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Chemical Synthesis of Yeast Mitochondrial ATP Synthase Membranous Subunit 8

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Abstract: Chemical synthesis of highly hydrophobic peptides and proteins remains a challenging problem. Strong interchain associations within the peptide–resin matrix have to be overcome. A synthetic strategy for solid phase peptide synthesis is proposed, mainly based on prolonged coupling time using aprotic polar solvent mixtures. A tailored chromatographic purification was required to obtain a sample sufficiently pure for structural analysis. In this work, the total chemical synthesis of the membrane-embedded yeast mitochondrial ATP synthase subunit 8 is described. The quality of the synthetic protein was checked by electrospray mass spectrometry, its tendency to adopt α-helical secondary structure is evidenced by circular dichroism spectroscopy. Copyright © 1999 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: membrane protein; ATP synthase F_0-domain; solid phase peptide synthesis; chromatography

INTRODUCTION

The assembly of multi-subunit protein complexes in biological membranes represents a current focus in structural biology. Very few three-dimensional structures of membrane-embedded proteins are known to date, despite considerable effort. Because of their strong hydrophobic character, one of the major problems encountered nowadays is the production of sufficient amounts of highly pure membrane proteins for structural analysis.

In this report, the authors focus their interest on the mitochondrial ATP synthase, the major enzyme responsible for the aerobic synthesis of ATP. ATP synthase exhibits a tripartite structure consisting of an extrinsic membrane complex (F_1), an intrinsic membrane domain (F_0) and a connecting stalk [1]. The F_0 sector consists, in the yeast *Saccharomyces cerevisiae*, of the three mitochondrially encoded proteins designated subunits 6, 8 and 9. Subunits 6 and 9 are directly involved in the function of the proton channel through the inner mitochondrial membrane. The role of subunit 8 (SU8) in the proton channel has yet to be defined in detail, but this subunit is known to be essential for the F_0 sector assembly in the ATPase complex [2–4]. Amino acid sequence comparison of fungal SU8 homologues lead to the recognition of three distinct domains within this small protein (48 residues): a highly conserved non-polar NH_2-terminal region, a central strongly hydrophobic region and a COOH-terminus bearing a conserved array of positively charged residues at positions 37, 42 and 47. Investigation of the SU8 three-dimensional structure will provide useful information for the understanding of its function in the assembly of the ATP synthase membrane anchored F_0 complex. This paper reports on the total chemical synthesis and purification of this very hydrophobic protein.

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MATERIALS AND METHODS

Solid Phase Peptide Synthesis

The primary sequence of SU8 is as follows:

^{1}MPQLVPFYFM NQLTYGFLLM ITLLILFSQF FLPMILRLYV SRLFISKL^{48}

Its chemical synthesis was performed on an Applied Biosystems Peptide Synthesizer (Model 431A) using the *t*-Boc strategy. *N*-α-protected leucine linked to 4-(oxymethyl)-phenyl-acetaminomethyl-copoly-(styrene-1%-divinyl-benzene) resin and other *N*-α-Boc-protected amino acids were from Novabiochem (Switzerland). DCC, HOBt, 1,2-ethanedithiol, DMSO from Sigma (Sigma-Chimie, France); DMF, dichloromethane (DCM), *N*-methylpyrrolidone (NMP), TFA, diisopropylethylamine (DIEA), methanol, acetic acid and acetonitrile were purchased from SDS (SDS-PEYPIN, France); HF was obtained from UCAR (Belgium). The *t*-Boc-*N*-α-amino acids were protected as follows: *p*-toluenesulfonyl for arginine, 2-chloro-carbobenzoxyl for lysine, benzyl for serine and threonine and 2-bromo-carbobenzoxyl for tyrosine. Elongation was done on Boc-Leu-PAM-resin (0.5 mmol; loading of starting resin: 0.4 mmol/g) according to the following operational cycle protocol: deprotection with 50% TFA/DCM, washing with DCM, neutralization with 10% DEA/DCM, washing with DCM, then coupling in NMP with 2 mmol preformed HOBt esters of side-chain protected Boc amino acids. DMSO and DIEA were added to increase the coupling efficiency by helping to break self-aggregation of peptides (see text about prolonged coupling times). To facilitate post-synthesis purification, unreacted peptide chains were blocked by acetylation using acetic anhydride.

Cleavage of the Protein from the Resin

The protein was cleaved from the resin by classical high HF treatment [5], with anisol and ethanedithiol as scavengers. Good splitting of the tosyl protection group of arginine was achieved by allowing a reaction time of 90 min at 0°C. HF was then rapidly evaporated *in vacuo*. After precipitation in diethylether, protein and resin were first triturated with acetonitrile and methanol (1:1), then with acetic acid to a final mixture of 1:1:1. After filtration and dilution with water, the cleaved protein was lyophilized.

Amino Acid Analysis

The peptide was hydrolyzed in 6 M HCl (150°C, 1 h) using a Pico-Tag work station (Millipore-Waters, France); amino acids were derivatized to their phenylthiohydantoin conjugates before RP-HPLC, using the Pico-Tag column. Amino acid sequences were confirmed by comparison with calibration analyses using 100 pmol amino acid standards supplied by the manufacturer.

Size Exclusion Chromatography

The crude mixture was loaded onto a Superdex Peptide HR 10/30 column (Pharmacia Biotech S.A., France) and eluted with 50% acetonitrile in water. Elution of the product was monitored at 280 nm.

Analytical and Semi-preparative HPLC Purification

The lyophilized product was dissolved in a mixture of 50% acetonitrile in water and analyzed by HPLC on a Waters 600E System Controller and Waters 996 Photodiode Array Detector using a Vydac 214TP column (4.6×250 mm) (Interchim, France). The elution was carried out with 0.05% aqueous TFA (= eluent A) and acetonitrile/isopropanol 80/20 (containing 0.05% TFA (= eluent B)) at a flow rate of 0.8 ml/min. Monitoring was performed at 215 and 280 nm. Semi-preparative HPLC was achieved with a Vydac 214TP column (10×250 mm); elution conditions are described in the text.

Mass Spectrometry

The crude lyophilized synthetic peptide was dissolved in a mixture of 50% acetonitrile in water and analyzed by combined liquid chromatography–electrospray mass spectrometry (EMS) on a Thermoquest Finnigan model LCQ instrument. A C_1 reversed phase column (TMS 250, 75×4.6 mm, 10-μm particles, TosoHaas, PA, USA) was used with the same eluents as for analytical HPLC. Eluent B varied in a linear gradient from 40 to 80% in 20 min at a flow rate of 0.8 ml/min. A flow diverter was inserted before the mass spectrometer, allowing an 80 μl/min flow rate to enter the electrospray source. The purity of the final product, isolated after preparative chromatography, was confirmed by EMS using direct infusion into the ion source (20 μM peptide solution infused at 4 μl/min).

CD Spectroscopy

CD spectra were recorded at 0.2 nm intervals over the wavelength range 190–260 nm on a Mark VI Jobin-Yvon dichrograph, calibrated using iso-androsterone (Roussel-Uclaf, France) in dioxane and camphorsulfonic acid (Sigma-Chimie, France) in water. The optical rotation was checked with cytochrome C (horse heart) and calmodulin (Fluka, Switzerland), lysozyme (chicken egg white, Merck, France). The protein was dissolved at a concentration of 500 μg/ml in a 1:1 (w:v) mixture of acetonitrile in deionized water. CD measurements were performed at room temperature in a 0.1 cm pathlength cell.

RESULTS AND DISCUSSION

Chemical synthesis of highly hydrophobic peptides and proteins remains a challenging problem. Strong intra- and inter-chain associations within the peptide–resin matrix and their effects upon reaction rates and coupling yields have to be overcome. For the membrane-embedded SU8, which shows a very strong hydrophobic character in its central part mainly between residues 15 and 35 (Figure 1), difficulties in chain assembly were expected. It was decided to use an acidic *t*-Boc based solid phase peptide synthesis methodology [7] for two reasons: first, with this option, the permanent side-chain protecting groups of functional amino acids are of less hydrophobic character than those utilized in basic Fmoc chemistry. Second, trifluoroacetic acid, used to cleave the temporary NH_2-terminal protecting group *t*-Boc, is a very efficient solvent for both peptide and resin during this important step in chain assembly [8]. Nevertheless, the major disadvantage of this chemistry option is that there is no instrumentation allowing 'real time' monitoring and feedback control, as it is possible by conductivity measurement of the cleaved Fmoc group in the corresponding step during the basic deprotection reaction. As it could not be predicted exactly where coupling rates might be poor due to hydrophobic intra- or inter-chain associations (the so called 'difficult sequences'), an orientation synthesis of SU8 on a small scale (0.1 mmol) was realized to check its feasibility and to locate low coupling yields.

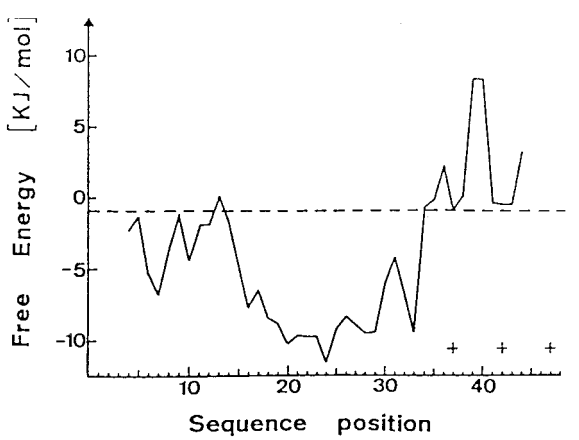

Figure 1 Hydrophobic profile of SU8, using the method of Von Heijne [6]. Residues 15–35, below the dotted line, are predicted to be positioned in the membrane; the three positive charges corresponding to residues Arg^{37}, Arg^{42} and Lys^{47} are indicated (figure courtesy of Dr J. Velours, [3]).

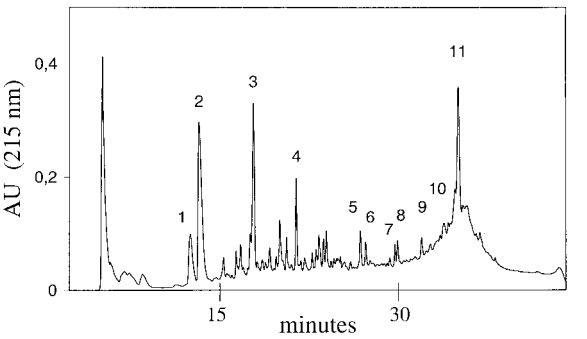

Figure 2 HPLC profile of the crude protein synthesized under standard conditions. SU8 corresponds to peak 11, the other peaks to various truncated acetylated peptides reflecting uncompleted coupling during the synthesis (number of amino acid residues from the C-terminus, that means in the order of sequence assembly): 1 (13aa), 2 (11aa), 3 (16aa), 4 (19aa), 5 (27aa), 6 (26aa), 7 (28aa), 8 (36aa), 9 (30aa) and 10 (31aa).

At several stages of the proteins chemical synthesis, from the C-terminal Leu^{48} to the N-terminal Met^{1}, the distribution of unique or newly incoming residues (Met^{34}, Gln^{29}, Thr^{22}, Gly^{16} and Asn^{11}) made it possible to check progressing chain elongation. Detection of these key residues in hydrolyzed samples of peptide–resin by amino acid analysis confirmed ongoing chain assembly at the corresponding steps (data not shown). The final product was cleaved from the resin, side-chain deprotected and examined with the aid of combined liquid chromatography and mass spectrometry analyses (Figure 2). The results revealed the presence of the 48-residue SU8 protein in the crude peptide mixture (peak 11). The other peaks (1–10) in the chromatogram correspond to truncated peptides caused

by premature stops of chain elongation due to incomplete coupling during the synthesis. The authors identified the major products indicating the presumed 'difficult sequences'. Incorporation of the following residues seemed to be particularly slow: Arg^{37}, Ile^{35}, Leu^{32}, Gln^{29}, Thr^{22}, Ile^{21}, Met^{20}, Leu^{18}, Phe^{17} and Gln^{12}. Thanks to this information, the authors were then able to modify the standard synthetic protocol in order to meet more favorable conditions for the assembly of this very hydrophobic protein.

The second synthesis was performed on a larger scale (0.5 mmol). To favor high coupling yields, double couplings were performed from the tenth residue on, including acetylation of the unreacted NH_2-termini to facilitate post-synthesis purification.

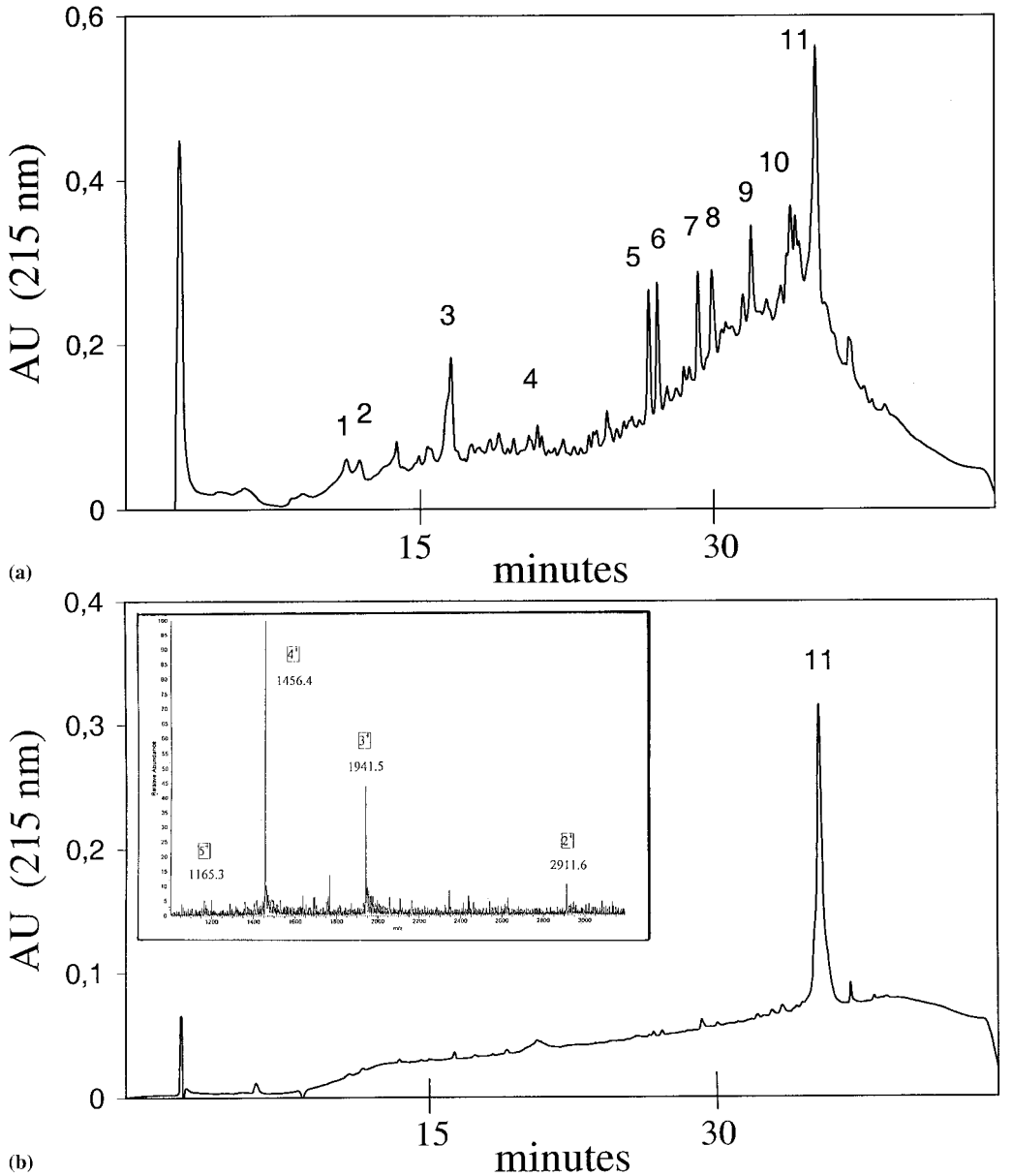

Figure 3 HPLC profiles of SU8 synthesized using the protocol adapted to its hydrophobic nature (see text). (a) and (b) show the crude and the purified final product, respectively. The elusion was carried out with 0.05% aqueous TFA (eluent A) and acetonitrile/isopropanol (80/20, containing 0.05% TFA, eluent B), where B varied in a 30-min linear gradient from 35 to 95%. The insert in (b) shows the EMS spectrum of the synthetic protein (peaks corresponding to the m/z ratio for the 2^+, 3^+, 4^+ and 5^+ protonated species, see text).

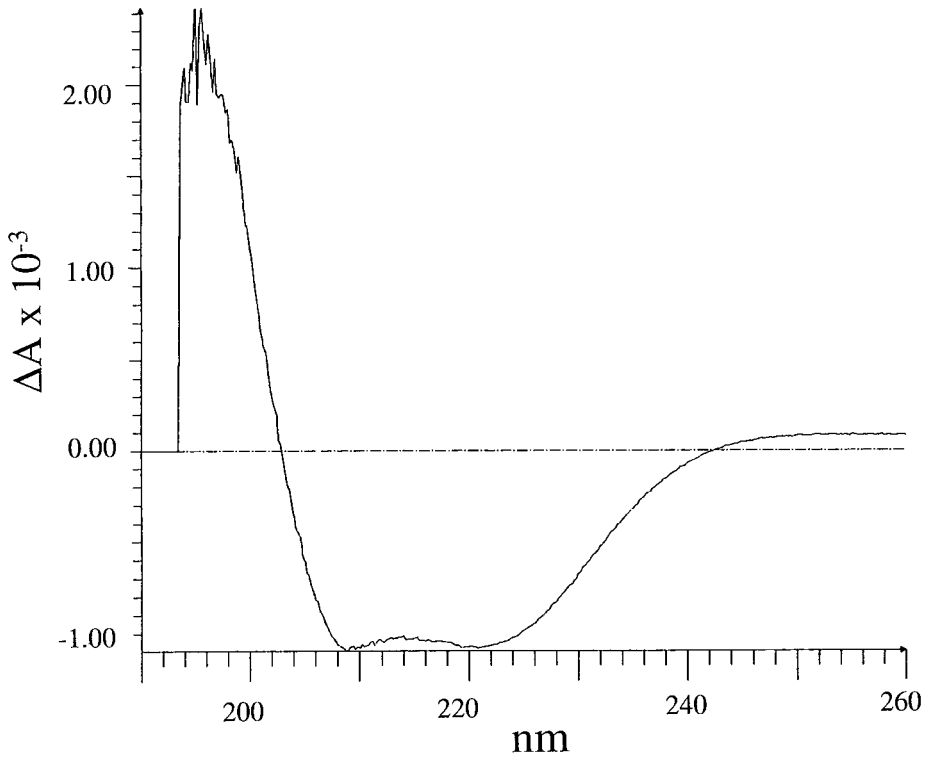

Figure 4 CD spectrum of the synthetic protein at a concentration of 500 μg/ml in a 1:1 mixture of acetonitrile in water. The spectrum was recorded at room temperature in a 0.1 cm path-length cell at 0.2 nm intervals over the wavelength range 190–260 nm.

Additionally, coupling times were prolonged during the coupling steps shown to be the most difficult: the authors allowed 20 min extra coupling time after addition of DMSO to the reaction mixture main solvent NMP, and a further 10 min after addition of DIEA. These aprotic polar solvents with strong H-bond acceptor properties are thought to discourage intra- and inter-chain association, that were taken to be responsible for the observed lowering in coupling yields. Figure 3(a) shows the decrease in proportion of major truncated products (peaks 1–4) in comparison with the first synthesis (Figure 2), and the proportionally higher amount of the desired product (peak 11). This clearly reflects the success of this methodology.

After the final peptide cleavage from the solid support, the crude product was submitted to gel filtration on a Superdex column in order to eliminate a major proportion of the smaller truncated peptide sequences resulting from the acetylation blocking of the unreacted amino groups. The fractions corresponding to the highest molecular masses were pooled and lyophilized (results not shown). The next purification step, semi-preparative reverse phase HPLC, lead to a large increase in synthetic peptide purity (Figure 3(b)). With respect to its very hydrophobic nature, a less hydrophobic C4-grafted silica column was used. Elution was performed with 0.05% aqueous TFA (eluent A) and a solvent mixture with high eluotropic strength for eluent B: 0.05% TFA in acetonitrile/isopropanol (80/20). Elution conditions for semi-preparative purification were found optimal when eluent B varied in a 20-min linear gradient from 80 to 90%. The high degree of purity of SU8, as it is required for structural analysis, was checked by EMS. The insert in Figure 3(b) shows the peaks corresponding to the mass/charge ratios for the multiple protonated species in the range of observation: 2^+ (2911.6 Da), 3^+ (1941.5 Da), 4^+ (1456.4 Da) and 5^+ (1165.3 Da). The measured molecular mass of 5821.4 ± 0.2 Da for the synthetic SU8 is very close to the calculated mass (5822.2 Da).

Classical algorithms for prediction of protein secondary structure [9] suggest an all-helical organization for the SU8 protein. Circular dichroism (CD) spectroscopy was used to investigate the nature and approximate amount of secondary structure

elements in the protein sequence. Testing different organic solvents, including trifluoroethanol, acetonitrile was finally chosen to dissolve the protein, since this solvent was used in post-synthesis purification. Figure 4 shows the CD spectrum of SU8 in a 1:1 mixture of acetonitrile in water. It is characterized by a positive band near 195 nm and two negative bands around 208 and 220 nm, suggesting a high percentage of α-helical elements composing the secondary structure of SU8 under these conditions [10].

CONCLUSIONS

In conclusion, the authors realized the total chemical synthesis and purification of the membrane bound yeast mitochondrial ATP synthase subunit 8. This small protein (48 residues) only contains three charged amino acids at its C-terminus, the rest being almost exclusively composed of non-polar residues (12 leucines, four isoleucines, seven phenylalanines, three tyrosines, etc.). The very hydrophobic character of this sequence demands special adapted protocols for its synthesis and purification. Prolonged coupling times in aprotic polar solvents (DMSO and DIEA) proved to diminish the expected intra- and inter-chain association inside the peptide–resin matrix during this crucial step in chain assembly. Isopropanol was of much benefit in the solubilization of the crude product and during the chromatographic purification steps (size exclusion followed by RP-HPLC). These optimized conditions could certainly be generalized for the synthesis and purification of other protein sequences containing large hydrophobic domains.

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REFERENCES

1. J. Weber, A.E. Senior (1997). Catalytic mechanism of F_1-ATPase. *Biochem. Biophys. Res. Comm. 1319*, 19–58.
2. J. Velours, M. Esparza and B. Guérin (1982). Amino acid composition of a new mitochondrially translated proteolipid isolated from yeast mitochondria and from the OSATPase complex. *Biochem. Biophys. Res. Comm. 109*, 1192–1199.
3. J. Velours, M. Esparza, J. Hoppe, W. Sebald and B. Guérin (1984). Amino acid sequence of a new mitochondrially synthesised proteolipid of the ATP synthase of *Saccharomyces cerevisiae*. *EMBO J. 3*, 207–212.
4. R.J. Devenish, T. Papakonstantinou, M. Galanis, R.H.P. Law, A.W. Linnane and P. Nagley (1992). Structure/function analysis of yeast mitochondrial ATP synthase subunit 8. *Ann. NY Acad. Sci. 671*, 403–414.
5. M. Mutter, G.C. Tuchscherer, C. Millert, K.H. Altmann, R.I. Carey, D.F. Wyss, A.M. Labhart and J.E. Rivier (1992). Template-assembled synthetic proteins with 4-helix bundle topology. Total chemical synthesis and conformational studies. *J. Am. Chem. Soc. 114*, 1463–1470.
6. G. Von Heijne (1981). Membrane proteins: the amino acid composition of membrane-penetrating segments. *Eur. J. Biochem. 120*, 275–278.
7. G. Barany and R.B. Merrifield in: *The Peptides*, E. Gross and J. Meienhofer, Eds., p. 1–284, Academic Press, New York, 1980.
8. M. Goetz, S. Geoffre, B. Busetta, C. Manigand, C. Nespoulous, D. Londos-Gagliardi, B. Guillemain and M. Hospital (1997). Synthesis and CD studies of an 88-residue peptide containing the main receptor binding site of HTLV-I SU-glycoprotein. *J. Pep. Sci. 3*, 347–353.
9. B. Rost and C. Sander (1993). Prediction of protein structure at better than 70% accuracy. *J. Mol. Biol. 232*, 584–599.
10. J.T. Yang, C.S.C. Wu and H.M. Martinez (1986). Calculation of protein conformation from circular dichroism. *Method Enzymol. 130*, 208–269.

