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Synthesis and secondary structure in membranes of the Bcl-2 anti-apoptotic domain BH4

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Abstract: Solid phase synthesis of BH4, the 26 amino-acid domain (^{6}RTGYDNREIVMKYIHYKLSQRGYEWD^{31}) of the anti-apoptotic Bcl-2 protein has been accomplished using Fmoc chemistry. The use of peculiar cleavage conditions provided high yields after purification such that tens to hundreds of mg could be obtained. A ^{15}N-labelled version of the peptide could also be synthesized for NMR studies in membranes. The peptide purity was not lower than 98% as controlled by UV and MALDI-TOF mass spectrometry. The secondary structure was determined in water, trifluoroethanol (TFE) and in lipid membrane using UV circular dichroism. The peptide shows dominant β-sheeted structures in water that convert progressively into α-helical features upon addition of TFE or membrane. The amphipathic character of the helix suggests that the peptide might have a structure akin to those of antimicrobial peptides upon interaction with membranes.

Keywords: solid phase synthesis; reverse-phase HPLC; MALDI-TOF mass spectrometry; circular dichroism; non-protected tryptophan; apoptotic peptides, ^{15}N-labelled amino acids

INTRODUCTION

Cell regulation via programmed cell death is an essential part of life in most organisms [1,2]. This apoptosis is highly regulated, enabling the body to control its cell population during developmental processes such as embryogenesis, tissue remodelling, maturing of immune and neuronal systems [1–3]. Apoptosis destroys normal cells at the end of their lifetime and ensures proper removal of pathogenic cells, such as cancer and auto-aggressive immune cells. If this tight regulation fails, pathological cells can escape their fate – the programmed cell death – thereby leading to diseases such as autoimmune disorders, neurodegeneration and cancer [2,3]. A common theme for all these diseases seems to be a serious distortion of the interplay between pro- and anti-apoptotic factors, an imbalance enabling, for instance, cancer cells to survive and to escape from the body protection system [1–3].

In apoptosis, protein–protein interactions tightly regulate two major pathways, the death-receptor pathway with caspases as the main executioners, and a mitochondrial pathway with the interplay of the Bcl proteins family. The latter contains three different groups of proteins: the first one exhibiting anti-apoptotic activity, while group II and III promote apoptosis [4,5]. In the mitochondrial pathway – a major execution track in mammalian cells – pro- and anti-apoptotic Bcl proteins meet on the mitochondrial membrane surface and tightly regulate the fate of a cell by controlling the release of the pro-apoptotic cytochrome c from mitochondria. Proteins of group I such as Bcl-2 or Bcl-x_L can interact with the pro-apoptotic members (Bax family) and may form hetero dimers in membranes [6]. However, it appears that proteins are located mainly outside the membrane and their insertion and complex formation is not well understood [7].

In cancer, a potential overexpression of anti-apoptotic Bcl-2 families or a reduced expression of apoptotic factors can presumably prevent the desired cell death. Sequence analysis of the antiapoptotic Bcl-2 proteins revealed four short conserved domains (BH1–BH4) and a hydrophobic *C*-terminal part that may anchor the anti-apoptotic proteins such as Bcl-2 in the outer mitochondrial membrane [2–7]. The group II of apoptotic proteins such as Bax and Bak, have a similar overall structure except for the BH4 domain (^{10}DNREIVMKYIHYKLSQRGYEW^{30}). This *N*-terminal extracellular amphiphatic BH4 domain thus appears as a key factor for the regulative anti-apoptotic activity of Bcl-2 proteins. This conserved BH4 domain could protect cells from death by interacting with the mitochondrial membranes, thereby blocking any action of the apoptotic family members, and preventing release of cytochrome c. However, the regulative mechanism of this domain is still unknown.

The synthesis and the purification of the BH4 domain have already been reported by Peherstorfer *et al.* [8]

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and by Shimizu *et al.* [7]. Peherstorfer investigated the ability of synthetic peptides derived from proteins of the Bcl-2 family (defined by the sequence 10–30) to interfere with the apoptotic process in LLC-PK_1 cells. The BH4 was synthesized by Genemed Synthesis (South San Francisco, CA) but no information about the synthesis and the purification was provided. The synthesis was checked by means of mass spectrometry and a purity of ca. 95% was reported. Shimizu performed the synthesis of the human Bcl-2 BH4 (amino acids 7–30) with a model 396 multiple peptide synthesizer (Advanced ChemTech) by using diisopropylcarbodiimide/1-hydroxybenzotriazole-activated, fluorenylmethoxycarbonyl-protected amino acids. The purity of this peptide was determined to be 95% by MALDI-TOF spectrometry. Again no information on the synthesis, purification and yield were given, no HPLC or MALDI-TOF spectra were shown. Moreover, Lee and co-workers [9] performed the synthesis of three BH4 (^{2}AHAGRSGYDNREIVMKYIHYKLSQRGYEWD^{31}, ^{6}RSGYDNREIVMKYIHYKLSQR^{26} and ^{10}DNREIVMKYIHYKLSQR^{26}) using standard 1-fluorenylmethoxycarbonyl chemistry and the purification using a preparative reverse-phase high performance liquid chromatography. The purities of the peptides were confirmed by analytical reverse-phase chromatography and mass spectral analysis. No further information about the synthesis and the purification were given. They studied by circular dichroism the secondary structure of those three peptides and they estimated from analysis of band intensities that the proportion of α-helix content was ca. 27%, when embedded in detergent (SDS) micelles. No structure was found in aqueous solution.

In order to elucidate its interaction with membranes on a molecular level the BH4 (6–30) domain of the Bcl-2 protein (^{6}RTGYDNREIVMKYIHYKLSQRGYEW^{30}) and BH4 (6–31) (^{6}RTGYDNREIVMKYIHYKLSQRGYEWD^{31}) were synthesized. This was done with the goal of higher yield and purity than those reported. The complete synthesis protocol was revisited and refined. Because SDS micelles poorly mimic the membrane medium, the secondary structure was investigated by dichroism circular in negatively charged lipid vesicles that best represent the mitochondrial membrane. Proper deconvolution of CD traces was performed using robust software. This is the first step towards a comprehension of the interaction between Bcl-2 protein and a model membrane. As will be shown below, the peptide is mainly β-sheeted in water and converts into α-helix upon addition of membranes or TFE.

MATERIALS AND METHODS

Chemicals

Fmoc-Asp(OtBu)-Novasyn TGA and Fmoc-Trp-Novasun resin, 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyl-uronium hexafluorophosphate (HBTU), *N*-hydroxybenzotriazole (HOBt) and *N*-α-Fmoc-amino acids were purchased from VWR-NovaBiochem (Läufelfingen, Switzerland). Amino acids were protected as follows: t-butyl (tBu) for threonine, aspartic acid, glutamic acids, serine; t-butoxycarbonyl (Boc) for lysines, tryptophan; trityl (trt) for histidine, asparagine, glutamine; 2,2,4,6,7-pentamethyl-dihydrobenzofuran-5-sulfonyl (pbf) for arginines. ^{15}N-labelled amino acids (valine and leucine) were obtained from Euriso-top, groupe CEA (Gif-sur-Yvette, France).

N-Methylpyrrolidone (NMP), piperidine, dichloromethane (DCM), dimethylformamide (DMF), diisopropylethylamine (DIEA) and anhydride acetic acid were purchased from SDS (Peypin, France); trifluoroacetic acid (TFA) was obtained from Applied Biosystems (Courtaboeuf, France); triisopropylsilane (TIS) from ACROS Organics (Geel, Belgium) and 1,2-ethanedithiol (EDT) from Aldrich (Saint Quentin Fallavier, France). 1,2-Dimyristoyl-sn-glycero-3-phosphocholine (DMPC) and 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPG) were obtained from Sigma (UK).

Peptide Synthesis

The syntheses were performed on an Applied Biosystems 433A Peptide Synthesizer (PE Biosystem, Courtaboeuf, France) using Fmoc strategy [10] both for the 25- and 26-residue peptides. The polyethylene glycol-polystyrene (PEG-PS) resin was preloaded with an unprotected tryptophan substituted at 0.18 mmol g^{-1} and a protected aspartic acid substituted at 0.22 mmol g^{-1}. Fastmoc chemistry was carried out in four major steps per cycle: (i) deprotection of Fmoc groups by piperidine, (ii) activation of added amino acid with HBTU/HOBt (37.9 g/13.6 g) in 200 ml of DMF, (iii) coupling by amide link formation with a solution of 35% DIEA in NMP and (iv) capping to prevent truncated peptide elongation with acetic anhydride/DIEA/HOBt (19 ml/9 ml/0.8 g) in 400 ml of NMP. Each deprotection step was monitored by conductivity.

Cleavage from the Resin

The final peptide mixture was cleaved from its resin and deprotected in 94% TFA including the following scavengers: 2.5% EDT, 2.5% milli-Q water, 1% TIS. The solution was prepared at 4 °C and typically 10 ml was mixed to 0.4 g of peptide containing resin. Total deprotection and cleavage were achieved after 120 min in a covered Erlenmeyer. The peptide solution was then filtered under vacuum. The crude peptide was precipitated by adding 100 ml of cold diethyl ether and the cloudy aqueous phase was collected and centrifuged in a bench-top machine at 8000 rpm for 10 min. After removal of the supernatant, 5 ml of water was added and the sample was lyophilized.

Purification and Analysis

The crude peptide was dissolved in distilled water with 0.1% TFA, bath sonicated for 15 min at 50 °C and purified by reverse-phase HPLC (Waters Alliance 2695 with photodiode array detector) using a milli-Q water/acetonitrile gradient. Both aqueous (A) and acetonitrile (B) solutions included 0.1% TFA. A semi-preparative Vydac (Hesperia, USA) C4 column

(300 Å, 5 μm, 250 × 10 mn) was equilibrated in 100% of A at a flow rate of 3 ml/min. Absorption was monitored at 225 and 280 nm, the best wavelengths being determined by the UV photodiode detector. 8–16 mg of crude peptide was dissolved in 1.8 ml of solvent A and the sample was loaded into a 2 ml loop, injected immediately onto the column at room temperature and eluted over 38 min going from 100% to 68% of solvent A. All peptides were collected using ~70% of A.

MALDI TOF Mass Spectrometry

Matrix assisted laser desorption and ionization time of flight (MALDI-TOF) mass spectrometry was performed on a Bruker REFLEX III in the reflectron mode with a 20 kV acceleration voltage and a 23 kV reflector voltage. α-Cyano-4-hydroxycinnamic acid (Sigma) was used as a matrix, prepared as a saturated solution of 50% acetonitrile/0.1% TFA in water. Peptide was mixed in a ratio 1:1 (v/v) with the matrix solution. Samples were prepared with the dried droplet method on a stainless steel target with 26 spots. External mass calibration was achieved with a mixture of eight peptides having masses ranging from 961 Da (fragment 4–10 of adrenocorticotropic hormone) to 3495 Da (β-chain of oxidized bovine insulin).

Circular Dichroism (CD)

CD-spectra (Jasco J-720 spectropolarimeter, USA) were recorded at 1 nm intervals over the 190–250 nm wavelength range using a 1 mm pathlength quartz cell (Hellma, Germany). Eight scans were performed. The experiments were run at 50 nm/min and the temperature was fixed at 298 K. The peptide secondary structure was determined in water, TFE and lipid membranes that mimic the mitochondrial negatively charged external membrane in the same concentration of 50 μM. To prepare lipid vesicles DMPC/DMPG(2:1) were co-dissolved in $CHCl_3/CH_3OH$ (3:1 v/v) that was evaporated under vacuum. The lipid film was then hydrated in distilled water and freeze-dried. The fluffy powder was re-suspended with buffer A: TKEB (10 mM Tris, 10 mM KCl, 0.5 mM EDTA, pH 7.4) to obtain large multilamellar vesicles. The solution was subjected to three freeze-thaw cycles followed by pulsed sonication for 5 min at power 7 using a probe-type sonicator Soniprep 150 (MSE, USA) under cooling to obtain small unilamellar vesicles (SUV) to reduce light scattering. Metal particles possibly coming from the sonicating tip were removed by centrifugation.

To estimate the peptide secondary structure content, an analysis of the relevant CD-spectra was carried out using the CDPro software (http://lamar.colstate.edu/~ssreeram/CDPro) developed by R. W. Woody and coworkers [11]. Measured CD traces, CD_{meas}, were converted to mean residue ellipticity $[\Theta](\mathrm{deg\,cm^2dmol^{-1}})$ using the relationship $[\Theta] = CD_{meas}(\mathrm{degree})/(C(\mathrm{mol\,l^{-1}})1(\mathrm{cm})NR.10)$, where NR is the number of residues per peptide, l the cell length and C the concentration. The basis set 4 of the CDPro software was used [12]. Analysis was performed using three methods, CONTIN, CONTIN/LL and SELCON 3 [13]. In general, CONTIN/LL, a self-consistent method with an incorporated variable selection procedure, produced the most reliable results.

RESULTS

Peptide Synthesis and Purification

Three different synthesis pathways were tested with the aim of providing sufficient amounts of pure BH4 peptide for biophysical studies. The solid phase synthesis was carried out using the FastMoc strategy in a simple coupling mode, with 1 mmol of resin and a ten-fold excess of amino acids for the synthesis of BH4 (6–30) and BH4 (6–31). As the structural studies require large amounts of ^{15}N-labelled peptides, the synthesis of the BH4 (6–31) ^{15}N-labelled was carried out on a medium range scale: 2.5 mmol of resin and with a four-fold excess of amino acids.

The first synthesis was carried out with 555 mg of resin that was preloaded with unprotected Trp. From 555 mg resin only 135 mg of crude peptide was obtained after cleavage (yield of 54%). The peptide was

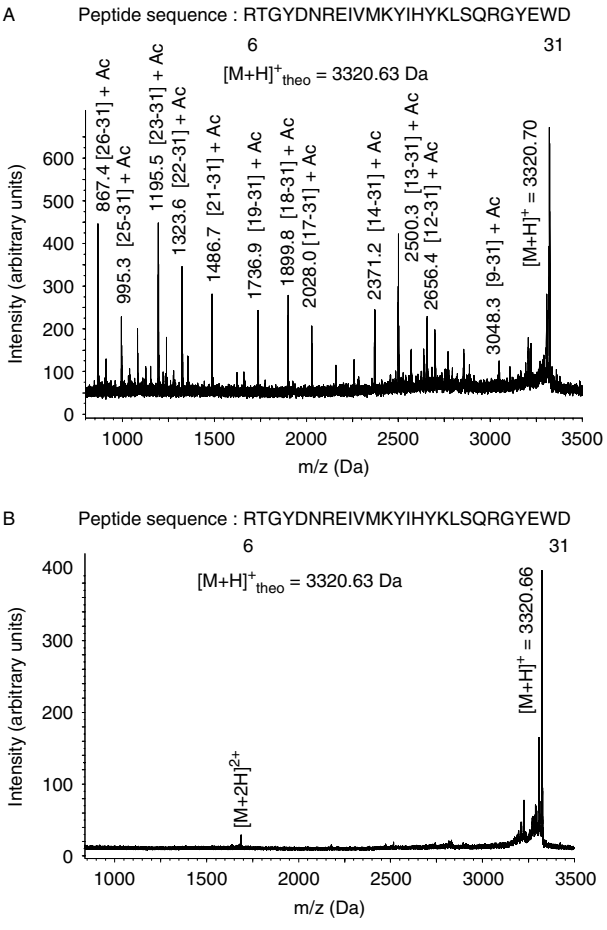

Figure 1 MALDI-TOF spectrum of (A) the crude reaction mixture containing the 26 amino acid long BH4 (6–31) domain of the Bcl-2 protein. The numbering in brackets [x-31] corresponds to truncated peptide segments. (B) BH4 (6–31) after purification by reverse phase HPLC. The second ionization product is observed at (M + nH)/(n), with a m/z of 1661 Da. The group of lines before the main peak comes from degradation by the MALDI laser.

characterized by MALDI-TOF (data not shown). Its main peak indicates a molecular mass of 3205.3 g.mol^{-1}, in agreement with the theoretical molecular weight of the 25 amino acids peptide of 3205.6 g mol^{-1}. In addition, there were several peaks, with one at m/z 3261 Da corresponding to the peptide with a tBu group (+56), one at m/z 3261 Da indicative of the peptide plus the resin linker (+164), and one at 3457 Da indicative of an attached pbf group (+252). The best purification by semi-preparative HPLC was achieved using a linear gradient with the following time intervals: eluent A varied in a 6 min linear gradient from 0% to 12%, then a 15 min linear gradient from 12% to 29% was applied and finally a 9 min linear gradient from 29% to 32% was used. BH4 (6–30) was eluted from the C4 reverse-phase HPLC column at a low percentage of acetonitrile (30%). The retention time was approximately 26 min. Absorption was monitored at 280 nm, the best wavelengths being determined by the UV photodiode detector. The BH4 (6–30) sequence contains five aromatic amino acids that have a high molar extinction coefficient and a high absorption peak at 280 nm. Only 1 mg of pure peptide (characterized by MALDI-TOF) was obtained.

In the second synthesis it was decided to make a 26 amino acid peptide, i.e. BH4 (6–31) extended to the next residue, aspartic acid (^{6}RTGYDNREIVMKYIHYKLSQRGYEWD^{31}). From 455 mg resin preloaded with aspartic acid, 188 mg of crude peptide was obtained after cleavage (74% yield). Figure 1 shows that the end capping segments are clearly seen, and the highest peak reflects BH4 (6–31), assuming that the full peptide was the principal product and no corresponding adducts were detected. The purification was carried out as before providing highly pure peptide. A typical elution profile is shown in Figure 2 with a major peak indicating pure BH4 (6–31) peptide. The high purity degree of BH4 (6–31) (around 99%), as required for structural analysis, was checked by HPLC (the chromatogram shows one peak integrated to obtain a degree of purity of 98%) and MALDI-TOF (Figure 1), no by-adducts are detected after purification. 67 mg of peptide was recovered giving a purification yield of ca 36%.

The final synthesis was performed with the aim of incorporating ^{15}N-labelled amino acids (valine and leucine) for NMR studies. The synthesis was carried out on a medium range scale (2.5 mmol of resin and with a four-fold excess of amino acids), using 1.136 g of an aspartic acid preloaded resin, from which 615 mg of crude peptide was recovered. Purification and characterization were carried out as before. A total 250 mg of pure peptide was obtained after purification that provided a yield of 41%. The final purity was 98% as checked by HPLC and MALDI-TOF.

Secondary Structure Analysis by Circular Dichroism of BH4 (6–31)

Circular dichroism was used to assess the secondary structure of the synthetic peptide under aqueous solution, TFE and upon interaction with small unilamellar vesicles (SUV). The peptide conformation was determined in water at pH 5, in TFE at pH 6 and upon interaction with SUV at pH 7.4. All experiments were performed at 25 °C.

The solid line in Figure 3 shows the CD spectrum in water and exhibits a minimum at 198 nm and a weak maximum at 230 nm. The weakness of the

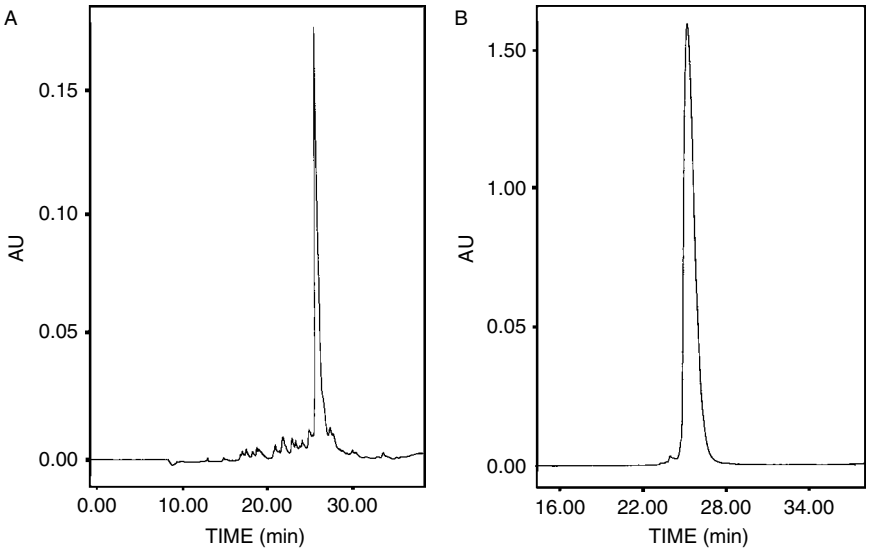

Figure 2 Photodiode UV chromatograms at 280 nm of (A) the crude peptide after cleavage from the resin, (B) the purified peptide. Both used reverse phase chromatography on a semi-preparative C4 column at a flow rate of 3 ml/min. The small peaks in (A) originate from truncated peptide segments. The major fraction was eluted after 26 min treatment with 30% of eluent B (see text).

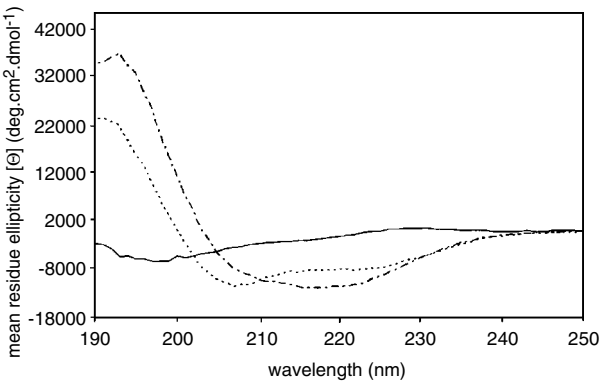

Figure 3 Circular dichroism spectra of BH4 (6–31) obtained in water (full line), in pure TFE (dash) and upon interaction with SUV of DMPC/DMPG 2 : 1 in a L/P molar ratio of 80/1 (dash-dot). The pH was 4.5 in pure water, 6 in pure TFE and 7.4 in the membrane buffer solution. Eight scans were accumulated at 25 °C. Scan speed was set at 50 nm/min.

signal is presumably due to the presence of a positive band at 225 nm arising from the four tyrosine residues that might interfere with a negative ellipticity band at 220 nm [14]. The tryptophan residue also has an irregular (depending of its environment) but high tendency to absorb in the entire spectral range (190–250 nm) [15]. It is well known that TFE promotes the α-helix conformation by strengthening the peptide H-bonds and may interrupt the hydrophobic interaction and then the ternary structure formation [16]. The dashed line represents the spectrum in TFE and displays minima at 208 and 222 nm and a maximum at 192 nm. No temperature-dependence is noticed in CD measurements indicated by no significant structural changes between 25 °C and 65 °C (data not shown). The dash-dot line in Figure 3 denotes the peptide spectrum in DMPC/DMPG vesicles. When small unilamellar vesicles are added in a ratio of 80/1 (L/P) a very broad minimum around 216 nm is detected and a maximum is reached at 193 nm, suggesting a mixture of α-helix and β-sheet conformations.

Deconvolution of CD traces was performed as described in the materials and methods to obtain accurate values of secondary structure elements using CDPro software [10] and an appropriate protein basis. The results reported in Table 1 show a higher peptide structure in membrane, with only 18% of random coil compared with ca 30% in solution (water and TFE). The secondary structure is mostly in β-sheet conformation (43%) in aqueous solution and turns into α-helix (38%) upon titration of SUV. As expected, TFE promotes highest contents of α-helix with 45%. Turns remain constant in the three media around 20%.

Table 1 Percentage of Secondary Structure Elements for the BH4 (6–31) Peptide in Water, TFE and upon Addition of DMPC/DMPG (2 : 1) SUV

Secondary structure	Water	SUV	TFE
α-Helix	5%	38%	45%
β-Sheet	43%	22%	10%
Turns	22%	22%	17%
Random coil	30%	18%	28%

Deconvolution of CD spectra was accomplished using basis 4 from the CDPro software and the CONTIN/LL algorithm (see text). Accuracy is estimated to be of ca 5%.

DISCUSSION

In this work, two main results appear: (i) an improvement in the synthesis and purification of the peptide BH4 in an effective way with a reproducible protocol and (ii) changes in the secondary structure from water to phospholipid model membranes. These different points will be discussed below.

High Yield and High Peptide Purity

The synthesis yield of the peptide BH4 (6–30) is 54%, whereas that of peptides BH4 (6–31) is 74%. Because the theoretical yield can be calculated to 77%, considering a 99% yield per step [17], it is seen that the BH4 (6–31) synthesis worked much better than that of BH4 (6–30). This first synthesis (6–30) was not very successful presumably because the resin was preloaded with unprotected tryptophan. Only 54% of peptide was cleaved from the resin. As suggested in the literature it is supposed that this low yield is not related to incomplete ester bond cleavage but merely to re-addition of the intermediate benzyl cation to the indole nucleus [18]. No further peptide was released from the resin after repeated treatment with trifluoroacetic acid. The *C*-terminal position of the tryptophan residue could favour the intramolecular process as this has already been suggested by Atherton and co-workers [18]. During the second synthesis a resin preloaded with aspartic acid was used to eliminate the risk of any side linkage to tryptophan. This residue was Boc protected to avoid any trouble [19]. The sequence contains some residues with delicate behaviour during the cleavage due to the presence of various nucleophilic functional groups in Trp, Met, Tyr, Arg. They are extremely susceptible to alkylation by cations produced during the cleavage process. Reaction of tryptophan, methionine with t-butyl cations results in modification of the product peptide. With the addition of scavengers such as EDT these side reactions can be suppressed. During the synthesis, the presence of thiols in the cleavage solution was necessary because it eliminated problems arising from cationic groups and reduced the number of adducts in the crude peptide. In general, the high yields of peptide obtained in the second and third synthesis trials are related to the dominant

hydrophilic character of the peptide sequence and the advanced methods recently developed in solid phase synthesis, such as the use of HBTU/HOBt activators and that of advanced solution cleavage procedures [17, 19–20]. Another crucial parameter involved in high yield is the cleavage time. During a long cleavage period protected groups such as pbf, are well removed, but the probability of a new complex between the linker and the indole ring of the tryptophan increases [21]. A period of 2 h was used here, providing the best compromise for the BH4 (6–31) peptide.

Structural Changes upon Binding to Vesicles

In contact with a model membrane that mimics the mitochondrial membrane, the β-sheet conformation of BH4(6–31) in water is converted into an α-helical structure. An α-helix was also reported by Lee and co-workers [9] upon addition of SDS micelles, whereas no structure was reported in water contrary to our results. The variance of the results in water is presumably due to the fact that deconvolution was not used to assess their conclusion. Although the results go in the same direction for structure in SDS and membranes, it must be noted that they worked on a longer peptide, BH4 (1–31), and estimated the helical content (27%) by comparison of 208 nm and 222 nm intensities, while our calculation showed 38% using solid deconvolution software. It must also be remarked that SDS micelles are less suitable for mimicking the mitochondrial membrane as they induce higher constraints by curvature and expose a smaller surface to the peptide. As the peptide is basic it can be proposed that the change in secondary structure going from water to a negatively charged surface is driven by electrostatic interactions, the flat membrane surface ensuring an additional structuring effect as opposed to small spherical micelles. TFE induced a higher α-helical structure than in SUVs; this is not surprising due to the well known role of this solvent in favouring hydrogen bonds and breaking aggregates. The helical character of BH4 upon binding to membrane gives an amphipathic character to the peptide where the distribution of hydrophobic and hydrophilic/charged residues may be located on different sides of the helix barrel. Interestingly this secondary amphipathicity is reminiscent of antibacterial peptides that are known to strongly interact with negatively charged membrane surfaces [22]. Further studies are being conducted in the laboratory to follow the possible perturbation effect of BH4 on membranes by solid state NMR.

The BH4 domain is thought to be the regulative part of anti-apoptotic Bcl-2 proteins. By looking thoroughly at the solution structure determined by solution NMR of the entire Bcl-2 protein in Tris and DTT buffer at pH 7.8 [23], the secondary structure of BH4 (6–31) in the protein is helical in majority (11–24) conferring to the peptide ca 50% of α-helix content. This is very close to the content found for the isolated peptide when interacting with membranes. It appears therefore that the BH4 part of Bcl-2 has an appropriate structure to interact favourably with negatively charged membranes. Because Bcl-2 proteins are presumably anchored to the membrane by their trans-membrane BH1 and BH2 domains, the contact of this amphipathic helix could ensure additional binding through electrostatic/amphipathic interactions that could in some cases lead to mitochondrial membrane instability or to compete for binding with pro-apoptotic proteins. However, additional experimental evidence are needed to assess this hypothesis.

CONCLUSION

Reproducible methods to prepare in large amounts and with very high purity the peptide corresponding to the BH4 domain of the anti-apoptotic human Bcl-2 have been developed herein. It must been remarked that starting the synthesis from a tryptophan residue presents some difficulties due to side-reactions on the indole ring and should be avoided whenever possible.

It must also been noticed that secondary structure changes upon membrane addition are better detected when proper lipidic membranes and deconvolution algorithms are used.

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