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Solubilization of V-ATPase transmembrane peptides by amphipol A8-35‡

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Abstract: Two transmembrane peptides encompassing the seventh transmembrane section of subunit *a* from V-ATPase from *Saccharomyces cerevisiae* were studied as complexes with APols A8-35 by CD and fluorescence spectroscopy, with the goal to use APols to provide a membrane-mimicking environment for the peptides. CD spectroscopy was used to obtain the overall secondary structure of the peptides, whereas fluorescence spectroscopy provided information about the local environment of their tryptophan residues. The fluorescence results indicate that both peptides are trapped by APols and the CD results that they adopt a β-sheet conformation. This result is in contrast with previous work that showed that the same peptides are α-helical in SDS micelles and organic solvents. These observations are discussed in the context of APol physical–chemical properties and transmembrane peptide structural propensity. Copyright © 2007 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: amphipols; fluorescence; circular dichroism; peptides; membrane-mimicking solvents; secondary structure

INTRODUCTION

In the past decades high-resolution NMR spectroscopy has become an important tool in the structural studies of transmembrane proteins and peptides [1]. An enormous challenge associated with the application of this technique to study these systems is related to the selection and setup of the environment in which the molecule is going to be embedded to perform the NMR experiment. The use of such a nonnative environment is needed, due to the difficulties of obtaining high-resolution structural information of peptides in their native environment by solid or liquid-state NMR. Despite recent advancements in solid-state NMR to achieve good quality structures [1], the procedure is still not standard. The methodology most often applied is by using membrane-mimicking solvents. The classical systems used to solubilize transmembrane proteins and peptides include detergent micelles (*e.g.* SDS or DPC) and organic solvents (*e.g.* TFE or DMSO) [2].

Recently, low-molecular weight amphipathic polymers called APols have been designed with the aim of keeping membrane proteins water-soluble under less dissociating conditions than are provided by detergents [3,4]. APols are comprised of a highly hydrophilic backbone grafted with numerous hydrophobic side chains (Figure 1(a)). They are not (or extremely weak) detergents, but can substitute detergents to stabilize transmembrane proteins in aqueous solution [3–6]. APols have been used recently to collect high-quality NMR spectra of a transmembrane protein [5].

In the past years, we have been working on resolving the structure of the transmembrane stator subunit *a* of the proton V-ATPase. This enzyme is a molecular rotor that pumps protons through the cell membrane at the expense of ATP. The structure of the rotor subunits has recently been elucidated for a related enzyme, the Na^+-V-ATPase [9]. However, no high-resolution structure is available for the stator subunit *a*. From mutagenesis experiments it is known that the seventh transmembrane segment from subunit *a* is of vital importance for proton translocation, due to the presence of three activity-related residues: H729, R735, and H743. Therefore, our focus is on structure determination of a number of peptides that mimic this segment by different spectroscopic methods in various environments: lipid bilayers, SDS micelles, and organic solvents (TFE and DMSO) [7,10–12]. On the basis of these studies we concluded that the seventh transmembrane domain exhibited an α-helical structure.

The goal of this paper was to examine the possible use of APol A8-35 as an alternative membrane-mimicking environment for our V-ATPase peptides. A8-35 (Figure 1(a)) is by far the best characterized APol to date [3,4,6,13,14]. It is a very mild surfactant that tends to biochemically stabilize membrane proteins

Abbreviations: A8-35, a specific form of amphipol; APol, amphipol; CD, circular dichroism; MTM7 and sMTM7, peptides mimicking the seventh transmembrane section of subunit *a* from V-ATPase; DTT, dithiothreitol.

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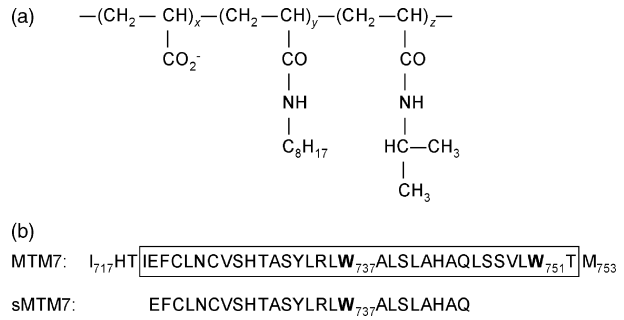

Figure 1 (a) Chemical structure of APol A8-35, with $x \approx 35\%$, $y \approx 25\%$, $z \approx 40\%$, and MW = 9–10 kDa [3]. (b) Primary sequence of peptides MTM7 and sMTM7. The tryptophan residues are highlighted in bold. The amino acid residues that are predicted to be part of the seventh transmembrane segment are boxed. The amino acid numbering is based on the sequence of the full-length subunit *a* from V-ATPase [7,8].

as compared to detergent solutions (e.g. References [3,4,15]) and has proven useful for refolding membrane proteins [16] as well as for structural investigations by NMR [5] or electron microscopy [17]. The structure of APol-trapped V-ATPase peptides was examined by CD spectroscopy, to obtain the overall secondary structure, and fluorescence spectroscopy, to analyze the local environment of tryptophan residues.

MATERIAL AND METHODS

Peptide Design

The peptides used in this study (MTM7 and sMTM7, Figure 1(b)) were previously designed for structural studies by CD, ESR, and NMR [7,11,12]. Both peptides encompass the transmembrane section of the seventh transmembrane domain of V-ATPase subunit *a*. While peptide MTM7 is composed of 37 amino acid residues and encompasses the full seventh transmembrane domain [7], peptide sMTM7 (25 amino acid residues) encompasses the cytoplasmic side of the proton channel [11,12]. The numbering of both peptides is based on the sequence of the full-length subunit *a* from the V-ATPase transmembrane section [8]. Peptides MTM7 and sMTM7 were produced on solid support using continuous flow chemistry by Pepceuticals Ltd., Leicester, UK. The final purity was tested by HPLC as well as mass spectrometry and was above 90%.

Amphipol A8-35

APol A8-35 (Figure 1(a)) is a derivative of polyacrylic acid [3]. The batch of A8-35 used for the present study (batch FGH20) was synthesized and purified according to published protocols [13,14].

Sample Preparation

Sample preparation was the same for peptide MTM7 and sMTM7. The peptide was initially dissolved in TFE at a concentration of 0.4 mM. For these samples CD and fluorescence spectra were obtained to verify the structure of the peptide. The TFE-peptide solution was then mixed with three different volumes of a 0.5 mM solution of APol A8-35 in TFE to obtain three peptide : A8-35 mass ratios: 1 : 40, 1 : 20, and 1 : 5. After 15 min of incubation, the samples were dialyzed (3.5 kDa mass cutoff) against a phosphate buffer (50 mM, pH 7.7) during two days at room temperature. To check chemical aggregation of the peptides via disulphide bridging an extra set of TFE-peptide samples was prepared with a molar excess of DTT. The final peptide concentration was 0.2 mM. For each experiment freshly prepared samples were used. To investigate the effect of the dialysis step on the formation of the peptide/A8-35 complex, two extra protocols were tested: (i) replacement of the dialysis by a freeze-drying step, where the TFE/peptide/A8-35 mixture was frozen at −70 °C followed by freeze-drying. Thereafter, the sample obtained was dissolved in buffer; (ii) replacement of the dialysis by a fast dilution of the TFE/peptide/A8-35 mixture directly in buffer (five-fold) followed by a concentration step.

Absorption Spectroscopy

Absorption measurements were carried out on a Cary 5E (Varian) spectrophotometer. The concentration of peptides MTM7 and sMTM7 was determined by measuring the UV absorbance at 280 nm, using an extinction coefficient of 12 740 $l.mol^{-1}.cm^{-1}$ and 7210 $l.mol^{-1}.cm^{-1}$, respectively [18].

Circular Dichroism Spectroscopy

Far-UV CD spectra were recorded on a Jasco J-715 spectropolarimeter, equipped with a Peltier thermal-controlled cuvette holder. The spectra were recorded using a 0.1-cm path length quartz cuvette, from 190 to 260 nm, with a 1.0-nm step resolution and a response time of 0.25 s. The spectra were collected and averaged over 50 scans. For thermal stability, experiments of the ellipticity from 190 to 260 nm were measured between 10 and 50 °C with a temperature interval of 5.0 °C. For each temperature the sample was allowed to equilibrate for 5 min. After reaching 50 °C, the temperature was lowered to 10 °C and a final spectrum was acquired to check for reversibility. All spectra were baseline corrected by using control samples without peptide. For all the experiments the optical density measured at 280 nm was below 0.5. The overall secondary structure of the peptides was determined by comparison with the published CD spectra of the α-helix, β-sheet, and unfolded conformations [19,20].

Fluorescence Measurements

Fluorescence spectra were recorded on a Fluorolog 3.22 (Jobin Yvon-Spex), using a 1-ml quartz cuvette with path lengths of 10 and 4 mm in the excitation and emission direction, respectively. Fluorescence spectra were recorded between 300 and 400 nm, using an excitation wavelength of 290 nm and using excitation and emission slits giving a 2-nm bandpass. Background fluorescence from sample without peptide was subtracted from each spectrum.

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RESULTS AND DISCUSSION

Circular Dichroism Spectroscopy

CD spectroscopy is a useful technique to determine the secondary structure of proteins and peptides under various conditions. The CD data in Figure 2 show that peptide sMTM7, the short version of V-ATPase subunit *a* TM7, adopts an α-helical conformation in TFE. However, when the peptide is transferred by dialysis to APol A8-35, a spectrum characteristic of β-sheet conformation is found. This conformation is present at all three peptide : A8-35 ratios studied (Figure 2). The minimum around 225 nm is not located at the typical minimum for a β-sheet conformation, which is normally found between 215 and 220 nm. This is possibly due to the contribution of some unfolded conformation present in both termini of the peptide. This kind of conformation gives a positive signal in the CD spectra in the region of 220 nm, which will result in a shift of the β-sheet minima.

Figure 3 shows the CD spectra of the longer peptide, MTM7. In TFE the peptide is in a characteristic α-helical conformation. After trapping with APol at peptide : A8-35 mass ratios of 1 : 40 and 1 : 20, a β-sheet conformation is again found. At a peptide : A8-35 mass ratio of 1 : 5, the peptide was not kept soluble (in this case the amount of APol A8-35 is too small) and a white precipitate was formed upon dialysis. The CD spectra of both peptides did not show significant changes as a function of temperature (data not shown). This observation suggests a thermodynamically stable β-sheet conformation.

To test whether the formation of β-sheet structure is due to the formation of disulphide bridges involving the cysteine residues present in peptides sMTM7 and MTM7, DDT was added to the initial TFE solution of both peptides. After transfer, the CD spectra of both peptide/APol complexes were similar whether DDT was added or not, indicating the presence of β-sheet conformation (data not shown).

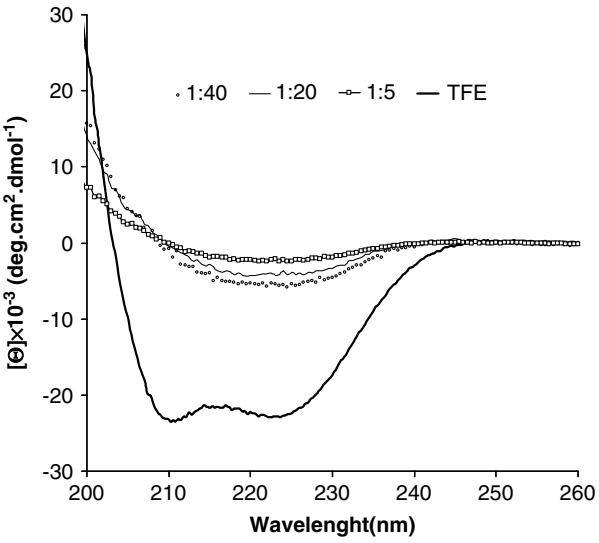

Figure 2 CD spectra of peptide sMTM7 in TFE and dissolved in APol A8-35 at peptide : A8-35 ratios of 1 : 40, 1 : 20 and 1 : 5.

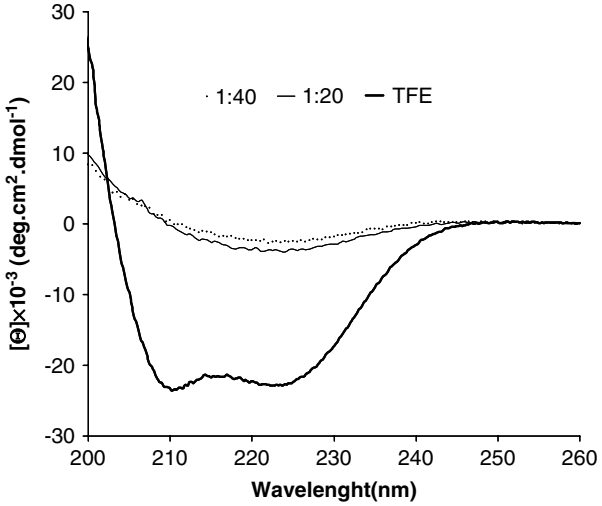

Figure 3 CD spectra of peptide MTM7 in TFE and dissolved in APol A8-35 at peptide : A8-35 ratios of 1 : 40 and 1 : 20.

Dialysis achieves the transfer of the peptide/APol mixture from a TFE to an aqueous solution in a progressive manner. To check whether the transition from the α-helical conformation observed in TFE to the β-sheet one was induced by this slow process, trapping was also performed either by freeze-drying the mixture of peptide and APol in TFE, followed by a resuspension into aqueous buffer, or by diluting the TFE solution rapidly into buffer (Materials and Methods). Both protocols yielded clear solutions of peptides in the β-sheet conformation (data not shown).

Fluorescence Spectroscopy

The fluorescence spectrum of sMTM7 trapped by APol A8-35 shows a fluorescence maximum of W737 at 330 nm (Figure 4). Compared to the position of this maximum for the peptide dissolved in TFE (340 nm), this suggests that residue W737 is located in a more apolar environment, meaning, very likely, that it is exposed to the hydrophobic core of an APol particle. In the case of peptide MTM7, fluorescence spectra are more complex due to the presence of two tryptophan residues (Figure 4). By comparison with the spectrum of peptide sMTM7, we assume that the two tryptophan residues (W737 and W751) in MTM7 experience different environments. One tryptophan shows a similar wavelength of the fluorescence maximum as found for peptide sMTM7 (330 nm). Thus this peak can be assigned to W737, located in an apolar region of APol A8-35. The other peak at 340 nm can be assigned to W751. This tryptophan is located in a more polar environment as compared to W737 and, probably since

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it is located at the far end of the *C*-terminal domain, more in contact with the aqueous phase. Upon addition of DTT, no changes in the fluorescence spectra were found (data not shown).

GENERAL DISCUSSION

Peptides MTM7 and sMTM7 are strongly hydrophobic and do not dissolve in aqueous solutions [11]. However, at sufficient high ratios of APol A8-35, both peptides become water-soluble. Nevertheless, it is remarkable that they both take up a β-sheet conformation in APol A8-35, whereas in TFE (Figures 2 and 3), DMSO, and SDS micelles, a mainly α-helical conformation was found [7,11,12]. Such a helical conformation would also be expected for the peptide sequences *in situ* in the native V-ATPase complex [8]. The β-sheet conformation probably arises from peptide aggregation, as observed, for instance, for the M13 coat protein in phospholipid bilayers [21].

While APols have been used during the last decade to stabilize a large variety of membrane proteins in aqueous solutions (for a review, Reference 4), only a couple of studies report about their interactions with peptides ([22]; and data quoted in {Table 1} of Reference 4). Both the nontransmembrane 56-residue transfer peptide derived from the *Serratia marcescens* haem acquisition protein [22] and a 23-residue mimic of the single transmembrane α-helix of human glycophorin A [4] exhibited, after transfer to APol A8-35 or an analog thereof, secondary structures similar to those observed in nondenaturing detergent solutions or once associated to lipid vesicles, namely ~30% α-helical in the first case and mostly helical in the second. In contrast, APol A8-35 fails to stabilize the helical structure of peptides sMTM7 and MTM7, thus

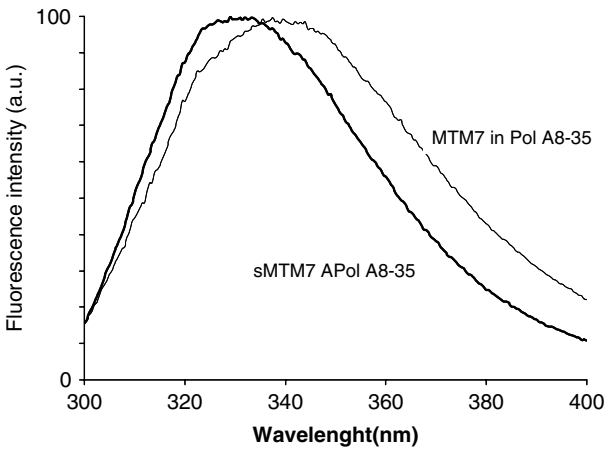

Figure 4 Fluorescence spectra of peptides MTM7 and sMTM7 dissolved in APol A8-35. The spectra at peptide : A8-35 ratio of 1 : 40 ratio are depicted. The fluorescence maximum of both spectra is normalized to 100. The other ratios show the same line shape.

it can be concluded that this APol does not provide a suitable membrane-mimicking environment for V-ATPase peptides. This observation can receive two types of explanations.

First, the formation of β-sheets could be the result of a kinetic trap, due to inappropriate transfer protocols. The bacterial outer membrane protein OmpF, for instance, which is perfectly water-soluble once trapped by APol A8-35 [3], will form aggregates under inappropriate transfer conditions [23]. Refolding membrane proteins, such as bacteriorhodopsin, will also aggregate in the presence of APol A8-35 if renaturation is not properly conducted [16]. However, transfer of sMTM7 and MTM7 was attempted here under a variety of conditions and always yielded β-stranded structures, suggesting that those may be the lowest-free energy state of these peptides in association with APol A8-35.

A second possibility would be that the formation of β-sheets results from the interaction with the anionic polymer of the arginine residue which, quite atypically, is present toward the middle of the putative α-helix. By driving the arginine side chain toward the surface of A8-35 particles, such an interaction could prevent the peptide from crossing the hydrophobic core of the particle, preventing the formation of an α-helix. Such a phenomenon could possibly be alleviated by resorting to nonionic [24] or cationic [25] APols.

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