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EXPRESSION AND STRUCTURAL ANALYSIS OF 14-3-3 PROTEINS

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INTRODUCTION

The 14-3-3 family of proteins was so named due to its migration on position on two-dimensional DEAE and gel electrophoresis (1). These proteins all have a molecular mass of around 30 kDa and exist as dimmers (2). To date, seven to eight mammalian brain isoforms of 14-3-3 have been described, named alpha-eta after their respective elution positions on HPLC (3). Five of these have been sequenced (4) and the alpha and delta isoforms are identical in primary structure to the beta and zeta isoforms respectively, but differ only in a post-translational modifications (5). The 14-3-3 family is highly conserved and individual isoforms differ by 1 to 5 mainly conservative amino acid substitutions. Isoforms have also been described from other mammalian tissues which are absent or present at low levels in the brain. These include an isoform found in T-cells (6) and one found in epithelial cells (7, 8). These have been named tau and epsilon respectively (5).

In this multi-disciplinary study we have used isoform-specific antibodies to analyse the domain structure of members of the 14-3-3 family after digestion with proteases. We concentrated on two isoforms of 14-3-3: tau, which is found at low levels in all tissues tested to date, and epsilon, which is found at high levels in brain and other tissues. Intact tau isoform and various deleted forms of tau were expressed in *E. coli*. Regions of the protein involved in dimerisation and membrane attachment were determined, and the nature of the phosphorilation by protein kinase C was analysed. In this way we have started to dissect the structure of 14-3-3 proteins and their function as regulators of protein kinase C.

RESULTS AND DISCUSSION

Using antisera specific for the N-termini of 14-3-3 isoforms described previously and an additional antiserum specific for the C-terminus of epsilon isoform, protease digestion of intact 14-3-3 showed that the N-terminal half of 14-3-3 (a 16 kDa fragment) was an intact, dimeric domain of the protein. This was confirmed by electrospray mass spectrometry.

Two isoforms of 14-3-3, tau and epsilon, were expressed in *E. coli* and secondary structure was shown by circular dichroism to be identical to wild-type protein. Expression of N-terminally-deleted epsilon 14-3-3 protein showed that the N-terminal 26 amino acids are important for dimerisation. Intact 14-3-3 is a potent inhibitor of protein kinase C, but the N-terminal domain does not inhibit PKC activity. Site-specific mutagenesis of several regions in the N-terminal of the tau isoform of 14-3-3 did not alter its inhibitory activity. 14-3-3 proteins are found at high concentration on synaptic plasma membranes. This binding is mediated through the N-terminal 12 kDa of 14-3-3. Intact 14-3-3 are phosphorilated by protein kinase C with a low stoichiometry, but truncated isoforms are phosphorilated much more efficiently by this kinase. This may imply that the proteins may adopt a different structural conformation, possibly upon binding to the membrane, which could modulate their activity.

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INFLUENCE OF BASIC RESIDUES ON THE C-TERMINAL REARRANGEMENT OF PEPTIDES IN GAS PHASE

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INTRODUCTION

The combination of mass spectrometry and soft ionization techniques have demonstrated its usefulness in protein sequencing due to its capability to handle complex mixture and sequence unknown and chemically modified peptides.

The interpretation of the Collision Activated Decomposition (CAD) spectra often tend to be complex, specially when intrinsic interactions regulate the fragmentation of peptides in gaseous phase. When basic amino acids are located within the peptide sequence or at the N-terminus, due to the C-terminal rearrangement signals appear in the CAD spectra originated by the interaction of the C-terminal hydroxyl group with the carbonyl group of the nearest amino acid (2). The peaks corresponding to this rearrangement has 18 mass units more than its correspondent b_n serie and sometimes they are the most intense daughter ion in the spectrum such that specific sequences at the C-terminus could be misassigned.

We designed and synthesized a set of peptides in order to study some factors that could affect the appearance of this fragmentation such as the position of the basic residue within the sequence, the nature of the amino acids involved in the rearrangement and the way in wich the different basicity of two isobaric amino acids such as lysine and glutamine could be helpful to differentiate them taking into account the intensities ratio of $b_{n-1}/b_{n-1}+18$.

MATERIALS AND METHODS

Mass spectra were obtained with a JEOL JMS-HX110HF two sector mass spectrometer operated with a JEOL JMA DA-5000 data system, ionization with a 4 KV Xenon beam produced positive ions that were analyzed at 10 kV accelerating voltage and resolution 1000. In the B/E linked-scan measurement Argon was used as a collision gas to decrease the intensity of the precursor ion to 50%.

All synthetic peptides were obtained using the multiple solid phase peptide synthesis. Dried and protected peptide-resin were treated with HF containing the appropriate scavenger mixture according to the **Low-High** procedure.

RESULTS AND DISCUSSIONS

The CAD spectra of peptides labeled with ^{18}O at their C-terminus shows signals with different isotopic ion distribution (3). The C-terminal ions show doublet signals, the N-terminal ions their natural isotopic distribution and the rearrangement ions a distribution different form the two pools previously mentioned allowing us an easier identification of this phenomenon and thus avoiding misassignments of the sequence.

Two set of pentapeptides were synthesized, one of them has a common amino acid at the C-terminus (Phe) and the unique change is at the penultimate amino acid, in the other set the penultimate amino acid was fixed (Ala) and the C-terminal was changed.

To facilitate the comparison between peptides we evaluated the intensity ratio b_4+18/b_4 because it shows the probability for both fragmentation to be formed. In the first set of peptides this parameter was similar for all peptides suggesting that the nature and the bulkiness of the side chain of the amino acid involved in the rearrangement do not have appreciable influences in this phenomenon with the exception of Pro as judge by the