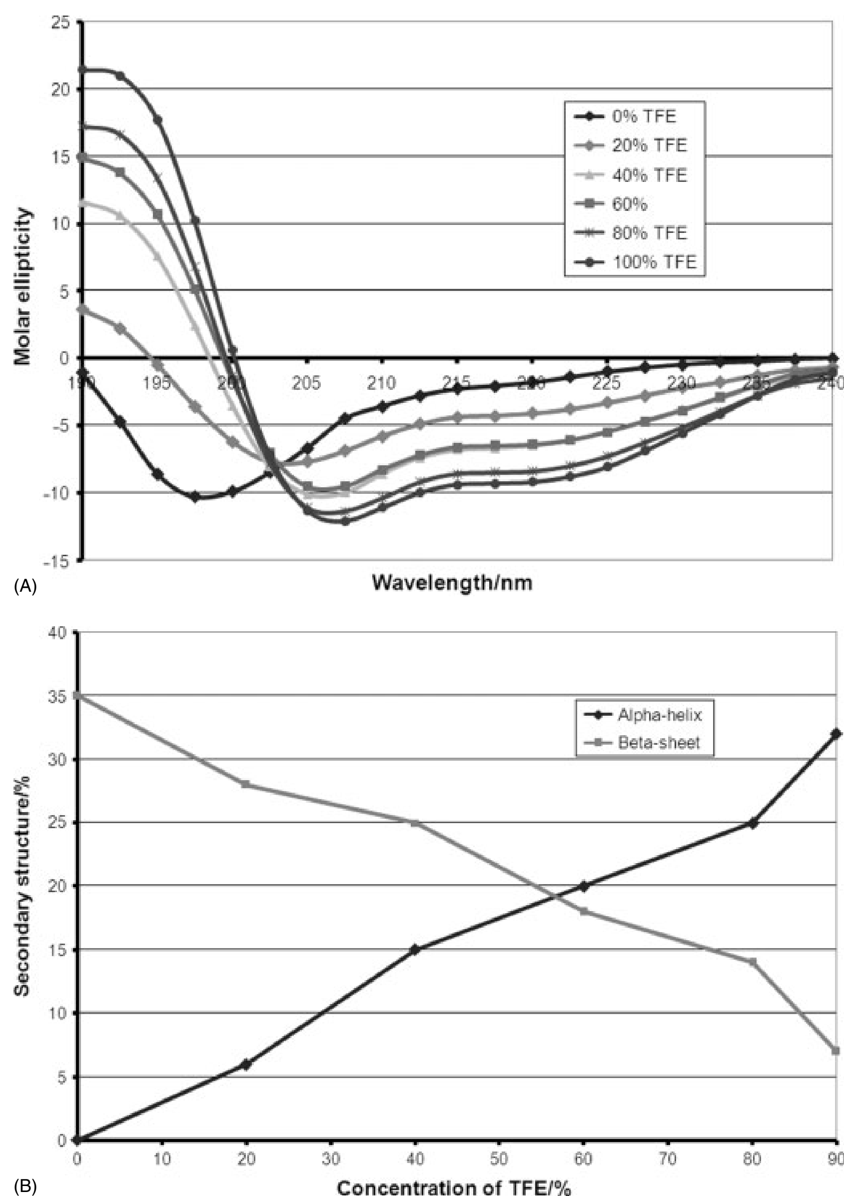

Figure 1. (A) Far-UV spectrum of the VB16_COWPX peptide as a function of TFE concentration. (B) Secondary structure content of the VB16_COWPX peptide.

no switch behaviour is observable. In both the cases, there was no cooperative, abrupt transition from sheet to helix. Figure 3(A) and (B) displays a linear rise of helical structure within the NPCRQAQDVSMWIVG peptide with a maximum amount of helical structures of around 20%.

Thus, the substitution of one single amino acid to Asn at position 1 leads to complete loss of the switch characteristics – despite the fact that all requirements known so far for the characterization of switch peptides have been fulfilled. Apparently some aspect of the Asn is responsible for the loss of the switch-like properties. The central question was, why and how does Asn destroy the conformational switch? One possible answer to these questions is that the dihedral angles between the Asn and the following residues adopt values that are not helix compatible and thus undermine the cooperativity of the molecular switch. In order to prove this hypothesis, NMR spectroscopic measurements of the hexapeptide TNPCRI were performed.

NMR Measurements on the Tetrad TNPCRI

For the NMR measurements, the amino acid Leu in the LPCR tetrad was substituted by Asn as described: the tetrad is further flanked by the amino acids Thr and Ile occurring in the natural gp120 sequence: T**NPCR**I.

The coupling constants derived from the 1 D spectrum [$^3J(H^N - H^\alpha)$ for $N = 6.85$ Hz, for $C = 7.04$ Hz, for $R = 7.04$ Hz and for $I = 8.02$ Hz] gave no evidence for a preferred secondary structure. Additionally, a low number of inter-residual and sequential ROEs (total number of ROEs: 56, intraresidual ROEs/$i - j/ = 0$: 50, sequential ROEs/$i - j/ = 1$: 6, medium range/$i - j/ = 2,3,4,5$: 0) indicated that the hexapeptide TNPCRI adopts no rigid structure, but is much more flexible. In fact, the structure calculations of the ten best structures do not even approach congruence, but are rather highly distributed.

The results discussed above explain the structural background for the loss of switch activity in the peptide.

 Copyright © 2010 European Peptide Society and John Wiley & Sons, Ltd.

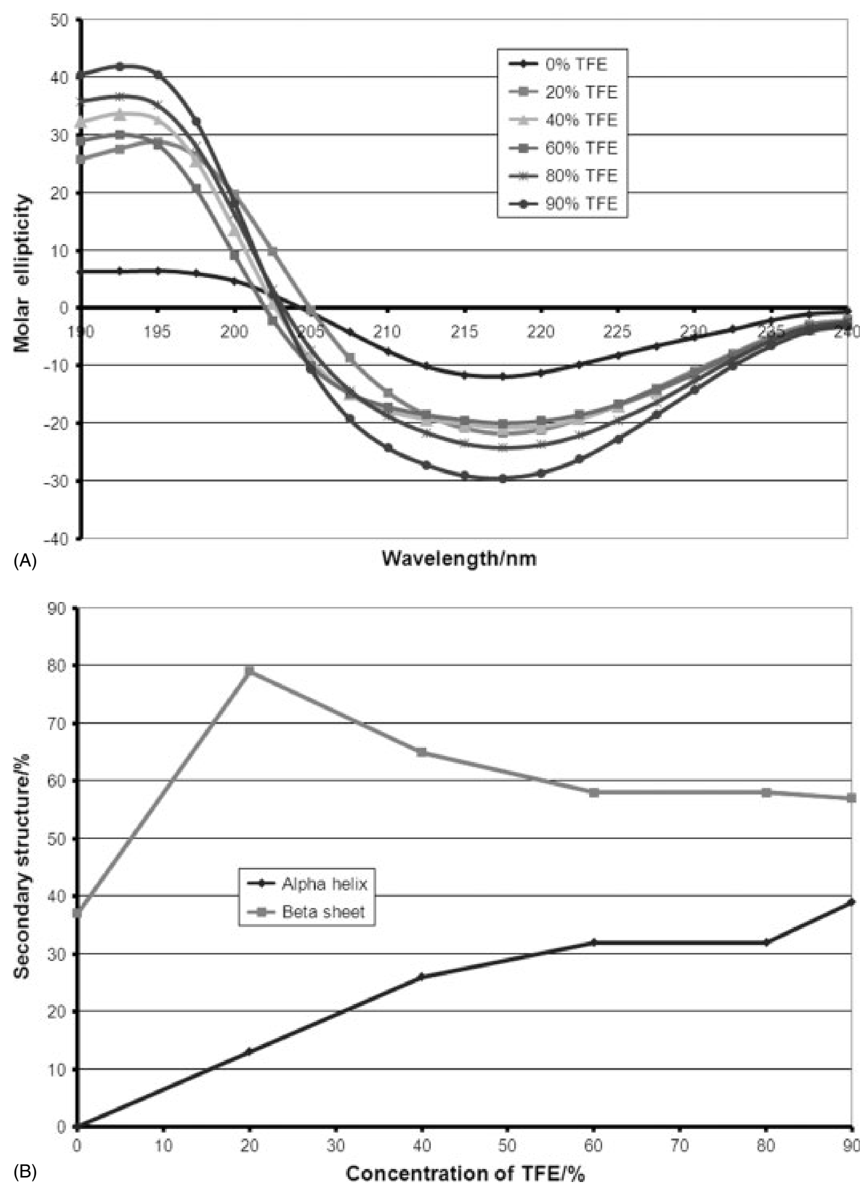

Figure 2. (A) Far-UV spectrum of the tetrad substituted peptide NPTKIKQFINMWQEV as a function of TFE concentration.(B) Secondary structure content of the NPTKIKQFINMWQEV peptide.

Earlier NMR investigations on the hexapeptide T**L**PCRI – containing the original, effective tetrad – have shown that its structure in solution is quite rigid between the Leu and the Arg residues [6]. On the other hand, the mutant T**N**PCRI is highly flexible. Thus, it is clear that a high probability for reverse turns is not alone a sufficient parameter for identification of 'successful' tetrads with respect to their control over the folding of subsequent residues. A further measurable parameter is a restricted range of φ and ψ angles.

CD spectroscopic experiments on the tetrad LPST helped refine the switch parameters further.

The Tetrad LPST

The tetrad LPST with a high probability of forming reverse turns ($P_t = 1.8 \times 10^{-4}$) provided two switch sequences. These are as follows: A1A1_BUFMA [4]: sodium/potassium-transporting ATPase alpha-1 chain precursor derived from *Bufo marinus* (LPSTLLGIRVAWEDR) and AGAL_COFAR: alpha-D-galaktoside-galactohydrolase from *Coffea arabica* (LPSTAVVN-ARDLWAHS).

Both peptides have strongly marked switch characteristics. Figure 4(A) and (B) show the changes in the CD spectrum and the secondary structure plot of the A1A1_BUFMA peptide as an example. The abrupt transition occurs at a concentration of *ca* 30% TFE within a narrow range between 20 and 40% TFE and the transition is completely finished at 40% TFE.

Further Considerations

At this point, the data presented led to new considerations with regard to search options for switch-inducing tetrads. Comparing the two sets of results, that is the presence among the switch 'positive' tetrads of two with a Leu at the *N*-terminal position (**L**PCR and **L**PST) and the two 'negative' tetrads (**N**PTK and **N**PGI), it can be suggested that difference arises from conformational

 Copyright © 2010 European Peptide Society and John Wiley & Sons, Ltd.

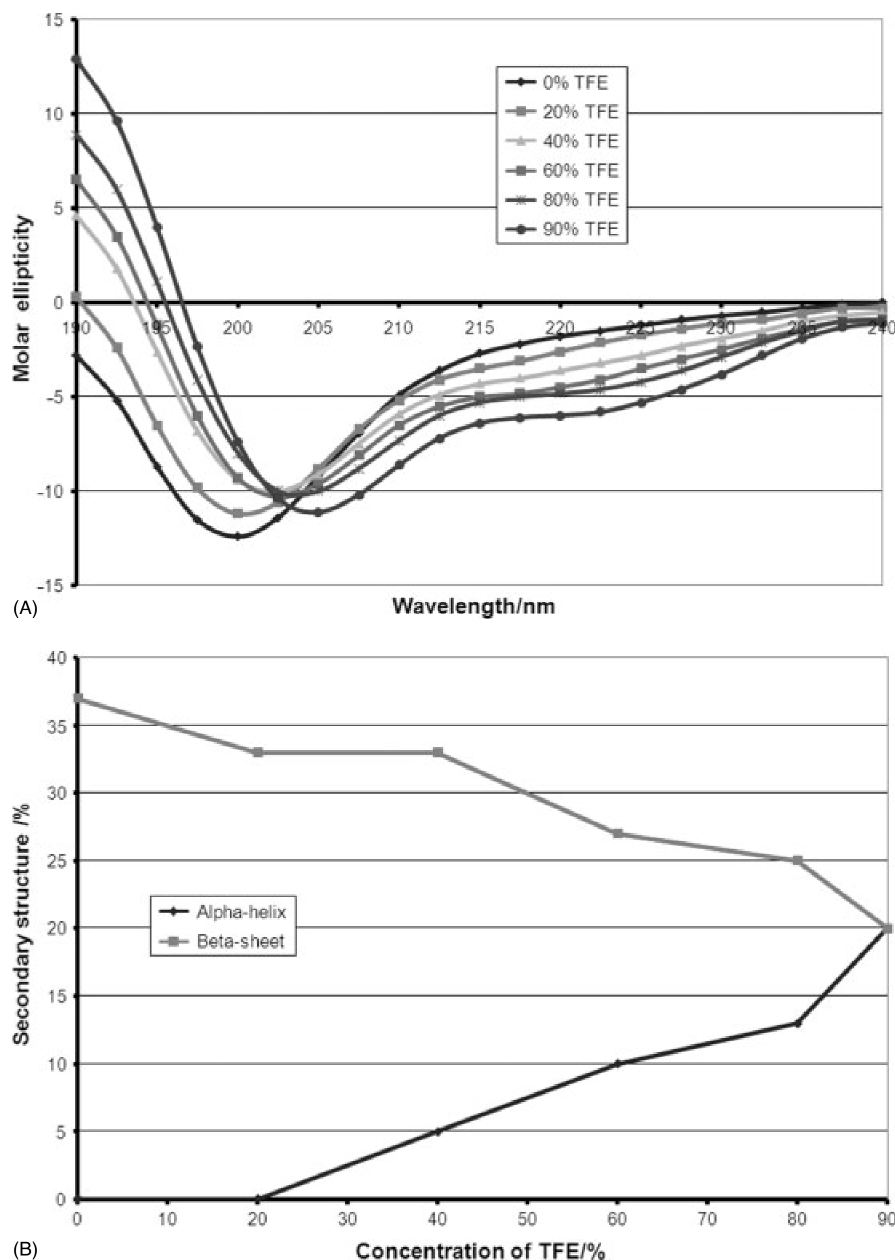

Figure 3. (A) Far-UV spectrum of the single amino acid substituted peptide NPCRQAQDVSMWIVG peptide as a function of TFE concentration. (B) Secondary structure content of the NPCRQAQDVSMWIVG peptide.

preferences of Leu and Asn residues. To check this hypothesis, we studied the most densely populated regions of Ramachandran space occupied by Leu and Asn residues within naturally occurring proteins more closely.

The comparison of the preferred dihedral angles for Asn and Leu in Ramachandran plots shows that Asn displays a much greater rotational freedom, having more local maxima concerning the φ- and ψ-angles and being more widespread on the Ramachandran plot [15]. Amino acids with this property could as a result destabilize the switch behaviour, while amino acids that adopt a more defined – rigid – position could support it. Pro in the second position has a particularly high probability of inducing a reverse turn [5] and appears to be an important component of the tetrad. The 'successful' tetrad LPCR has a Cysteine residue at the third position; LPST a serine. A comparison of the Ramachandran plots for both amino acids shows that their distribution is very similar [15].

The observed features allowed us to formulate the following three principles for the search and characterization of switch-inducing tetrads:

1. The amino acid in the first position should occupy the same or very similar Ramachandran space as Leu.
2. The Pro residue in the second position should be retained.
3. The amino acid in the third position should occupy the same or similar Ramachandran space as Cys or Ser.

Adding these criteria as further parameters for the identification of switch peptides allowed us to detect and characterize new families of switch-inducing tetrads.

 Copyright © 2010 European Peptide Society and John Wiley & Sons, Ltd.

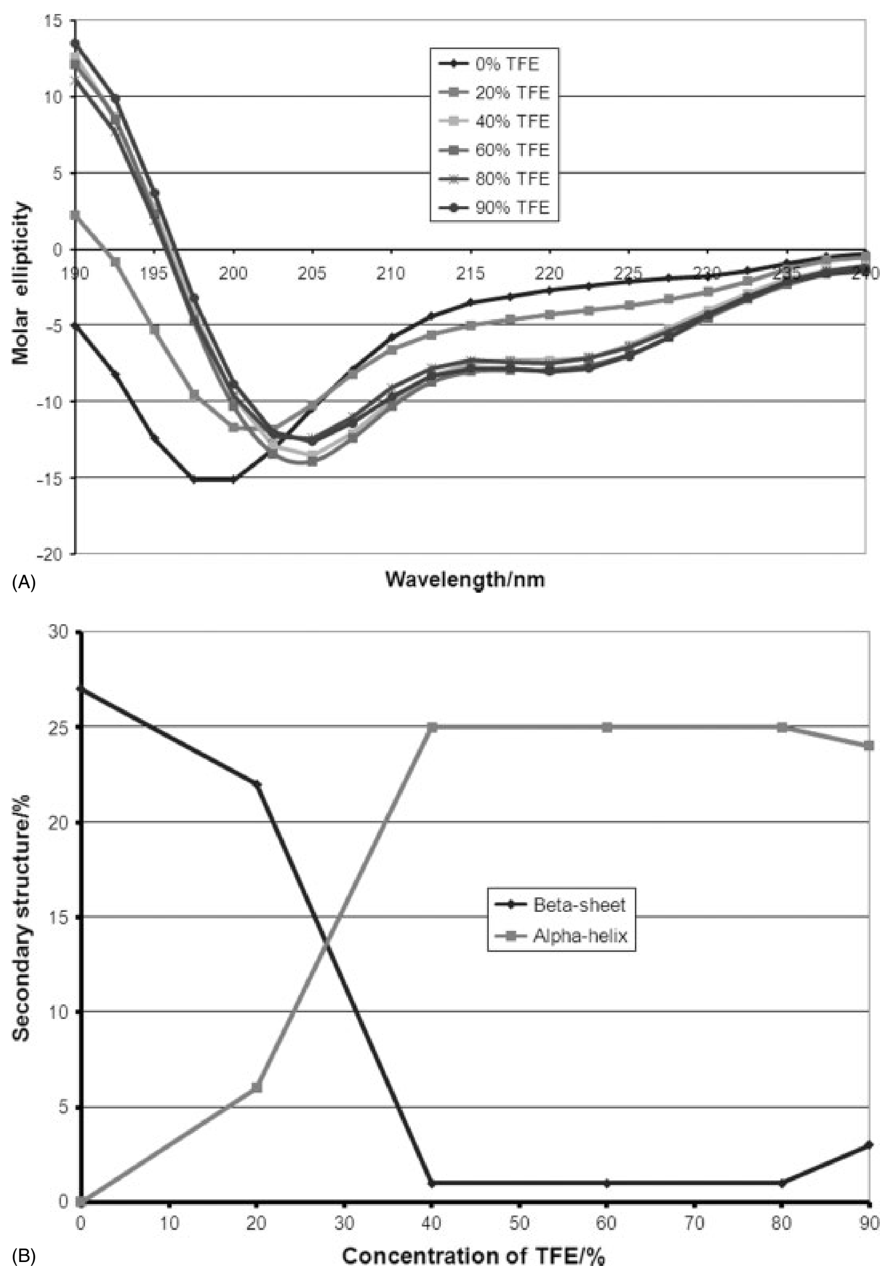

Figure 4. (A) Far-UV spectrum of the A1A1_BUFMA peptide as a function of TFE concentration. (B) Secondary structure content of the A1A1_BUFMA peptide.

The Tetrad VPSR

The tetrad VPSR yielded the following candidates for switch peptides (see Table 1): RPOB-CAUCR derived from *Caulobacter crescentus* (VPSRMNVGQIFETHLGWACA), RPOB-AGRT5 [5] from *Agrobacterium tumefaciens* (VPSRMNVGQILETHLAWACA) and RPOB-SYNJB taken from *Synechococcus spec.* (VPSRMNVGQVYECLLGWAAE).

All three sequences derive from the β-subunit of DNA-directed RNA polymerase.

CD Investigations of the VPSR Tetrad

The measurements on the peptides RPOB-CAUCR, RPOB-AGRT5 and RPOB-SYNJB all produced positive results with respect to switch behaviour.

Figure 5(A) and (B) shows the CD spectrum and secondary structure plot of the peptide RPB_AGRT5 as an example. It displays a very abrupt, early transition. The structural change from β-sheet to helix is complete. In the CD spectrum, an isodichroic point is present at 202 nm.

In order to prove the completeness of the newly developed parameters, we designed two artificial switch peptides (**LPST**VASMIGH**W**VLM [6] and **VPSR**INAQLSLWQMG [7]) bearing no resemblance to the natural sequences except the tetrads LPST and VPSR.

The secondary structure plot of the first artificial peptide shows a switch behaviour with an abrupt and sharp transition from β-sheet structure to helix at 25% TFE (Figure 6(A) and (B)). As can be seen in the CD spectra of the second artificial peptide (Figure 7(A)), there is an isodichroic point at *ca* 208 nm. The

 Copyright © 2010 European Peptide Society and John Wiley & Sons, Ltd.

Journal of PeptideScience

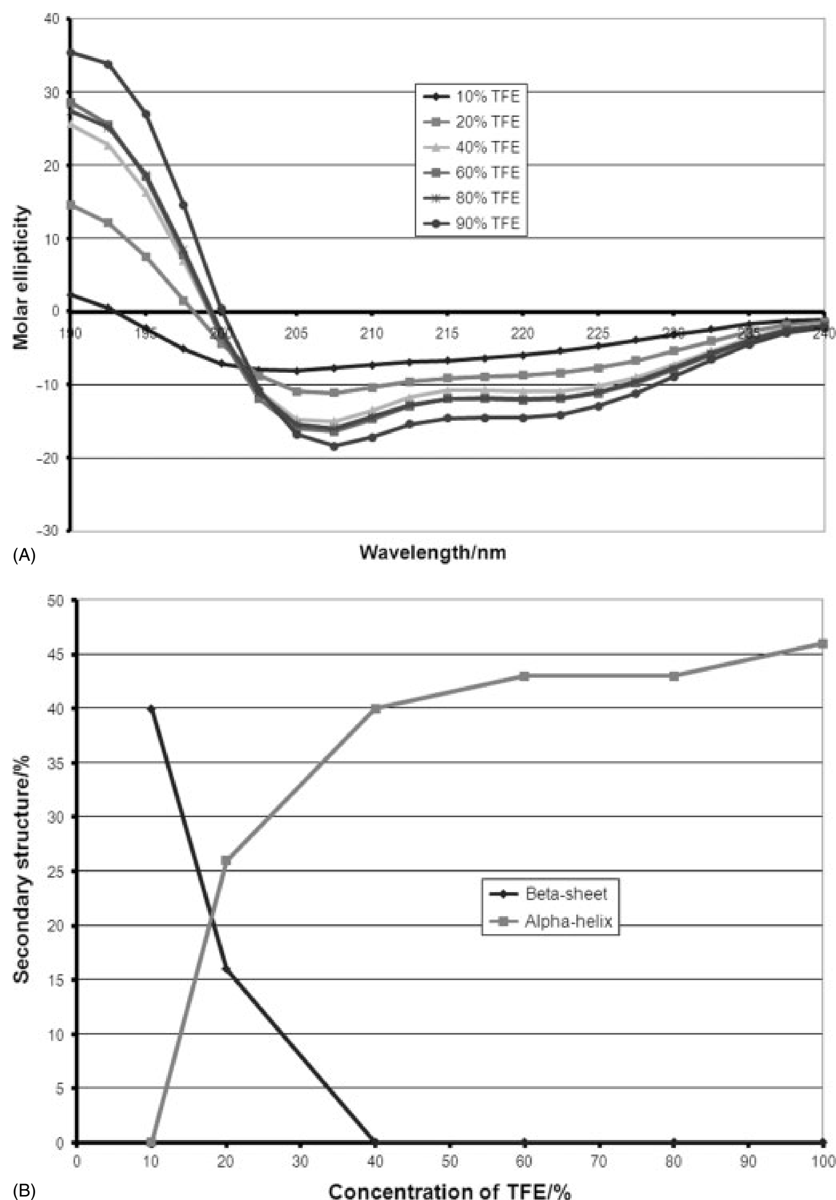

Figure 5. (A) Far-UV spectrum of the RPOB_AGRT5 peptide as a function of TFE concentration. (B) Secondary structure content of the RPOB_AGRT5 peptide.

secondary structure plot shows that this constructed peptide also displays a strong switch behaviour; the transition occurs at *ca* 35% TFE (Figure 7(B))

The obtained results presented here clearly show that it is possible to identify new families of switch peptides. For full characterization of the tetrad VPSR and to verify the postulate set concerning the rigidity of φ- and ψ-angles, NMR measurements were carried out on the VPSR tetrad. For this purpose, the peptide GVPSRM was synthesised with the naturally occurring flanking residues of RPOB_CAUCR, Gly at the *N*-terminus and Met at the *C*-terminus, and measured under conditions analogous to those for the hexapeptide TNPCRI.

NMR Spectroscopic Investigation of the Hexapeptide GVPSRM

In comparison with the results from the NMR measurements on the flexible hexapeptide TNPCRI, this displayed significant differences. The coupling constants, derived from the 1 D spectrum, were as follows: $^3J(H^N - H^\alpha)$ for $V = 7.43$ Hz, for $S = 6.26$ Hz, for $R = 7.43$ Hz and for $I = 7.73$ Hz. The coupling constant for the amino acid Ser with a value of around 6 Hz indicates the presence of a helix-compatible φ angle. Additionally, the ROESY spectrum of the peptide GVPSRM had almost double the number of ROESY cross peaks compared to hexapeptide TNPCRI. In the latter peptide, there were only six inter-residual cross peaks and all of them were sequential, whereas the peptide GVPSRM exposed 34 sequential and 12 medium-range cross peaks, distributed around the central part of the molecule. The six medium-range cross peaks between the Val residue in the second position and the Ser in the fourth position were especially meaningful.

Figure 8 shows a view of the superposition of the ten best (energetically most favourable) conformations of the peptide that were calculated from the results of all spectra. From both, the coupling constants and the ROESY data, it is obvious that

 Copyright © 2010 European Peptide Society and John Wiley & Sons, Ltd.

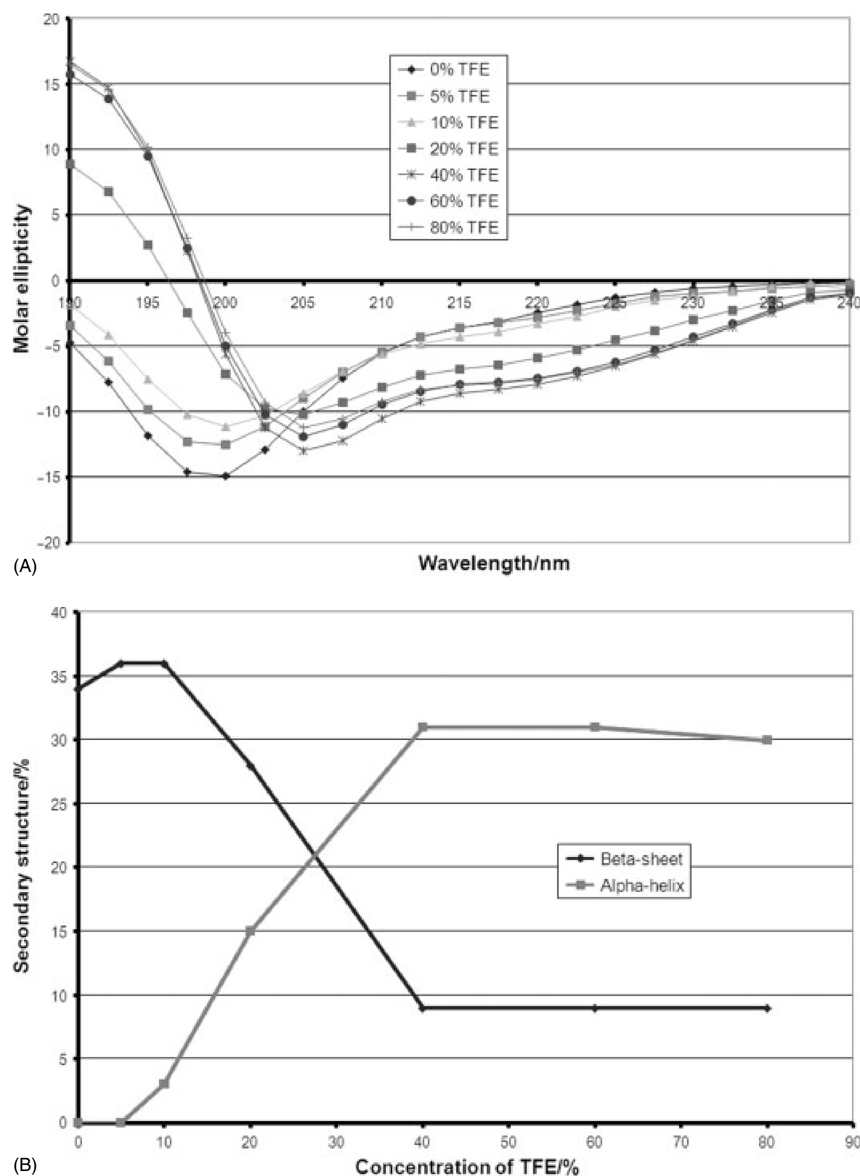

Figure 6. (A) Far-UV spectrum of the artificial peptide LPSTVASMIGHWVLM as a function of TFE concentration. (B) Secondary structure content of the LPSTVASMIGHWVLM peptide.

the region between the second and the fourth amino acid is important for the structural arrangement of the sequence, and the hexapeptide GVPSRM shows a high rigidity in the region between Val(2) and Ser(4). The Ramachandran plot (Figure 9) of the involved amino acids Val and Ser gives evidence of the 3_{10}-helix-compatible φ angles, all in the range between -70° and -100°. The Pro between both residues possesses a fixed φ angle anyway ($\sim-60^\circ$) due to the fact that it is rather an *N*-alkylated amino acid, with a ring formed by its side chain, which is directly connected to the *N*-atom of its main chain. Interestingly, the ψ-angles of the Ser are also located in a very narrow area, indicative of a high rigidity and low conformational and rotational freedom for this residue.

Discussion

The work presented here indicates that there exist other naturally occurring families of polarity-driven conformational switches beside the LPCR family and refines new physico-chemical characteristics of helix-inducing tetrads within polarity-triggered switch peptides.

The first attempts to find new switch families resulted in an intriguing failure, the tetrad NPGI, which would not support switch behaviour. The Pro-Gly motif is known to form type-II β-turns, [16–25] which are not helix compatible. This might explain the lack of the switch function of the initially chosen peptides with the *N*-terminal tetrad NPGI. However, using a strategy of tetrad/single amino acid substitutions within established conformational switch peptides showed that it is the Asn that is co-responsible for the loss of switch activity. The NMR investigations of the hexapeptide TNPCRI offered a structural reason for this phenomenon. Compared with the hexapeptide already structurally defined with NMR [6], which was distinguished by its rigid structure and 3_{10}-helix-compatible φ angles, the Asn-substituted tetrapeptide was not able to adopt fixed angles and was much more flexible, showing a high degree of rotational

Copyright © 2010 European Peptide Society and John Wiley & Sons, Ltd.

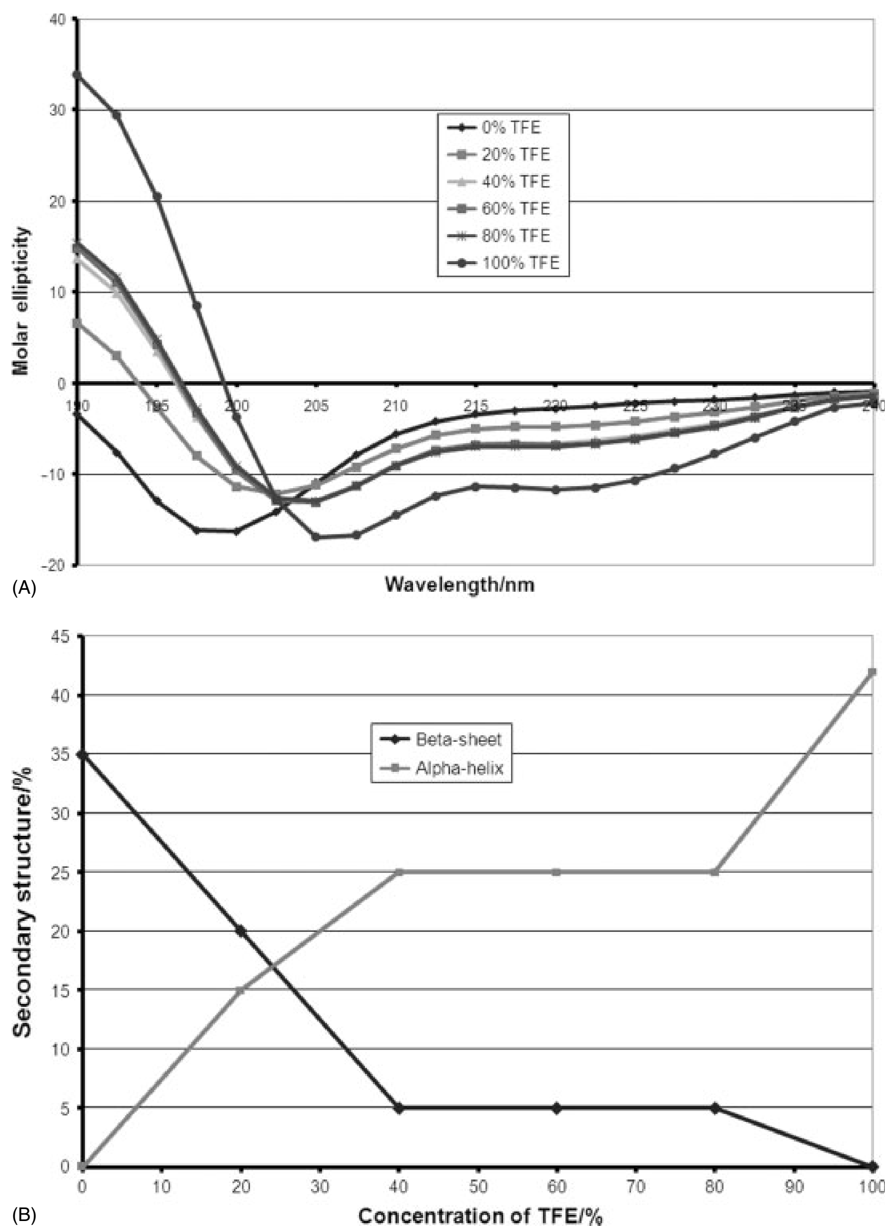

Figure 7. (A) Far-UV spectrum of the artificial peptide VPSRINAQLSLWQMG as a function of TFE concentration. (B) Secondary structure content of the VPSRINAQLSLWQMG peptide.

freedom. It would seem that a critical point for the success of a tetrad in governing the subsequent residues is its conformational rigidity.

An intra-molecular H-bond between the carbonyl group of the γ-position of Asn and the backbone amide group two residues downstream in Asn-turns, typically consistent of Asn-Pro motif, has been discussed by Marraud *et al*. as prohibiting the formation of a classical β-turn [26]. Given the previous assumption that a high probability of forming a turn is a critical point for characterizing switch peptides and helix initiation sites; this might be another explanation for the incapacity of the tetrads NPGI and NPTK to act as helix initiation sites. On the other hand, one would expect the presence of the H-bond to give the peptide a rigid, helix incompatible conformation, rather than to cause increased flexibility.

An explanation for the high flexibility of the hexapeptide TNPCRI, which seems to be more likely, is the fact that Asn (as well as Asp) is often found in the partially allowed Ramachandran spaces [27] as well as in the region for left-handed helices (α-L) [28]. Occupying left-handed φ- and ψ-angles makes it impossible to build up right-handed helical structures, such as a 3_{10}-helix and explains the high flexibility observed within the TNPCRI peptide.

The tetrads found here show a high probability of forming β-turns according to the Chou–Fasman method. This led to the hypothesis that it might be an important and necessary parameter for characterizing switch peptides and helix-inducing tetrads. However, a high probability of β-turns does not seem to be a sufficient criterion for the efficacy of such tetrads. On the one hand, using the Chou–Fasman method for the search of new helix-inducing tetrads might not be entirely appropriate, as it is a pure empirical method for predicting secondary structures within proteins based on statistical analysis of the frequencies of certain amino acids occurring within a helix,

 Copyright © 2010 European Peptide Society and John Wiley & Sons, Ltd.

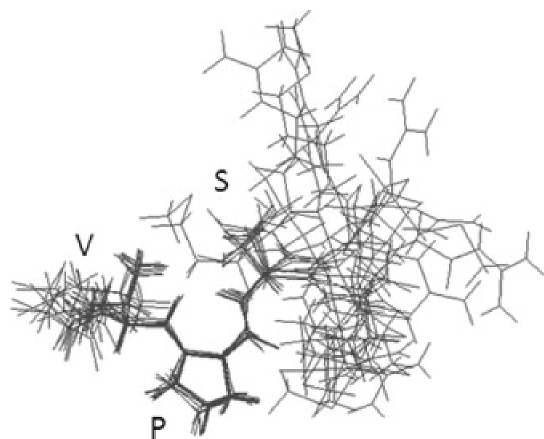

Figure 8. Overlay of the peptide backbone of ten calculated structures of the hexapeptide GVPSRM.

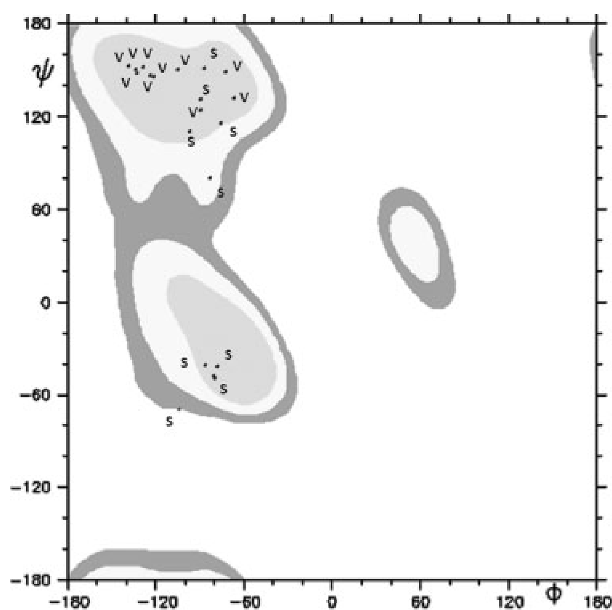

Figure 9. Ramachandran plot of the superpositioned amino acids Val 2 and Ser 4 of the hexapeptide GVPSRM.

β-sheets and β-turns. Additionally, the Chou–Fasman method does not distinguish between the different types of β-turns; rather, it displays probabilities of all nine types of β-reverse turns known so far [16,22–25].

Neither the first hexapeptide TLPCRI, which was revealed as an inverse γ-turn, nor the GVPSRM peptide fit into the classification of Lewis [22]. β-Turns always involve four sequential amino acid residues, whereas the distance between the α-C_i and the α-C_{i+3} is less than 7 ´Å and the tetrapeptide is not allowed to be in helical conformation [23–24]. Within the GVPSRM peptide, only three amino acids take part in the rigid structure: Val2, Pro3 and Ser4, rather displaying an inverse γ-turn structure [24,29] with even a rigid ψ-angle of the Val2 residue (between -120° and 150°) and a more or less fixed φ-angle of Ser4 (between -80° and -100°).

The most important parameter, which determines the success of a tetrad with respect to the switch behaviour of a peptide, therefore seems to be the conformational restriction and a high rigidity fixing helix-favourable torsion angles. Thus, for further search of tetrads, which are capable of inducing 3_{10}-helices [22] and switch behaviour, it is suggested that the search also be extended for tetrads with a high probability for γ-turns. The Pro residue plays an important role in this context. Guruprasad and Rajkumar showed that Pro is always found at position *i* in γ-turns, although it prefers position $i + 1$ or position *i* in an inverse γ-turn [30]. Indeed, a Pro in the second position is the single most important determinant of reverse turns of any kind.

With the identification of the switch-inducing tetrads LPST and VPSR, it could be shown that besides the LPCR family other natural families of switch peptides exist and that these are present in diverse proteins. Through the successful characterisation of switch peptides and the refined description of the parameters of helix-inducing tetrads, the work presented here should contribute to significant advancement in our understanding of the basic principles of this unusual folding behaviour. Future searches for further natural switch sequences should be decisively simplified thereby.

Expanding the spectrum of known physiological switch functions could provide a deeper view into the mechanism of protein–protein or protein–membrane interactions and could extend our knowledge of cellular and extra-cellular recognition processes.

It should be noted that the purely synthetic, that is, designed switches discussed here do not have the strength of some of the natural switches such as those from the LAV strain of HIV or the tomato polygalacturonase peptide, which start and end with over 50% secondary structure. On the other hand, a number of the naturally occurring switches are equally weak, that is, start and end with secondary structure contents of only 20–30%. The significance of these differences has not been established at this point, although one could speculate that the requirements of the binding site or receptor are likely to play a decisive role.

It is known that the LAV peptide from gp120 of HIV-1 is absolutely necessary for the successful binding of gp120 to the CD4 receptor [1] and thus has crucial biological relevance. It would also be important to find out whether other polarity-driven switch-like natural sequences are connected with some biological function. Also, it would be interesting to determine whether particular tetrad families tend to be associated with particular functions. Such knowledge would enable the application of switch sequences as tools in the field of protein engineering. The design of artificial peptides that show switch-like behaviour in a predictable manner (caused by protein–protein or protein–membrane interactions), opens, e.g. the possibility of coupling switch peptides to medically active substances in order to allow these to be transported to specific target cells.

Acknowledgements

We thank Dr Rüdiger Pipkorn for the peptide preparations, Prof. Dr Paul Rösch and Dr Kristian Schweimer from the University of Bayreuth for permitting us to carry out the NMR spectroscopic measurements on the TNPCRI peptide and for guidance with the evaluation of the data. We also would like to thank Dr Michael Sattler and Dr Lorenzo Corsini from the EMBL in Heidelberg for the performance of additional NMR measurements and for providing computer programs for the evaluation of the data.

References

1 Reed J, Kinzel V. A conformational switch is associated with receptor affinity in peptides derived from the CD4-binding domain of gp120 from HIV I. *Biochemistry* 1991; **30**: 4521–4528.

Copyright © 2010 European Peptide Society and John Wiley & Sons, Ltd.

2 Reed J, Kinzel V. Primary structure elements responsible for the conformational switch in the envelope glycoprotein gp120 from human immunodeficiency virus type 1: LPCR is a motif governing folding. *Proc. Natl. Acad. Sci. U.S.A.* 1993; **90**: 6761–6765.

3 Gehenn K, Pipkorn R, Reed J. Successful design and synthesis of a polarity-triggered beta → alpha conformational switch using the side chain interaction index (SCII) as a measure of local structural stability. *Biochemistry* 2004; **43**: 607–612. DOI: 10.1021/bi0301744.

4 Graf von Stosch A, Kinzel V, Pipkorn R, Reed J. Investigation of the structural components governing the polarity-dependent refolding of a CD4-binding peptide from gp120. *J. Mol. Biol.* 1995; **250**: 507–513. DOI: S0022283685703944. [pii].

5 Chou PY, Fasman GD. Empirical predictions of protein conformation. *Annu. Rev. Biochem.* 1978; **47**: 251–276. DOI:10.1146/annurev.bi.47.070178.001343.

6 Lindemann A, Kinzel V, Rosch P, Reed J. Structure of the LAV6 peptide: a nucleation site for the correct receptor-induced refolding of the CD4-binding domain of HIV1 gp 120. *Proteins* 1997; **29**: 203–211. DOI: 10.1002/(SICI)1097-0134(199710)29 : 2<203::AID PROT8>3.0.CO;2-D. [pii].

7 Graf von Stosch A, von der Lieth CW, Reed J. Molecular dynamics study of the proposed beta-hairpin form of the switch domain from HIV1 gp120 alone and complexed with an inhibitor of CD4 binding. *Proteins* 1999; **34**: 197–205. DOI: 10.1002/(SICI)1097-0134(19990201)34 : 2<197:AID-PROT5>3.0.CO;2-#. [pii].

8 Schweizer S, Reed J. Effect of variation of the strength of the aromatic interactions of tryptophan on the cooperative structural refolding behavior of a peptide from HIV 1. *Biophys. J.* 2008; **95**: 3381–3390 DOI: S0006-3495 (08) 78480-7 [pii] 10.1529/biophysj.107.126375.

9 Gehenn K, Stege J, Reed J. The side chain interaction index as a tool for predicting fast-folding elements and the structure and stability of engineered peptides. *Anal. Biochem.* 2006; **356**: 12–17. DOI: S0003-2697 (06) 00445-3 [pii]10.1016/j.ab.2006.06.021.

10 Rajan R, Balaram P. A model for the interaction of trifluoroethanol with peptides and proteins. *Int. J. Pept. Protein Res.* 1996; **48**: 328–336.

11 Llinás M, Klein MP. Charge relay at the peptide bond. A proton magnetic resonance study of solvation effects on the amide electron density distribution. *J. Am. Chem. Soc.* 1975; **97**: 4731–4737.

12 Singh J, Thornton JM. *Protein Side-Chain Interactions*. IRL Press: Oxford, 1992.

13 Kyte J, Doolittle RF. A simple method for displaying the hydropathic character of a protein. *J. Mol. Biol.* 1982; **157**: 105–132. DOI: 0022–2836 (82) 90515-0. [pii].

14 Reed J, Reed TA. A set of constructed type spectra for the practical estimation of peptide secondary structure from circular dichroism. *Anal. Biochem.* 1997; **254**: 36–40. DOI: S0003-2697 (97) 92355-1 [pii] 10.1006/abio.1997.2355.

15 Kleywegt GJ, Jones TA. Phi/psi-chology: ramachandran revisited. *Structure* 1996; **4**: 1395–1400.

16 Hutchinson EG, Thornton JM. A revised set of potentials for beta-turn formation in proteins. *Protein Sci.* 1994; **3**: 2207–2216.

17 Karle I, Gopi HN, Balaram P. Infinite pleated beta-sheet formed by the beta-hairpin Boc-beta-Phe-beta-Phe-D-Pro-Gly-beta-Phe-beta-Phe-OMe. *Proc. Natl. Acad. Sci. U.S.A.* 2002; **99**: 5160–5164. DOI: 10.1073/pnas.022616499022616499. [pii].

18 Syud FA, Stanger HE, Mortell HS, Espinosa JF, Fisk JD, Fry CG, Gellman SH. Influence of strand number on antiparallel beta-sheet stability in designed three- and four-stranded beta-sheets. *J. Mol. Biol.* 2003; **326**: 553–568. DOI: S0022283602013049. [pii].

19 Roy RS, Gopi HN, Raghothama S, Karle IL, Balaram P. Hybrid peptide hairpins containing alpha- and omega-amino acids: conformational analysis of decapeptides with unsubstituted beta-, gamma-, and delta-residues at positions 3 and 8. *Chemistry* 2006; **12**: 3295–3302. DOI: 10.1002/chem.200500742.

20 Budesinsky M, Sebestik J, Bednarova L, Baumruk V, Safarik M, Bour P. Conformational properties of the Pro-Gly motif in the D-Ala-l-Pro-Gly-D-Ala model peptide explored by a statistical analysis of the NMR, Raman, and Raman optical activity spectra. *J. Org. Chem.* 2008; **73**: 1481–1489. DOI: 10.1021/jo702297y.

21 Giddu S, Subramanian V, Yoon HS, Satyanarayanajois SD. Design of beta-hairpin peptides for modulation of cell adhesion by beta-turn constraint. *J. Med. Chem.* 2009; **52**: 726–736. DOI: 10.1021/jm8008212.

22 Lewis PN, Momany FA, Scheraga HA. Chain reversals in proteins. *Biochim. Biophys. Acta* 1973; **303**: 211–229.

23 Richardson JS. The anatomy and taxonomy of protein structure. *Adv. Protein Chem.* 1981; **34**: 167–339.

24 Rose GD, Gierasch LM, Smith JA. Turns in peptides and proteins. *Adv. Protein Chem.* 1985; **37**: 1–109.

25 Venkatachalam CM. Stereochemical criteria for polypeptides and proteins. V. Conformation of a system of three linked peptide units. *Biopolymers* 1968; **6**: 1425–1436. DOI: 10.1002/bip.1968.360061006.

26 Abbadi A, Mcharfi M, Aubry A, Premilat S, Boussard G, Marraud M. Involvement of side functions in peptide structures – the Asx turn – occurrence and conformational aspects. *JACS* 1991; **113**: 2729–2735.

27 Chou PY, Fasman GD. Prediction of protein conformation. *Biochemistry* 1974; **13**: 222–245.

28 Srinivasan N, Anuradha VS, Ramakrishnan C, Sowdhamini R, Balaram P. Conformational characteristics of asparaginyl residues in proteins. *Int. J. Pept. Protein Res.* 1994; **44**: 112–122.

29 Milner-White EJ, Ross BM, Ismail R, Belhadj-Mostefa K, Poet R. One type of gamma-turn, rather than the other gives rise to chain-reversal in proteins. *J. Mol. Biol.* 1988; **204**: 777–782.

30 Guruprasad K, Rajkumar S. Beta- and gamma-turns in proteins revisited: a new set of amino acid turn-type dependent positional preferences and potentials. *J. Biol. Sci.* 2000; **25**: 143–156.

 Copyright © 2010 European Peptide Society and John Wiley & Sons, Ltd.