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Does a synthetic peptide containing the leucine-zipper domain of c-myb form an α-helical structure in solution?

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Abstract

We have examined a synthetic peptide containing the putative leucine zipper domain of the chicken c-myb proto-oncogene using circular dichroism (CD) spectroscopy. The peptide adopts an α-helical structure only at low temperatures and in the presence 2,2,2-trifluoroethanol.

Key words: c-myb; Proto-oncogene; Leucine zipper; Circular dichroism spectroscopy

1. Introduction

The leucine zipper is a sequence motif common in many transcription factors, which mediates dimerization of proteins via hydrophobic interaction [1]. In a leucine zipper domain, four to five leucines are spaced by six amino acids, the structure of the dimer is an α-helical coiled-coil [2]. A coiled-coil leucine zipper requires not only the conserved leucines at position d in a given sequence $(abcdefg)_n$, also hydrophobic residues in helical position a and charged amino acids in the positions e and g required to generate an specific dimerization via amphipatic helices [3].

After the first publication on the C/EBP leucine zipper dimerization motif [4], a variety of other proteins have been reported to contain a leucine zipper domain. Most of these proteins exhibit sequence-specific DNA-binding activities. Usually this activity is mediated structurally by a distinct DNA-binding motif, such as helix-loop-helix (e.g. c-myc, MyoD [5]), basic-region (e.g. c-fos, c-jun [6]) or homeodomain (e.g. athb-1, athb-2 [7]) flanked in near vicinity by a leucine zipper domain able to form homodimers or heterodimers with other proteins. In this regard, the putative leucine zipper of the c-myb protein is rather unique, since it is substantially separated (~ 200 amino acids) from the DNA-binding domain [8].

The proto-oncogene c-myb encodes a protein of 75 kDa which is believed to act as an transcription factor. Analysis of its gene product revealed, that it consists of three functional domains responsible for DNA-binding, transcriptional activation and negative regulation [9]. The c-myb protein is phosphorylated in a cell-cycle dependant manner [10], phosphorylation abolishes its specific DNA-binding activity which was shown to reside in a tripartite imperfect repeat of ~ 51 amino acids at the N-terminus [11]. In each of these repeats three tryptophans are spaced by 18 or 19 amino acids, a motif conserved in myb proteins of all species. Furthermore, the DNA-binding activity is regulated by the redox status of a conserved cysteine at position 130 [12]. A transcriptional activation and a negative regulatory domain have been mapped adjacent to the carboxy-terminus of the DNA binding domain [9]. Whereas an acidic patch in the transcriptional activation domain, characteristic for transcriptional activators, is thought to interact with other factors, a putative leucine zipper region is located in the negative regulatory domain [13]. Recently it was reported, that this zipper mediates in vivo homodimer formation of myb proteins which interferes with DNA binding [14]. This negative autoregulation of c-myb activity was demonstrated in co-transfection assays and by cross-linking synthetic peptides containing the murine c-myb leucine zipper. A mutant peptide, in which leucines 3 and 4 were replaced by proline, failed to cross-link and to dimerize to the wild type protein. However, the authors did not provide a direct proof, that the leucine zipper adopts an α-helical structure necessary for dimerization. We therefore examined the putative leucine zipper domain of chicken c-myb with circular dichroism spectroscopy. We could demonstrate that an α-helical

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This paper is dedicated to Prof. Dr. Günther Maass on the occasion of his 60th birthday.

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structure is adopted only at low temperatures and in the presence of 10% 2,2,2-trifluoroethanol.

2. Materials and methods

The peptides MIVHQGTILDNVKNLLEFAETLQFIDSFLNTS (amino acids 375–407 of the chicken c-myb protein) and HYNDEDPEKEKRIKELELLLMSTENELKG (amino acids 283–311) were synthesized on 9050 peptide synthesizer using standard Fmoc-chemistry. Crude peptides were purified by preparative HPLC on a Vydac C18 column. Identity and purity was checked with HPLC (Vydacx C18) and ESI mass spectrometry (Perkin-Elmer, Sciex API III).

Circular dichroism (CD) spectra were recorded in a buffer of 30 mM potassium phosphate, pH 7.2, 1 mM EDTA, 0.1 mM dithiothreitol, 0.01% (w/v) lubrol (PEDL) and 10% (v/v) 2,2,2-trifluoroethanol at 4°C in 0.01 cm cuvettes in a Jobin Yvon Dichrograph R.J. Mark III at a concentration of 1 $\mu g/\mu l$. The spectra were analyzed using the program CIRCULAR (kindly provided by Dr. F. Peters and Dr. J. Greipel, MHH). The temperature-induced denaturation of the zipper peptide was studied by recording the circular dichroism at 220 nm. The samples were heated from 4°C to 50°C with a rate of 10°C · h^{-1}, and cooled back to 4°C with the same rate.

Peptide cross-linking was performed as follows: 1–3 μg peptide in 1 ml PTDL buffer (30 mM potassium phosphate, pH 7.2, 10% (v/v) 2,2,2-trifluoroethanol, 0.1 mM dithiothreitol, 0.01% (w/v) lubrol) were incubated on ice with 0.0025% (v/v) glutardialdehyde for 60 min. 100 μl of a saturated solution of glycine were added, the mixture was then kept at room temperature for 30 min. The samples were precipitated with trichloro-acetic acid and separated on a 17.5% SDS-tricine-PAGE gel. The peptides were visualized with silver-staining.

3. Results and discussion

The leucine zipper of c-myb proteins differs in several aspects from 'classical' leucine zippers such as in GCN4, C/EBP, c-jun and c-fos proteins: it is not located adjacent to a DNA-binding domain, it does not possess a high density of charged amino acids (e.g. 35% in the GCN4 leucine zipper, 16% in the case of the c-myb leucine zipper), in contrast, 50% of its amino acids are nonpolar. Furthermore, leucines in position 1d and 2d of the putative c-myb leucine zipper there are a methionine and an isoleucine rather than leucine.

A substitution of a leucine by methionine or isoleucine at position d is tolerable, but leads to a decrease in the dimerization capacity, with methionine being the most and isoleucine being the least acceptable alternative to leucine [15]. Methionine and isoleucine in position 1 an 8 of the putative leucine zipper are conserved in the human, murine and chicken c-myb proteins as shown in a compilation (Fig. 1). Furthermore, a recent report on the dimerization specificity of leucine zippers [3], highlights the importance of salt bridges of amino acids in the g and e position (cf. Fig. 1) in dimer formation. There are no charged amino acids at these positions in the c-myb protein. However, as the c-myb leucine zipper has an unusual high content of 50% unpolar amino acids, the interface of a dimer would be formed by mainly hydrophobic interactions and not strengthened by salt bridges but rather weakend by one repellent electrostatic interaction (Fig. 2). Thus, it can be anticipated that this structure should have only a weak propensity to form a dimeric coiled-coil structure. Our CD data support this hypothesis: the polypeptide does not show much evalutative secondary structure in aqueous solution. We then recorded a thermal denaturation profile in the absence of trifluoroethanol (Fig. 4). The polypeptide denatures irreversibly at 28°C with a pretransition at 21°C. However the addition of 10% 2,2,2-trifluoroethanol, known to favour stabilization of secondary structure, allows the polypeptide to form an α-helical structure at 4°C, as demonstrated in the CD-spectrum (Fig. 3). An α-helix content of 90% was calculated according to the procedure of Chen et al. [16].

Since we could detect an α-helical structure in the presence of trifluoroethanol in the CD-experiment, we

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                  1111111222222233333334444444555
position          defgabcdefgabcdefgabcdefgabcdefg

human MYB         MIVHQGTILDNVKNLLEFAETLQFIDSFLNTS
                  ********************************
murine MYB        MIVHQGTILDNVKNLLEFAETLQFIDSFLNTS
                  *****  ***************** ********
chicken MYB       MIVHQSNILDNVKNLLEFAETLQLIDSFLNTS
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Fig. 1. Comparison of the putative leucine zipper regions of human, murine and chicken MYB proteins. Identical positions are marked with an asterix, positions d are underlined.

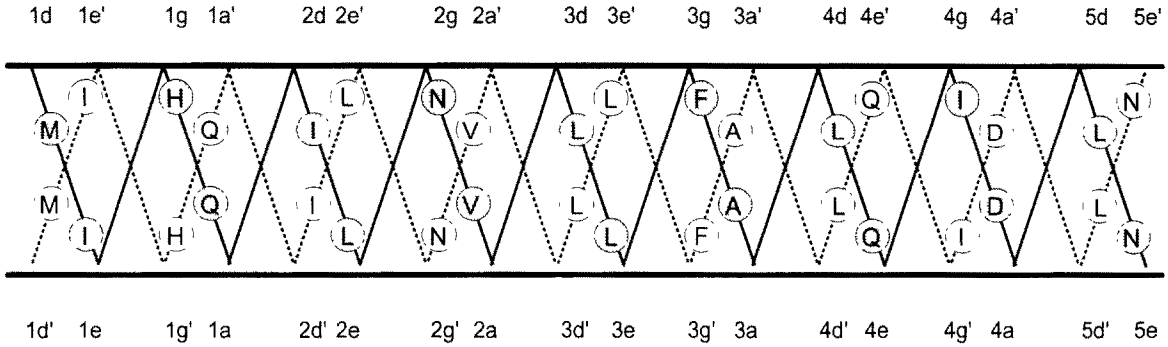

Fig. 2. Schematical delineation of the interface of two peptides in the structure of a parallel, two-stranded coiled-coil. The single letter code of amino acids is used. Hydrophobic interaction is expected between the a,a′ and d,d′ positions, no electrostatic interactions are found between g,e′- or g′,e-positions. Repellent interaction is postulated at aspartatic acids in positions 4a and 4a′.

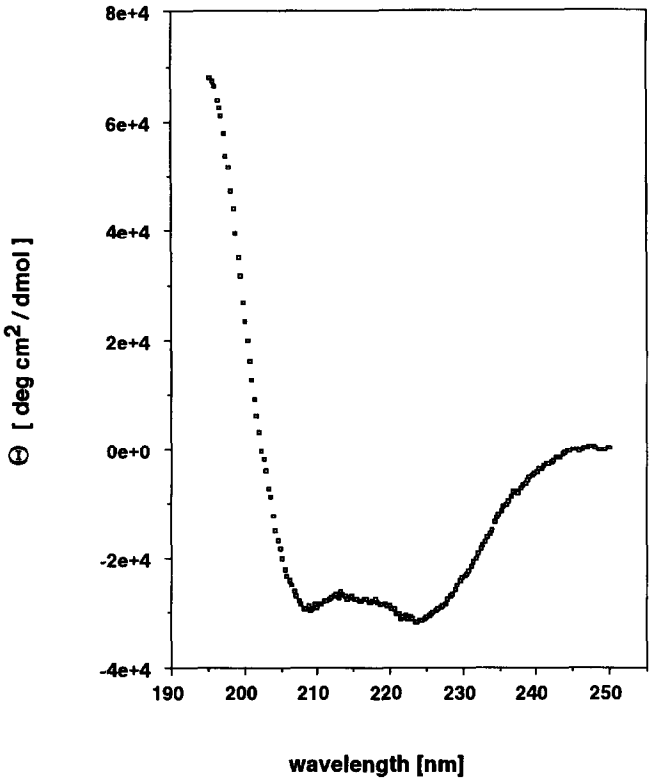

Fig. 3. CD-spectrum of 1 μg/μl zipper peptide in PEDL-buffer with 10% (v/v) 2,2,2-trifluoroethanol at 4°C. 90% α-helical content can be calculated from this spectrum using the procedure of Chen et al. [16].

asked the question, whether we could detect dimers of the synthetic leucine zipper in solution on a gel filtration column, on native PAGE gels or in a chemical cross-linking experiment under conditions where the peptide is α-helical, i.e. in the presence of 10% trifluoroethanol. While the peptide eluted from a Sephadex G50 column (1 × 50 cm) as a monomeric species and migrated in a native 6% PAGE gel as a monomer, (data not shown), we were successful to cross-link the putative leucine zipper peptide with glutardialdehyde to dimers, trimers and higher multimers (Fig. 5), whereas the polar peptide HYNDEDPEKEKRIKELELLLMSTENELKG was not cross-linked. In conclusion, we do not detect dimer formation under equilibrium conditions (gel filtration, native gel electrophoresis) but under non-equilibrium conditions (cross-linking) we detect dimers, trimers and multimers. It is possible, that these dimers are coiled-coils, but it is more likely that they are aggregates, formed via hydrophobic interactions, a result of the high content of unpolar amino acids in this region.

A heptad repeat of leucines occurs widely in many proteins for probabilistic reasons, reflecting the role of the abundant amino acid leucine in stabilizing hydrophobic surfaces of α-helices [17]. This motif can be easily extended, when substitutions in the leucine positions are allowed. The hydrophobic area defined by such 'mini-zippers' may be important for intermolecular interactions [18] or serve as a structural requirement for the tertiary structure of a protein without the necessity to form parallel dimers of a two-stranded α-helical coiled coil structure as determined for the basic-region leucine zipper of GCN4 [2].

We have emphasized that the putative leucine zipper of the c-myb protein deviates in many aspects from the leucine dimerization motif found in other DNA-binding proteins, namely in its unusual amino acid composition and its distance to the DNA-binding domain. Furthermore, we show, that a synthetic peptide containing the leucine zipper region of c-myb does not dimerize in solution under conditions where it adopts an α-helix. The CD-data and the fact that the peptide is easily denatured, suggest that the α-helical structure of the isolated leucine zipper is not stable under physiological conditions. On the other hand, it is conceivable that the trifluoroethanol-induced stabilizing effect on the α-helical structure of the isolated peptide is somehow compensated by the flanking amino acids in the full length c-myb protein. A highly hydrophobic but unstructured area might be necessary for the tertiary structure of the protein.

In conclusion, we show that a peptide containing the putative leucine zipper region of the chicken c-myb protein can adopt an α-helical structure only at very re-

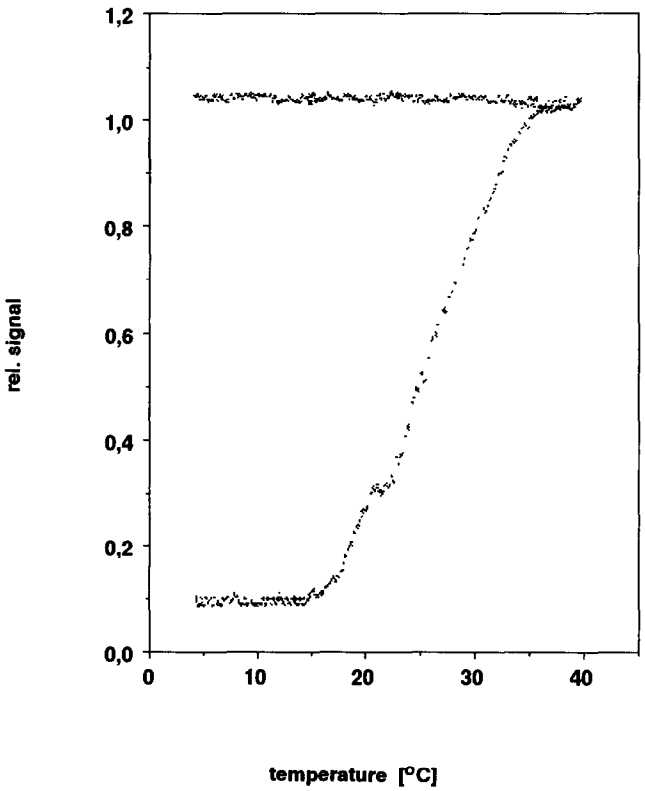

Fig. 4. Thermal denaturation profile of the zipper peptide. The relative signal is plotted versus the temperature. A small peak at 21°C can be interpreted as a pre-transition, total denaturation is achieved at 28°C. Shifting the temperature back from 38°C to 4°C yields no regaining in structure.

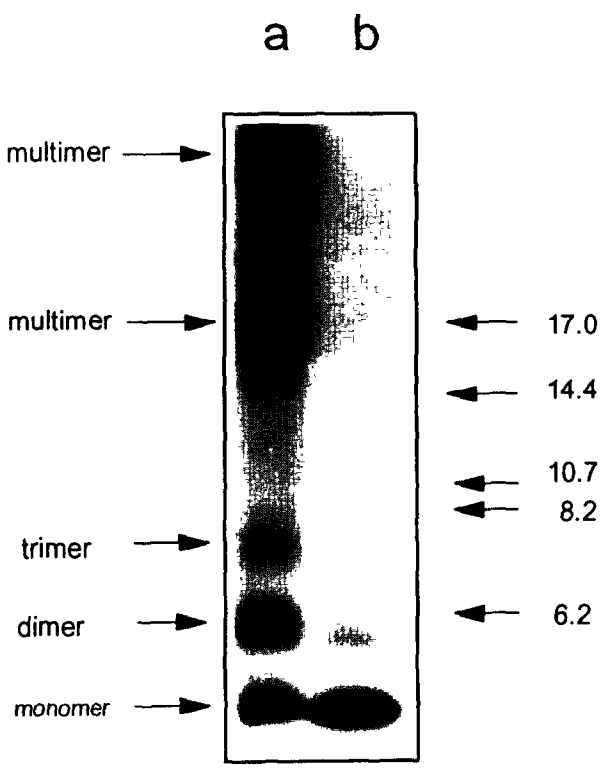

Fig. 5. Chemical cross-linking of the leucine zipper peptide with glutardialdehyde. Lane a, 3 μg/ml peptide; lane b, 1 μg/ml peptide. Molecular weight positions and monomer, dimer, trimer and multimeric species are marked by arrows. The apparent molecular weight of the peptide is smaller than determined by calculation.

stricted conditions: low temperature and the presence of a stabilizing agent. We can not rule out the possibility that this region is involved in intramolecular interactions, but we find little evidence that this sequence is involved in c-myb homodimer formation via a coiled-coil structure.

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