Secondary structure of an armadillo single repeat from the APC protein

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Abstract The armadillo domain is a repeating sequence motif of a variety of proteins with different functions. Here we describe the structure of a synthetic single armadillo repeat solved by two-dimensional nuclear magnetic resonance spectroscopy. Our results indicate α-helical secondary structural elements in half of the residues.

Key words: ^{1}H-NMR; Nuclear Overhauser effect; APC protein; Armadillo repeat

1. Introduction

The repeating 42 amino acid armadillo (arm) sequence motif was originally identified in a segment polarity gene product from *Drosophila* [1]. Arm motifs were also found in several unrelated proteins of different species (for review see [2,3]). All known arm proteins contain seven to twelve copies of repeats arranged in tandem with very short or no intervening sequences. Some proteins of the arm family like armadillo, smgGDS (exchange factor for Ras related small G proteins) and the yeast protein SRP (suppressor of RNA polymerase I mutations) solely consist of arm repeats with short flanking unique amino-terminal and carboxy-terminal domains. Otherwise in arm proteins like APC (Adenomatous Polyposis Coli) and p120^{cas} the arm motif represents only a short sequential section of the whole protein sequence.

Mutations in the APC tumor suppressor gene were found in the germline of patients with familial adenomatous polyposis coli and also in the majority of sporadic colorectal tumors [4–8] Mutations in the 2843 amino acids APC protein are mostly frameshift deletions or insertions leading to translation stops. Most of the APC gene alterations are located downstream of a seven tandem arm repeat region (residues 452–767, see Fig. 1) Until now only very little is known about structural aspects of the APC protein except for a coiled coil domain formed by the N-terminal amino acids 1–55 [9].

Despite the high sequence identity of more than 30% among the arm repeat regions of different proteins the definition of a common biological role of the arm sequence motif on the basis of experimental data is limited to a mediation of specific protein-protein interactions in signal transduction pathways analogous to the role of SH2 and ankyrin. Biochemical results indicate that single amino acids in the arm region are necessary for the association with other proteins [10]. For armadillo and plakoglobin there is genetic and biochemical evidence implicating separate individual functions of small groups of arm repeats or even individual repeats [11,12]. Nevertheless, the level of similarity between single arm repeats and between various arm proteins is sufficient to confirm a structural relationship.

We investigated structural determination of a synthetic single arm repeat by ^{1}H-NMR and CD spectroscopy. The protein **LWNLSARNPKDQEALWDMGAVSMLKNLIHSKHK-MIAMGSAAA** corresponds to amino acid sequence position 684–726 of the APC protein (see Fig. 1). The sequence of the sixth armadillo repeat was chosen because of its high consensus with armadillo repeats of other proteins.

To obtain indications about the putative secondary structure of the synthetic peptide we applied different secondary structure prediction methods (Tripos Associates, Inc., St. Louis, USA) [13–18]. All algorithms showed α-helical secondary structure in more than 50% of the peptide sequence.

2. Materials and methods

2.1. Peptide synthesis

The peptide was synthesized by solid-phase peptide synthesis using standard procedures. The product was purified by reversed-phase high-performance liquid chromatography (RP-HPLC) to a purity of 95% confirmed by capillar electrophoresis and electrospray mass spectrometry. The synthetic protein was freeze-dried and stored at −20°C. For denaturation/renaturation experiments 10 mg of the peptide were dissolved in 1 ml H_2O containing 6 M urea. The sample was incubated at room temperature for 30 min. For renaturation the sample was dialysed against each 1000 vols. of 4 M urea for 4 h, against 2 M urea for 4 h, and twice against H_2O for 12 h at 4°C. The renatured peptide was freeze-dried and stored at −20°C.

2.2. CD spectroscopy

10 mg of peptide were dissolved in 550 μl buffer 1 (10 mM sodium-citrate, 50 mM NaCl, pH 4.0), for measurements the solution was diluted 1:100. CD spectra were recorded from 250 nm to 190 nm at 20 nm/min on a Jasco J710 spectropolarimeter, calibrated using d_{10}-camphorsulfonic acid, against a reference sample without protein. Spectra were taken as an average of ten scans recorded with a bandwidth of 1 nm, a 0.25 nm step size and a 1 s time constant. Subsequent to a baseline correction, the observed ellipticity was converted to a mean residue ellipticity, [θ] (degree cm^2 dmol^{-1}), using the relationship $[\theta] = \theta/(\ell cN)$, where θ is the observed ellipticity, ℓ the path length in mm, c is the molar concentration and N is the number of residues in the peptide.

2.3. NMR spectroscopy

For NMR experiments 10 mg of peptide were dissolved in 550 μl buffer 2 (10 mM sodium citrate-d_4, 50 mM NaCl, pH 4.0) containing 10% D_2O or in buffer 3 (10 mM sodium citrate-d_4, 50 mM NaCl in 100% D_2O, pH 4.0).

Diversified 2D NMR experiments were recorded on a Bruker AMX500 spectrometer working at a proton resonance frequency of 500 MHz: double quantum filtered correlated spectroscopy DQF-COSY [19], nuclear Overhauser enhancement spectroscopy NOESY [20] with mixing times 150, 200 and 500 ms, total coherence spectroscopy clean-TOCSY [21]. The spectra were acquired in the phase-sensitive mode with quadrature detection in both dimensions using the time-proportional phase incrementation technique (TPPI) in f_1 [22]. Solvent suppression was performed by continuous coherent irradiation prior to the first excitation pulse and during the mixing time in the NOESY experiment.

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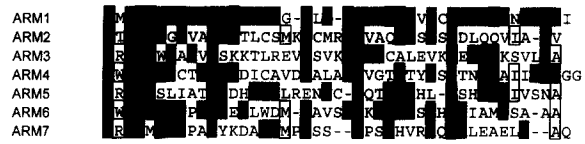

Fig. 1. Consensus for the seven arm repeats. Identity residues are in closed boxes, shaded boxes show 50% homology.

The following spectral parameters were used: frequency width 6330 Hz, data size 1K×2K data points, sinebell-squared filter with a phase shift of π/4 was applied prior to Fourier transformation. Sample temperature was kept at 298 K with a standard Bruker VT2000 temperature-control unit using a continous stream of cooled dried air. Entire 2D NMR spectra were processed using the software package NDEE [23] on a Silicon Graphics workstation. Chemical shift values are reported in ppm from 2,2-dimethyl-2-silapentanesulfonic acid (DSS) as an internal reference.

2.4. Structure calculation

Interproton distance restraints for structure calculations were derived from comparing peak volumes of 150 and 200 ms mixing time NOESY spectra to internal references and classifying restraints into three qualitative groups (strong, 1.8–2.7 Å; medium, 1.8–4.0 Å; weak, 1.8–5.5 Å). Structures were calculated using a standard distance geometry/simulated annealing protocol in XPLOR 3.1 [24]. A total of 189 NMR restraints were used for structure calculations.

3. Results

If it is assumed that a CD signal minimum at 222 nm is almost exclusively dependent on the helical character of a protein, it is possible to approximate the α-helicity by the method of Holzwarth and Doty [25]. The negative ellipticity at 222 nm in the CD spectrum recorded at 296 K in buffer 1 gave an estimate of ~15% α-helix for the APC arm peptide.

The first set of 2D spectra was recorded at 298 K in buffer 2 (see section 2). Identification of as many spin systems as possible (representing protons within individual amino acid residues) was made via 2D-double quantum filtered correlation spectra (DQF-COSY) [19] and a total correlation spectrum (CLEAN-TOCSY) [21] recorded with a 100 ms mixing time. Sequential assignment was performed using nuclear Overhauser enhancement spectra (NOESY) [20] with a 150 and 200 ms mixing time. A region of the CLEAN-TOCSY and the NOESY spectra illustrating part of the sequential assignment in the NH-Cα-region is shown in Fig. 2. At this stage ~80% of the peptide's resonances had been assigned in H_2O solution.

Additional DQF-COSY, CLEAN-TOCSY and NOESY spectra were recorded in D_2O solution at 298 K. The assignment was completed by superimpositioning and systematic comparison of these spectra using the graphic display and assignment programm of NDEE [23]. For results of the assignment procedure see Table 1.

Hα-HN NOEs of sequential residues lead from Asn-3 to Asn-8 interrupted by the following Pro-9. From Lys-10 to Asn-26 the entire chain could be traced via Hα-HN NOEs. Sequential Hα-HN NOEs were also found for the regions Leu-27 to His-29 and Ser-30 to Ala-41 (see Fig. 3). The presence of $d_{\alpha N}(i,i+3)$ and $d_{\alpha\beta}(i,i+3)$ NOEs as well as successive strong $d_{NN}(i,i+1)$ and weak $d_{\alpha N}(i,i+1)$ NOEs [26] identifies two helices (see Fig. 4) containing residues 8–16 (helix I) and 20–26 (helix II), approximately. Fig. 4 also summarizes the sequential and medium range NOE connectivities used to identify secondary structural elements.

Subsequently, we used the chemical shift data to perform a secondary structure estimation according to the chemical shift index CSI strategy by Wishart and Sykes [27]. The results are shown in Fig. 4. The procedure depends on a simple correlation between chemical shifts of Cα proton resonances of consecutive amino acids and local secondary structure. Cα proton resonances shifted more than 0.1 ppm upfield relative to the corresponding random coil resonances indicate local α-helical structure; Cα proton resonances shifted more than 0.1 ppm downfield compared to the corresponding resonances in a random coil structure indicate local β-sheet structure. Only reso-

Table 1
^1H chemical shifts and assignments of the arm peptide at pH 4.0, T = 298 K

Residue	HN	Hα	Hβ	Hγ	Others
Leu-1		3.97	1.65	1.57	Hδ* = 0.91
Trp-2	8.64	4.58	3.27		
Asn-3	8.28	4.60	2.69, 2.6		Hδ21 = 6.84, Hδ22 = 7.51
Leu-4	8.02	4.23	1.58	1.69	Hδ1* = 0.86, Hδ2* = 0.92
Ser-5	8.20	4.40	3.88		
Ala-6	8.11	4.32	1.38		
Arg-7	8.16	4.29	1.84, 1.73	1.59	Hδ* = 3.15
Asn-8	8.37	4.92	2.87, 2.71		Hδ21 = 7.62, Hδ22 = 6.94
Pro-9		4.38	2.31	2.01	Hδ1 = 3.81, Hδ2 = 3.75
Lys-10	8.23	4.22	1.83, 1.76	1.46, 1.40	Hε* = 2.99, Hδ* = 1.68
Asp-11	8.10	4.64	2.88, 2.82		
Gln-12	8.19	4.22	2.11, 1.98	2.31	Hε21 = 7.43, Hε22 = 6.84
Glu-13	8.18	4.12	2.06, 1.98	2.41	
Ala-14	8.07	4.19	1.29		
Leu-15	7.90	4.22	1.58	1.45	Hδ1* = 0.88, Hδ2* = 0.81
Trp-16	7.85	4.64	3.30		
Asp-17	8.09	4.62	2.72, 2.63		
Met-18	8.04	4.35	2.12, 2.02	2.62, 2.51	
Gly-19	8.28	3.93			
Ala-20	8.01	4.29	1.38		
Val-21	8.00	4.02	2.12	0.98, 0.95	
Ser-22	8.21	4.37	3.86		
Met-23	8.21	4.45	2.11, 2.06	2.62, 2.53	
Leu-24	8.02	4.27	1.59	1.68	Hδ1* = 0.85, Hδ2* = 0.93
Lys-25	8.12	4.19	1.82, 1.68	1.38	HE* = 2.99, Hδ* = 1.49
Asn-26	8.18	4.67	2.86, 2.78		Hδ21 = 7.57, Hδ22 = 6.91
Leu-27	8.03	4.32	1.68	1.59	Hδ1* = 0.85, Hδ2* = 0.92
Ile-28	7.95	4.04	1.83	1.43, 1.16	Hδ* = 0.82
His-29	8.46	4.52	3.33, 3.21		
Ser-30	8.21	4.44	3.90, 3.86		
Lys-31	8.40	4.31	1.81, 1.75	1.43	Hε* = 2.99, Hδ* = 1.70
His-32	8.43	4.68	3.27, 3.17		
Lys-33	8.38	4.30	1.76, 1.72	1.44	Hε* = 3.00, Hδ* = 1.69
Met-34	8.46	4.50	2.06, 2.01	2.59, 2.54	
Ile-35	8.17	4.17	1.85	1.47, 1.19	Hδ* = 0.91
Ala-36	8.37	4.35	1.38		
Met-37	8.36	4.49	2.12, 2.03	2.64, 2.56	
Gly-38	8.45	4.00			
Ser-39	8.22	4.47	3.89		
Ala-40	8.33	4.36	1.41		
Ala-41	8.05	4.23	1.39		
Ala-42	8.16	4.29	1.38		

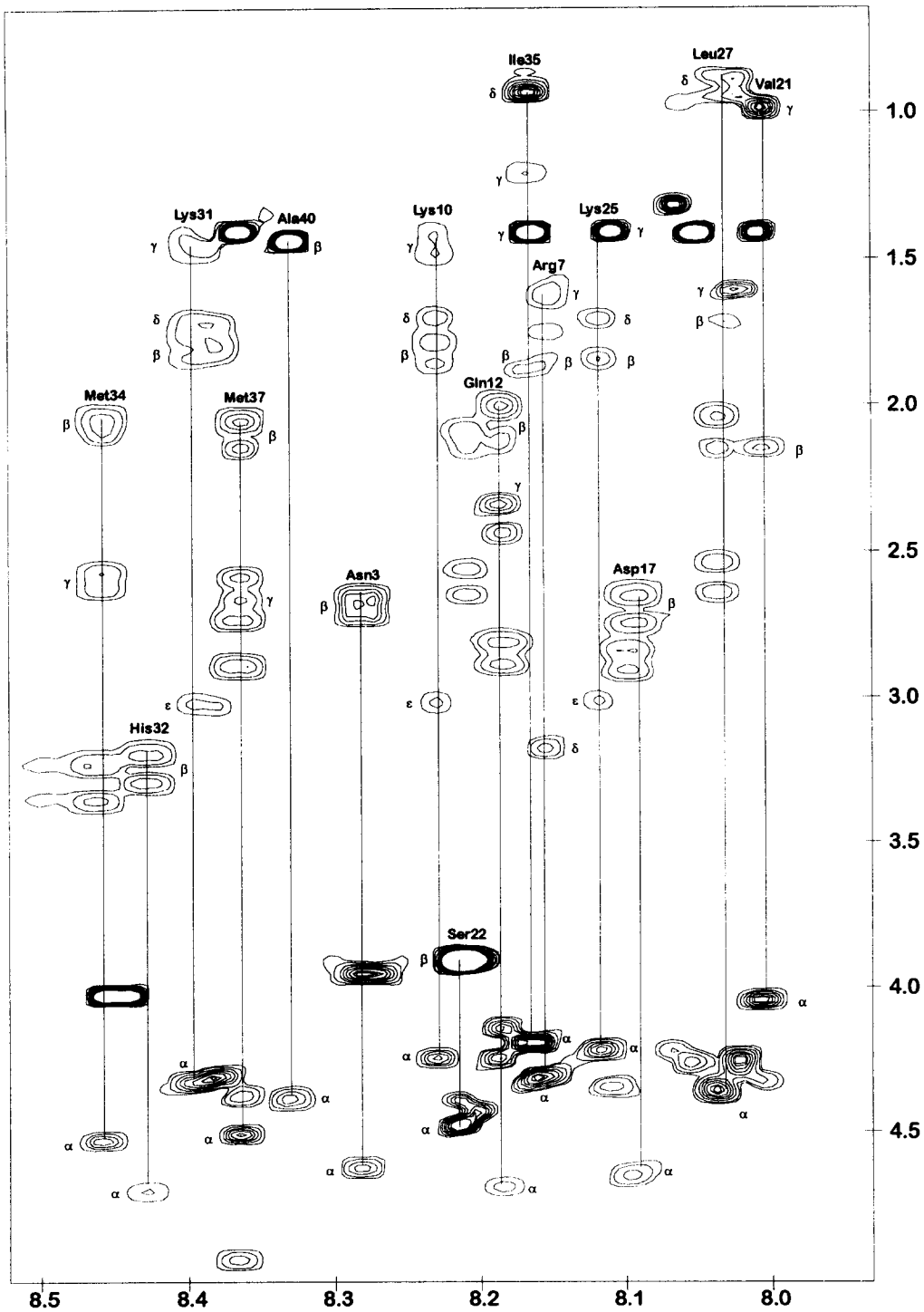

Fig. 2. Part of a 500-MHz CLEAN-TOCSY spectrum; mixing time 100 ms; 298 K. The spin system assignment is indicated.

nances with the same sense chemical shift deviation for a stretch of more than three sequential residues should be taken into account. In agreement with the secondary structure predictions this method estimates a short helical region (Lys-10 to Ala-14).

Computational calculations (see section 2) were started from initial random coil conformations. Structures were optimized by simulated annealing protocol and energy minimization [24]. Structure determination depends on 189 NOE restraints including 132 sequential and 51 medium range NOEs (i to $\leq(i+4)$) (see Fig. 4). Long-range NOEs (i to $\geq(i+5)$) which contain further information about the tertiary structure of the protein could not be observed. From 40 calculated structures, 10 converged structures were selected having the smallest restraint violations and energies. These 10 structures are well defined. For residues 8–16 and 20–26 the rms deviations from the mean calculated structure for the backbone atoms were 1.125 and 1.135 Å (see Table 2). Stereochemistry of the structures, as judged by the ϕ and ψ angles in a Ramachandran plot and by energy calculation, is acceptable, since 43% of all residues lie within the most favoured region

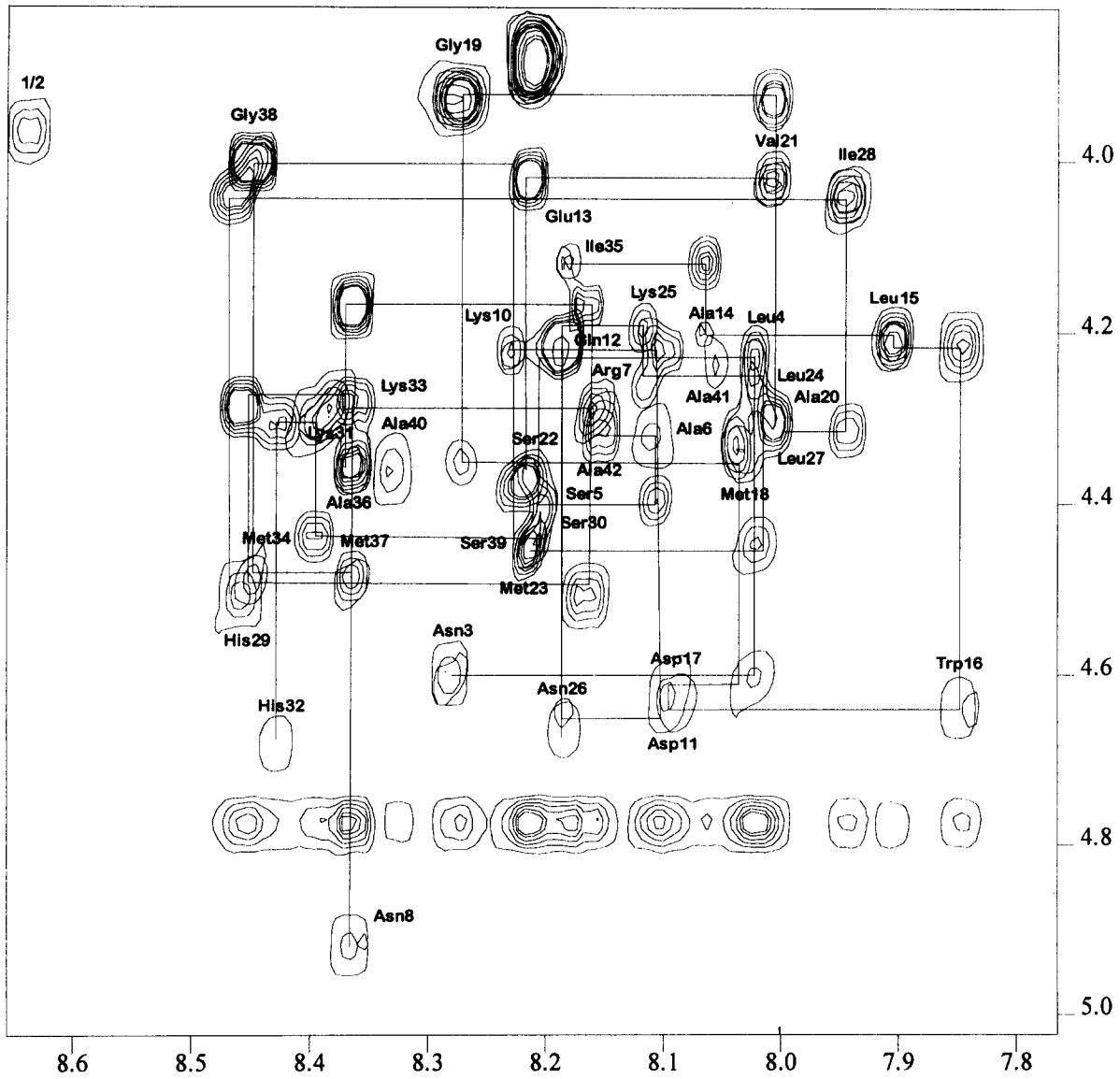

Fig. 3. Sequential αN connectivities used in the assignment of the arm repeat. Spectra shown are a NOESY (200 ms mixing time) and a CLEAN-TOCSY by superimposition, recorded at 298 K. The lines demonstrate the chain tracing procedure.

of the Ramachandran ϕ,ψ plot and 50% in the additional allowed regions [28]. On superimposing the ten structures after least-squares fitting of residues 8–16/20–26, the C-and N-terminus fan out in a variety of directions (see Fig. 5).

A comparison of the NOEs between the structural elements of the renatured peptide and the non-denatured peptide are quite similar (data not shown). These results could be established by computational calculations of 40 structures and superimposing the mean structures of these two peptides.

4. Discussion

The solution structure of a synthetic peptide corresponding to a single arm sequence motif was solved by two-dimensional nuclear magnetic resonance spectroscopy. The analysed peptide is the sixth of seven tandem arm repeats of the tumor suppressor protein APC.

The peptide shows 43% sequence homology to other APC arm repeats and at least 37% homology to repeats of further known arm proteins [2]. Thus, the presented results may be considered as a first step towards the structural analysis of other proteins containing the arm motif.

Table 2
Structural statistics and atomic rms differences

Deviations from idealized geometry	
Bonds (Å)	0.0032 ± 0.00034
Angles (°)	0.57 ± 0.052
Impropers (°)	0.38 ± 0.05
NOEs (Å)	0.057 ± 0.0048
XPLOR potential energies (kJ/mol)	
F_{bonds}	28.41 ± 6.4
F_{angles}	252.69 ± 46.3
$F_{improper}$	31.49 ± 8.36
F_{vdW}	125.17 ± 36.4
F_{NOE}	130.05 ± 22.7
F_{total}	567.80 ± 117.1
Atomic rms differences to mean structure (Å, residues 8–16/20–26)	
Backbone	1.125 ± 0.53/1.35 ± 0.15
All heavy atoms	1.902 ± 0.97/1.867 ± 0.37

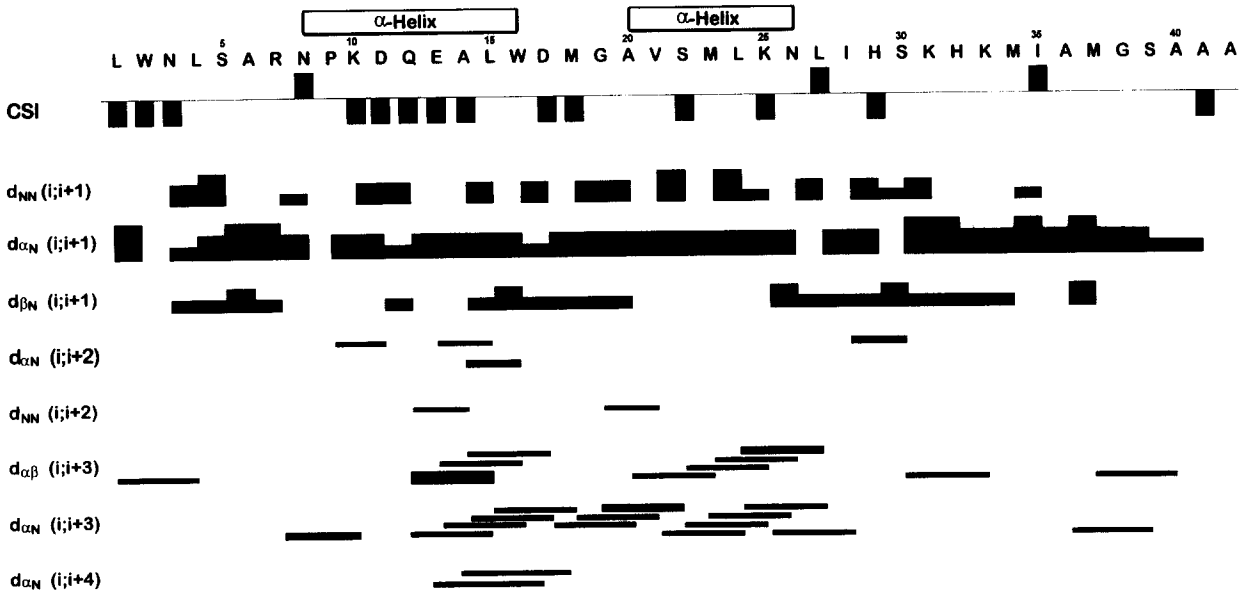

Fig. 4. Sequence of the analyzed arm repeat with a survey of sequential and medium range NOEs which are relevant for secondary structure determination. The derived secondary structure is shown at the top. The thickness of the lines corresponds to the magnitudes of the NOEs, i.e. weak, medium and strong.

All secondary structure predictions of this peptide provide a helical structure for more than 50% of the residues. Our NMR data deduced from chemical shift values and structurally related NOE connectivities result in two stable α-helices of 16 of the 42 amino acids. According to sequential and medium-range NOEs α-helical character is especially apparent between residues Asn-8 and Trp-16, interrupted by three amino acids and continues up to residues Ala-20 to Asn-26 (see Fig. 5). The N-terminal 7 amino acids and the C-terminal 16 amino acids of the single arm peptide show no indications to form any specific secondary structural element. The unfolded character of the C-terminus suggests a structure dependence of the sixth arm repeat from the entire arm motif. The tertiary structure could not be further defined since no long-range NOEs could be observed.

The CD data also indicate a tendency of the single peptide to form helical elements in solution. According to the helix calculation procedure by Holzwarth and Doty [25] the amount of amino acids forming helical structure is about 15%. According to to Gans et al. [29] endgroup effects of short helical elements reduce the total amount of α-helix estimated by CD spectra. This could be a possible explanation for the low helical value found by our CD measurements.

Comparing the structures of the non-denatured and renatured protein after urea denaturation, we noticed that they are similar, with the same topology of folding. The structural identity is in good agreement with a stable conformation for a single repeat under the applied experimental conditions. The refolding ability of the peptide indicates a stable secondary structure.

A comparison with known tertiary structures of other proteins with repeating sequence motifs or repetitive structural

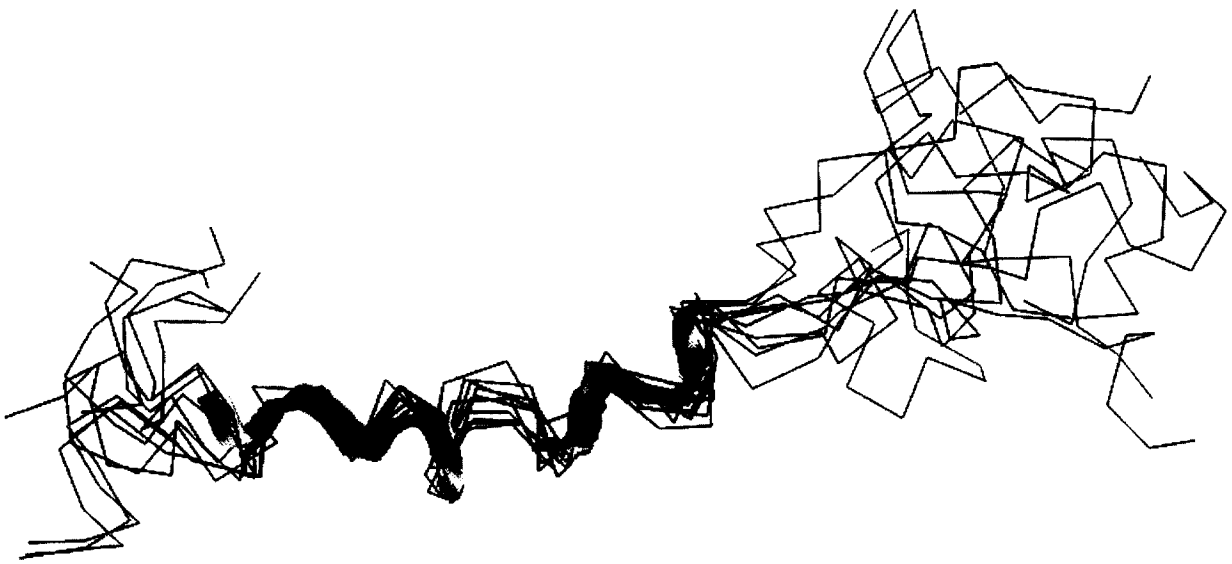

Fig. 5. Superimposition of the backbone atoms for 10 refined structures with a ribbon running through the lowest energy structure.

units seems appropriate to predict a possible tertiary structure of the complete arm domain based on the structure of the single repeat. Several repeating sequence motifs and repetitive structural units such as the ankyrin repeat and the leucine-rich repeat have been described. Individual ankyrin repeats are not capable of folding independently [30]. Structural subdomains of ankyrin consist of six repeats whereas at least two six-repeat domains are required for interaction with other proteins. Despite the ankyrin repeat the leucine-rich motif is an example of a sequence motif forming an independent structural unit [31]. Leucine-rich repeat proteins contain tandem arrays of β-sheet-loop-α-helix-loop units [32]. Another example for a repetitive sequential unit is the WD-40 repeat. This motif consists of four different β-sheets each belonging to an individual tertiary structural domain (for review see [33,34]).

The fact that at least seven tandem repeats are present in any arm protein is a hint of the necessity for more than one arm motif for proper folding in vivo. Taken together with our results this hypothesis is consistent with recent biochemical results showing that exons rather than individual arm motifs encode functional domains of arm proteins [10], although the possibility that single arm motifs form individual secondary structural units and thereby an overall tertiary structure which is similar to the leucine-rich repeat structure or to the coiled-coil structure still cannot be excluded. In addition, the folding of the repeating arm motifs into an as yet undescribed tertiary structure is possible as well. Only the structural analysis of the complete arm domain may show a properly folded tertiary structure.

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