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Pro-apoptotic bax-α1 synthesis and evidence for β-sheet to α-helix conformational change as triggered by negatively charged lipid membranes

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Abstract: Solid phase synthesis of Bax-α1, the 25 amino acids domain (^{14}TSSEQIMKTGALLLQGFIQDRAGRM^{38}) of the pro-apoptotic Bax protein has been accomplished using Fmoc chemistry. A new fast and harmless protocol is described for complete TFA removal from the purified peptide powder leading to a final purity greater than 98% as controlled by ^{19}F-NMR, UV and MALDI-TOF mass spectrometry. Secondary structure was determined in various solution and membrane media using UV Circular Dichroism. In water solution, Bax-α1 is present as a mixture of β-sheet and unstructured (random coil) conformations. A marked change from β-sheet to α-helix secondary structures is observed upon interaction with negatively charged phospholipids vesicles whereas neutral lipid membranes have no significant effect on the aqueous peptide conformation. Results are discussed in terms of Bax binding to mitochondrial membranes. Copyright © 2006 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: solid phase synthesis; TFA removal; ^{19}F NMR; UV circular dichroism; electrostatic interaction; apoptotic peptides

INTRODUCTION

Understanding the regulation of the programmed cell death -apoptosis- is a challenge that can promote valuable information to treat diseases including autoimmune disorders, neuro-degeneration and cancer [1–4]. In general, pro- and anti-apoptotic members of the Bcl-family meet at the mitochondrial membrane, and arbitrate a life or death decision for the cell [2,5–7]. Once this internal pathway is activated the outer mitochondrial membrane gets permeabilized, thereby inducing the lethal and irreversible release of cytochrome C [8,9]. Normally, the anti-apoptotic Bcl-2 protein and its homologues prevent this process by keeping the pro-apoptotic proteins such as Bax and its homologues under control, thereby maintaining the integrity of the mitochondrial membrane [9–12]. Nevertheless, the process of such interplay is still poorly understood, but clearly the ability of Bax to bind at the mitochondrion outer membrane is a key step to induce apoptosis. Membrane proteins, such Bax, Bcl-XL or Bcl-2, possess helical anchor segments to promote insertion into the hydrophobic core of the mitochondrion membrane; they are usually located at the protein *C*-Termina part (CT). Substitution of the Bcl-XL CT by the Bax CT inhibits the insertion capability of Bcl-XL protein while the opposite leads to massive binding to the mitochondrion [13,14]. On the other hand, deletion of the protein *N*-terminal part (NT) impairs the binding of Bax to mitochondria, whereas a fusion of the NT terminus of Bax with a cytosolic protein results in the binding of the chimeric proteins to mitochondria, both in a cell-free assay and *in vitro* [15].

To elucidate both the conformation and the membrane insertion capability of this 'anchor' peptide, we decided to use an optimized solid phase peptide synthesis method [16] to produce reasonable amounts of highly pure Bax NT segment. During the purification process we faced the problem of elevated amounts of TFA contaminating the purified peptide, which was removed by implementing a special procedure. A circular dichroism (CD) study was thus undertaken in different media, including negatively charged phospholipid vesicles that mimic the mitochondrion outer membrane, to follow the Bax-α1 peptide structural modifications.

MATERIALS AND METHODS

Chemicals

Fmoc-Asp(OtBu)-Novasyn TGA 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-labeled amino acids (valine and leucine) as required for NMR structural

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studies 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).

Peptide Synthesis

The synthesis was performed on an Applied Biosystems 433A Peptide Synthesizer (PE Biosystem, Courtaboeuf, France) using the Fmoc strategy. The polyethylene glycol-polystyrene (PEG-PS) resin was preloaded with an unprotected methionine substituted at 0.23 mmol g^{-1}. Fastmoc chemistry was carried out according to reported procedures [16] 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 measurements.

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 added to 0.5 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. Adding 100 ml of cold diethyl ether precipitated the crude peptide and the cloudy aqueous phase was collected and centrifuged in a bench-top apparatus at 8000 rpm for 10 min. After removal of the supernatant, 10 ml of water/acetonitrile in a 60 : 40 v/v ratio was added and the solution was lyophilized.

Purification and Analysis

The crude peptide was dissolved in distilled water with 0.1% TFA and purified by reverse phase-high performance chromatography (RP-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 nm) was equilibrated in 100% of A at flow rate of 3 ml min^{-1}. Absorption was monitored at 225 nm. 5 mg ml^{-1} of crude peptide was dissolved in solvent A and the sample was loaded into a 2-ml loop, injected immediately onto the column at room temperature and eluted over 31 min going from 100% to 45% of solvent A. All peptides were collected using ~60% 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 the 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).

^{19}F Nuclear Magnetic Resonance

NMR experiments were carried out at room temperature on a Bruker Avance DPX 400 NB spectrometer. ^{19}F NMR spectra were acquired at 376.5 MHz, using a single pulse sequence. Typical acquisition parameters were as follows: spectral window of 8.8 kHz, $\pi/2$ pulse width of 13 μs. A recycle delay of 10 s was used and 45 scans were recorded with deuterium (D_2O) lock. Quadrature detection was used and a line broadening of 2 Hz was applied prior to Fourier transformation.

Model Membrane Preparation

Stock solutions of 10 mM dimyristoylphosphatidylcholine/dimyristoylphosphatidylglycerol (DMPC/DMPG) with different molar ratios were prepared by co-dissolving the desired amount of phospholipids in $CHCl_3/CH_3OH$ (3 : 1 v/v), which was evaporated under vacuum. The lipid film was hydrated with distilled water and lyophilized. The fluffy lipid powder was re-suspended in Tris buffer (10 mM Tris, 10 mM KCl, 0.5 mM EDTA, pH 7.4) to obtain large multilamellar vesicles (MLVs). The solution was subjected to 10 freeze-thaw cycles to homogenize the size of the vesicles. Large unilamellar vesicles (LUV) were then produced by size extrusion method, where the solutions were passed subsequently through 100 nm pore size polycarbonate filters under nitrogen pressure.

Circular Dichroism (CD)

CD-spectra (Jasco J-810 spectropolarimeter, USA) of peptide solutions upon titration with large unilamellar vesicles (LUVs, 100 nm diameter) of DMPC containing different amounts of DMPG were recorded between 190–250 nm, using a 1-mm path-length quartz cell (Hellma, Germany). Samples were allowed to equilibrate 15 min between each addition of LUVs. In order to estimate the peptide secondary structure content, an analysis of the relevant CD-spectra was carried out using the CDPro program [17–19]. The analysis was performed using the self-consistent method Contin-LL and the basis 10 that contains 56 reference proteins.

RESULTS

Peptide Synthesis and Evaluation

The synthesis of Bax-α1 (^{14}TSSEQIM KTGALLLQGF IQDRAGRM^{38}) was carried out on a medium range scale (0.25 mmol of resin and with a fourfold excess of amino

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acids), using 1.1 g of methionine preloaded resin. A mass of 1.9 g of dried peptide/resin was recovered. If the coupling efficiency were 100%, 0.25 mmol of peptide with protected groups (MW 6908.1 g mol^{-1}) would have yielded a crude protected peptide mass of 1.7 g. Here, 0.9 g of crude protected peptide was synthesized (i.e. ~0.13 mmol). Consequently, the coupling efficiency of the synthesizer corresponds to 97% per amino acid. After 2 h of cleavage in the appropriate solution of TFA and scavengers (*cf* methods), and precipitation in cold ether, 320 mg of crude peptide was obtained, which was close to the expected mass of 360 mg (0.13 mmol at 2752 g mol^{-1}). As it will be discussed below, residual TFA is still present as a counter ion for basic residues and seriously alters the crude peptide mass, which turned out to be only 250 mg. The crude peptide was characterized by MALDI-TOF spectroscopy (Figure 1(A)). The main peak indicates a molecular mass of 2751.3 g mol^{-1}, in agreement with the theoretical molecular weight of the 25 amino acids peptide of 2752 g mol^{-1}. In addition, there were several peaks, corresponding to truncated peptides stopped by the acetic acid capping during the synthesis. HPLC purification by semi-preparative C4 reverse-phase column was achieved using a linear gradient with the following time intervals applied: eluent B varying in a 15-min linear gradient from 0 to 32%, a 6-min linear gradient from 32% to 40%, a 2-min plateau at 40%, a 2-min linear gradient form 40% to 55% and finally a 2-min plateau at 0% was used to wash and equilibrate the columns for the next injection. 1.8 ml of crude peptide at 5 mg ml^{-1} in solvent A was injected per run at a flow rate of 3 ml min^{-1}. Bax-α1 was eluted from the column at 40% acetonitrile after 24 min. A typical elution profile is shown in Figure 2 with a major peak indicating pure Bax-α1 peptide. The high degree of purity of Bax-α1 (around 98%), as it is required for structural analysis, was checked using pure peptide by HPLC and MALDI-TOF (Figure 1(B), Figure 2 dashed line). Injection was done with the same gradient but using an analytic C4 reverse-phase column in order to improve the separation and detect any by-adduct. 0.5 ml at 5 mg ml^{-1} in solvent A was injected per run at a flow rate of 1 ml min^{-1}. No by-adducts have been noticed after purification. Total mass after purification was 85 mg giving a total synthesis yield of *ca* 12%.

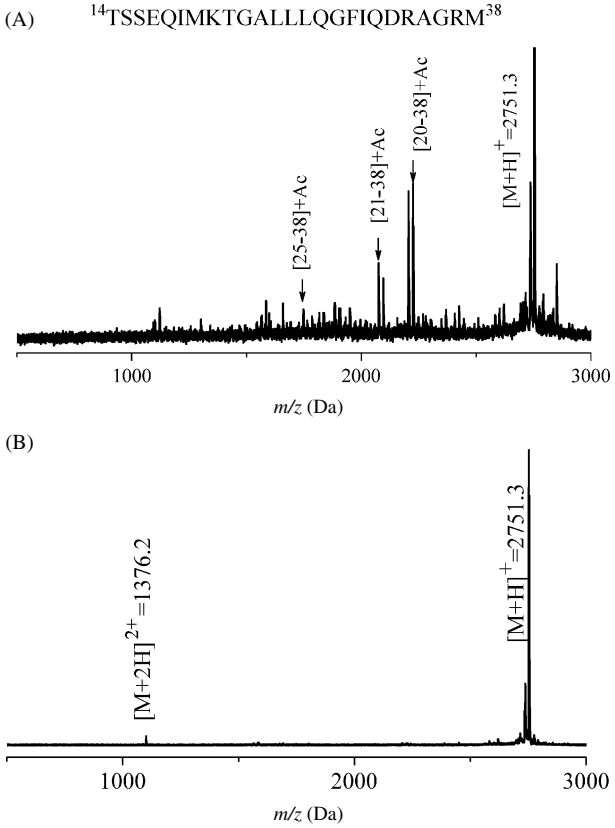

Figure 1 MALDI-TOF spectrum of (A) the crude reaction mixture containing the 25 amino acid long Bax-α1 peptide. The numbering in brackets [x-38] corresponds to truncated peptide segments. (B) Bax-α1 after purification by reverse phase HPLC. The second ionization product is observed at (M + nH)/(n), with a m/z of 1376 Da. The massif before the main peak comes from degradation by the MALDI laser.

TFA Removal by Counter Ion Exchange

During the cleavage step, TFA interacts strongly with the peptide: as the sequence of Bax-1 includes three basic amino acids (one Lys and two Arg), it is an ideal counter ion to equilibrate these charges. Remarkably, the interaction is so strong that TFA cannot be removed after solvent evaporation and large errors in mass determination can thus be made. To elucidate this behavior in a systematic way, solution state ^{19}F NMR was performed to accurately measure the amount of TFA and then calculate the ratio of counter ions per basic amino acids. 65 µl of TFE reference solution in $H_2O/D_2O/CH_3CN$ (40 : 35 : 25) at 10 mM in a capillary was thus inserted in 5 mm diameter NMR tube filled with a 600 µl solution of Bax-α1 (1 mM) in the same solvent mixture. In Figure 3, the top spectrum monitors out the TFA presence with a typical single resonance at 77 ppm while the reference TFE triplet resonances (due to J^{HF} couplings) appear at 75.7 ppm. Nevertheless, areas of the two peaks are directly comparable as TFE and TFA both contain the same amount of fluorine, i.e., three per molecule. Therefore, the area ratio provides a measure of the TFA concentration in Bax-α1 solution. A TFE/TFA ratio of 1 : 5 was calculated from the NMR spectrum (Figure 3), corresponding to a mass of 0.37 mg of TFA (*ca* 23% of the initial mass). Because 1.62 mg of powder from the synthesis/purification was weighed for the experiment, only 1.25 mg represents that of the pure peptide. Hence, the TFA/peptide molar ratio is 7 : 1 corresponding approximately to two counter ions per basic amino acid. As the TFA counter ions induce dramatic mass error and could be harmful for

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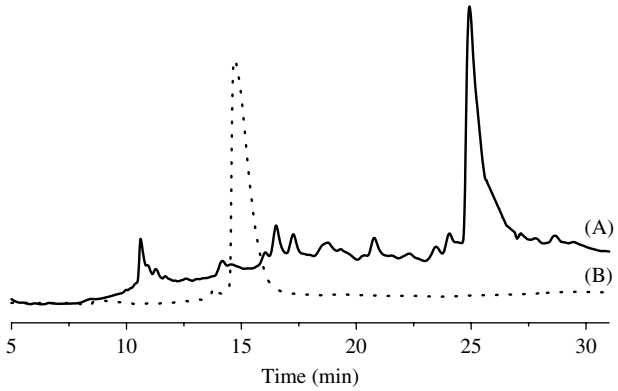

Figure 2 Photodiode UV chromatograms at 225 nm of the crude peptide (A) after cleavage from the resin (solid line) using a reverse phase chromatography on a semi-preparative C4 column at a flow rate of 3 ml min^{-1}. Photodiode UV chromatograms at 225 nm of the purified peptide (B) (dashed line) using a reverse phase chromatography on an analytic C4 column at a flow rate of 1 ml min^{-1}. See text for gradient settings. Please note that the Y-scale is not directly comparable between the two chromatograms due to the different injection volumes and flow rates.

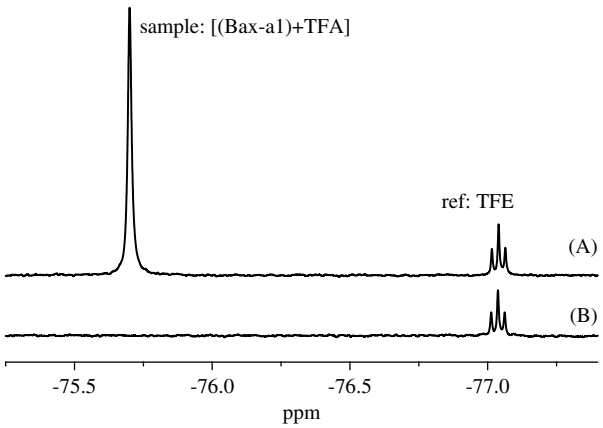

Figure 3 Solution ^{19}F NMR spectrum of Bax-α1 peptide in 600 µl H_2O/D_2O/ACN (40 : 35 : 25) at 1 mM within internal reference of TFE solution (65 µl in H_2O/D_2O/ACN (40 : 35 : 25) at 10 mM in a capillary) before treatment with HCl (A, top spectrum), after treatment (B, bottom spectrum). Triplet resonances arise from the J^{HF} couplings of the three fluorines in TFE and single resonance from the three fluorines in the TFA counter ions of Bax-α1. The sample/reference area ratio is 1 : 5. Experiment was done at room temperature on a 400 MHz Bruker spectrometer.

study in delicate media or in spectroscopic experiments (e.g. Infra-Red), it has to be replaced by a softer counter ion. Threefold molar excess of HCl per TFA was used in Bax-α1 solution under ice cooling during 20 min. Treatment with HCl acid, which has a pKa value (−7) lower than that of TFA (0.5), induces re-protonation of the TFA anions to form the free acid, which can easily be removed by freeze-drying. From 10 mg of peptide/TFA weighed, 7.8 mg of product was recovered after treatment, which indeed corresponds to the expected mass of the pure peptide released from TFA counter ions. Finally, mass spectroscopy (not shown) demonstrated that there was no peptide degradation induced by the procedure and ^{19}F NMR (Figure 3, bottom spectrum) reported the absence of residual TFA.

Secondary Structure Analysis by Circular Dichroism

The membrane-mediated conformational behavior of Bax-α1 was characterized by CD. The results for the peptide before and upon titration with LUV of varying surface charge are shown in Figure 4. In phosphate buffer, the membrane-free peptide exhibits a broad minimum between 222 nm and 208 nm with a weak maximum at 190 nm, indicating a mixture of secondary structure elements. Deconvolution using protein basis 10 and the self-consistent method contin-LL [17–19] lead to 13% of α-helix, 22% of β-turn, 31% of β-sheet and 33% of random coil structures (Table 1). Titration with zwitterioninc, pure DMPC LUV ranging from 10 to 100 lipid to peptide (L/P) molar ratio, induces no significant changes in the peptide secondary structure on comparing with the membrane-free state, (Figure 4, top panel). To evaluate any effect on Bax-α1 conformation induced by electrostatically driven association with membranes, DMPC LUVs containing increasing amounts of the negatively charged DMPG lipid were used. Adding LUVs composed of DMPC/DMPG at a 9 : 1 molar ratio, to Bax-α1 in phosphate buffer induces significant changes in the CD spectrum (Figure 4, middle panel), clearly depending on the L/P ratio. The titration with LUVs induces an isodichroic point at 206 nm and the presence of two marked negative dichroic bands at 208 and 222 nm, typical for helical structures. Nevertheless, the intensities remain low in comparison to those obtained for pure α-helix or β-sheet structure. The deconvolution results of these CD patterns as a function of the L/P ratios are shown in Table 1. Most remarkably, the α-helix fraction in the peptide structure increased on increasing the L/P ratio to reach up to 28% for L/P = 100. The importance of electrostatic interactions in binding is even more pronounced when increasing the DMPG contents in the LUVs up to 33 mol%. In Figure 4, bottom panel, the α-helical features dramatically increase, as visible in the strong increase of the typical dichroic band intensities. Deconvolution (Table 1) reveals a major α-helix population (60%) at a L/P ratio of 100 that had already almost reached at L/P = 30.

DISCUSSION

Besides the success in synthesizing the Bax-α1 peptide, the major outcome from this study is the clear evidence of a conformational change from a β-sheet secondary

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Table 1 Secondary structure of the Bax-α1 peptide[a] upon titration by DMPC LUV[b] containing increasing amount of DMPG

	L/P	α Helix[c]	β Sheet[c]	β Turn[c]	Random coil[c]
Phosphate buffer pH 7	—	13	31	23	33
DMPC	10	13	31	22	34
	30	13	31	22	34
	50	13	33	22	32
	100	12	36	21	31
DMPC/DMPG 9 : 1	10	14	30	23	33
	30	18	27	22	33
	50	19	28	22	31
	100	28	21	21	30
DMPC/DMPG 2 : 1	10	27	30	19	24
	30	54	16	11	19
	50	56	15	10	19
	100	60	15	8	17

[a] Concentration varying from 50 μM (L/P = 0) to 37 μM (L/P = 100).
[b] LUV of 100 nm diameter.
[c] Deconvolution of CD spectra was accomplished using basis 10 from the CDPro software and the CONTIN/LL algorithm [17–19]. Accuracy is estimated to be few percents.

structure to an α-helix in one of the peptides, upon interaction with model membranes that mimic the mitochondrial outer membrane. Also, a minor but nonetheless important result, is the new protocol to completely remove TFA ions from the synthesized peptide. These results will be discussed sequentially.

Electrostatically Driven Membrane Binding Induces Bax-α1 Helix Formation

While Bax-α1 peptide population is mainly partitioned between β-structures and unstructured conformations in buffer or uncharged vesicles, electrostatic interactions induce a pronounced transition from β-sheet to α-helical structures. Clearly, helix formation requires binding of the peptide to anionic phospholipids. Because no structural change happened in the presence of neutral lipid vesicles, no insertion by hydrophobic forces seems to occur, which too could have driven helical formation [20,21]. Here, not only the presence of the anionic PG lipid seems to be important, but also the absolute fraction of DMPG in the membrane. Correlating the helical fractions obtained in the presence of various DMPC/DMPG ratios with corresponding L/P ratios (Table 1), one remarks that ten negative charges per peptide (9 : 1 PC/PG and L/P = 100) lead to a helix content of *ca* 30% whereas 60% α-helix is obtained with 33 negative charges per peptide (2 : 1 PC/PG and L/P = 100). Of course the

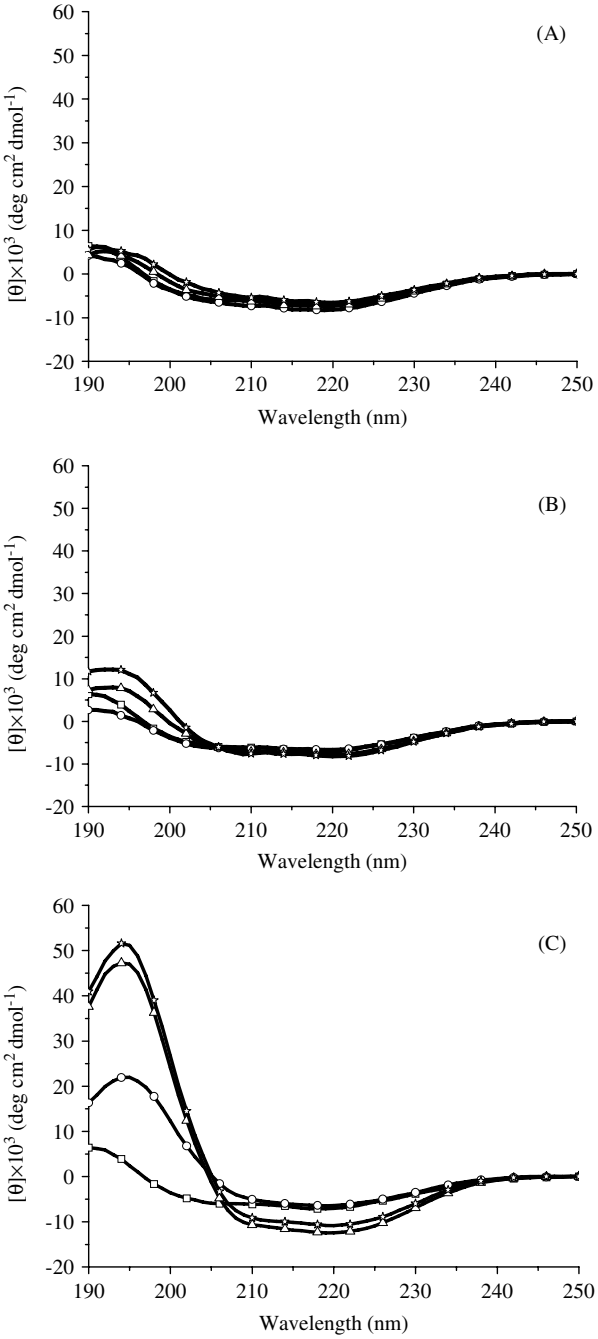

Figure 4 CD spectra of Bax-α1 peptide upon titration by DMPC LUV containing increasing amount of DMPG. Top panel (A) corresponds to LUV of pure DMPC, middle panel (B) to LUV of DMPC/DMPG in molar ratio in ratio 9 : 1 and bottom panel (C) to LUV of DMPC/DMPG in molar ratio 2 : 1. The lipid to peptide molar ratios (L/P) depicted in the three panels are 100 (star), 50 (triangle), 10 (circle) and pure peptide in phosphate buffer at pH 7 (square). 4 scans accumulated at 25 °C between 190 nm and 250 nm.

increase in helicity is not linear with the surface charge density (in principle, only half of the charges are available for the interaction, the others being in the inner vesicle monolayer) but this suggests that membrane patches of negative charges will clearly be in favor of

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a strong interaction with helix stabilization for Bax-α1. This suggests that two major factors govern this process: (i) the absolute concentration of anionic lipids, and (ii) the size of the available membrane surface for optimal association between peptide and negatively charged lipids.

At this level of the discussion it is interesting to consider the solution structure determined by solution NMR for the entire Bax human protein in Tris and DTT buffer [22]. At pH 6 the secondary structure of Bax-α1 (residues 14–38) within the whole protein is helical at 76% (residues 17–36). Because this segment alone exhibits mostly random coil or β-state in buffer (*vide supra*), it must therefore require stabilization either by hydrophobic or electrostatic interactions with other segments of the whole protein. Our data show no real interaction with uncharged liposomes whereas anionic lipids stabilize the helix. If the ability of Bax to bind the mitochondrion is mostly provided by its NT part [13,15], the helical conformation must play an important role and so do anionic lipids at the outer mitochondrion membrane. Recent results [23], on the interaction of Bax with negatively charged membranes showed the following results (i) in the presence of lipids the percentage of secondary structure elements as found from CD is essentially unchanged (67% α-helices, 23% β-structures and 10% random), (ii) the membrane prevents Bax from thermal denaturation and (iii) the *N* terminus is still available for apoptosis activity as probed by antibody binding capability. For the latter result, which is questioned by the authors, it is reported that activity is surprisingly obtained both with neutral or negatively charged membranes. Replacing our results in this context would suggest that the entire Bax protein would loosely bind to neutral membranes by preserving most of its solution structure by offering, nonetheless, its *N* terminus for activity whereas with negatively charged membranes, as in the outer mitochondrial membrane, strong electrostatic interaction with the Bax-a1 would happen, resulting in a larger exposure of the *N* terminus helix for greater activity as it could be guessed from the immunoprecipitation gel pictures reported by Andrews and coworkers [23]; the overall secondary structure is nonetheless preserved, although the topology of the helices could be changed upon membrane interaction. A structural study, at atomic resolution and upon interaction with membranes, is clearly required to understand further the mode of action.

A New Protocol to Efficiently Remove TFA During the Purification

It has been reported that TFA is considered a contaminant for various reasons. Firstly, we point out the dramatic error in mass weighing. Indeed, depending on the number of basic amino acids number in the peptide sequence, a nonnegligible amount of TFA, for instance, 23% in mass in our case, leads to serious inaccuracy as in molar concentration calculation, peptide/lipid ratio, etc... Moreover, light spectroscopy as IR is sensitive to TFA molecules since this salt leads to serious signal distortions and misinterpretation in spectra deconvolutions [24,25]. Also of importance in the course of membrane interactions is the fact that a peptide capped with TFA counter ions will not interact in the same manner with negatively charged membrane. The reverse situation could be obtained. Replacement of TFA counter ions by HCl has already been used for years [25], but was never addressed precisely. Here, we describe a simple fast and accurate method that uses 19F-NMR to ascertain that TFA is completely removed.

CONCLUSION

The peptide corresponding to the first helix of the pro-apoptotic human Bax protein has been synthesized with very high purity. TFA salt has been drastically removed in order to avoid any problem for further studies. UV CD in the presence of membranes of different nature has been efficiently used to monitor Bax-α1 secondary structure changes from a mostly β-sheet in water to a mainly α-helix upon association to negatively charged biological model membrane systems. The propensity to stabilize a helical structure for the *N* terminus may be an important parameter when Bax interacts with the mitochondrial membrane during apoptosis.

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