Volume 311, number 3, 256–258 FEBS 11672 October 1992

The secondary structure of influenza A M2 transmembrane domain

A circular dichroism study

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Received 7 September 1992

Using circular dichroism, this study investigated the secondary structure of the influenza A M2 transmembrane domain. When reconstituted into 1,2-dioleoyl-*sn*-glycero-3-phosphocholine liposomes, the M2 transmembrane domain was found to adopt a predominantly α-helical secondary structure which was unaffected by both temperature and the addition of 1-aminoadamantane hydrochloride. Reconstitution into 1,2-dioleoyl-*sn*-glycero-3-phosphoglycerol liposomes resulted in a marked decrease in helical content.

Spectrophotometry; Circular dichroism; Influenza M2 protein; Ion channel

1. INTRODUCTION

The influenza A virus M2 protein has recently been the focus of extensive research. This protein is expressed on the cell surface during viral synthesis [1] and has been identified in viral particles [2]. It is a disulphide-bonded homotetramer [3] of 97 amino acids and is important during two separate phases of the cell cycle [4]. Immediately after viral endocytosis, and concomitant with pH-induced fusion between the endosomal and viral membranes, mediated by hemagglutinin (HA) [5], M2 is thought to conduct protons into the interior of the virus, leading to uncoating, the release of ribonucleoprotein from the matrix protein (M1), and eventual nuclear infection [6]. When HA is transported to the cell surface in post-Golgi vesicles [7], M2 may also facilitate influenza synthesis and assembly by countering any vesicular acidification. The relative importance of these events, which occur at different stages of the infectivity cycle, may depend on the particular viral strain [4]. Amantadine (1-aminoadamantane hydrochloride), the only registered drug used in the prophylaxis and treatment of influenza infections, is thought to operate by impeding proton flow during these events, however, it is not yet clear if the mechanism of this action involves an alteration in the secondary structure of the membrane-spanning domain or physical blockade. Importantly, influenza A is thought to become resistant to amantadine via single amino acid substitutions in the membrane-spanning domain of M2 [8].

Regarding the thesis that M2 orchestrates proton flow, it has recently been shown that, when expressed in *Xenopus laevis* oocytes, it has an associated ion channel activity selective for monovalent cations [9]; using a two-electrode voltage-clamp procedure the Pinto study observed Na^+ and K^+ currents. In addition, a further electrophysiological study directly identified proton translocation when the transmembrane domain of M2 was incorporated into single-channel, voltage-clamped, planar lipid bilayers [10]. Both studies reported current negation upon addition of amantadine.

The premise that membrane-spanning regions are often α-helical is generally based on theoretical predictions and observations of such in bacterial photosynthetic reaction centres [11–13]. In addition, when the M2 transmembrane domain is modelled as an α-helix the residues which are substituted in amantadine-resistant mutants all appear on the same face of the helix [14]. Thus it is an attractive assumption that the M2 transmembrane domain is α-helical, however, several caveats exist. An amantadine-resistant influenza virus has been isolated that contains a proline substitution in the transmembrane domain, which is not usually thought to be compatible with a helical conformation [8]. In addition, Pinto et al. [9] observed that deletion of four transmembrane residues and addition of one extra residue still permitted ion channel activity.

The present study describes an investigation of the secondary structure of the native influenza A M2 transmembrane domain, and the influence of amantadine at different concentrations, using circular dichroism (CD).

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The results are discussed in terms of the recent electrophysiological data available concerning the mode of action of the M2 protein.

2. EXPERIMENTAL

In order to investigate the secondary structure of the influenza A M2 transmembrane domain, a synthetic 25 residue peptide (sequence: SSDPLVVAASIIGILHLILWILDRL), corresponding to the predicted transmembrane sequence common to several strains of influenza A [15], was incorporated into 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) liposomes [16] and examined using CD. The peptide was synthesized in the MRC Cellular Immunology Unit, Oxford, UK, purified by reverse-phase HPLC and subsequently sequenced in the WelMet Unit, University of Edinburgh, UK. The lipid was chosen for this study because phosphatidylcholine is the most common phospholipid headgroup in mammalian systems and oleic acid represents the most frequently occurring fatty acyl chain. The possible effects of temperature, addition of amantadine, and liposomal lipid type used, were also investigated. M2 was also reconstituted into 1,2-dioleoyl-*sn*-glycero-3-phosphoglycerol (DOPG) liposomes.

CD spectra were recorded on a JASCO J-600 spectropolarimeter; analysis of the spectra in terms of secondary structure content was undertaken using the CONTIN procedure of Provencher and Glöckner [17], the methods of Chang et al. [18] and of Siegel et al. [19].

The liposomes were prepared in a 10 mM Tris-HCl, 0.1 mM EDTA buffer system, pH 7.4. Two concentrations of amantadine were added to the reconstituted liposomes, 4 and 12 μg/ml. These were designated low and high concentration, respectively. All chemicals were supplied by Sigma Chemical Co., UK.

Liposomal and control protein concentrations were determined colorimetrically following the Lowry modification of Markwell et al. [20], using bovine serum albumin as standard.

3. RESULTS AND DISCUSSION

The far UV CD spectrum at 20°C of the M2 transmembrane peptide incorporated into DOPC liposomes is shown in Fig. 1. Under these conditions, satisfactory data could be obtained down to 200 nm; below this wavelength, the noise levels precluded accurate measurements of ellipticity. The spectrum shows the characteristic double minima at 223 nm and 209 nm of α-helices [18]. Estimates of 92, 79 and 100% α-helix were obtained using the methods of Provencher and Glöckner [17], Chang et al. [18] and Siegel et al. [19], respectively. Although the exact values of these estimates should be treated with caution, in view of the fact that data could only be collected down to 200 nm instead of 190 nm, which is preferred [17,18], it is clear that under these conditions the M2 peptide exists largely, if not completely, in an α-helical form. In addition, estimates of helix approaching 100% have been obtained both when M2 peptide is dissolved in 0.4% (w/v) sodium dodecyl sulphate (SDS) and 50% (v/v) 2,2,2-trifluoroethanol (TFE) (see Table I). These solvents maximize possible secondary structure conformation. Such results are in direct contrast to those obtained when M2 is dissolved in buffer. In aqueous solution, the M2 peptide did not apparently adopt any stable secondary structure; the low value of the molar ellipticity at 225 nm (Table I) is typical of a random coil. The DOPC liposomal measurements were repeated at 37°C, and no change was observed in the spectrum when compared with the 20°C data.

On addition of amantadine to the liposomes, to give final concentrations of 4 or 12 μg/ml, there were no significant changes in the CD spectra (see the molar ellipticity values in Table I). Both low and high drug concentrations were used in order to examine any possible concentration dependence [21]. These results are significant in that they clearly demonstrate that amantadine itself has no discernable effect upon the helical nature of the M2 peptide, as was previously mooted [22]. This, along with recent findings that amantadine does indeed inhibit ion flow through M2 [9,10], augments the thesis that the efficacy of amantadine is due to its channel-blocking abilities and not to any conformational interference.

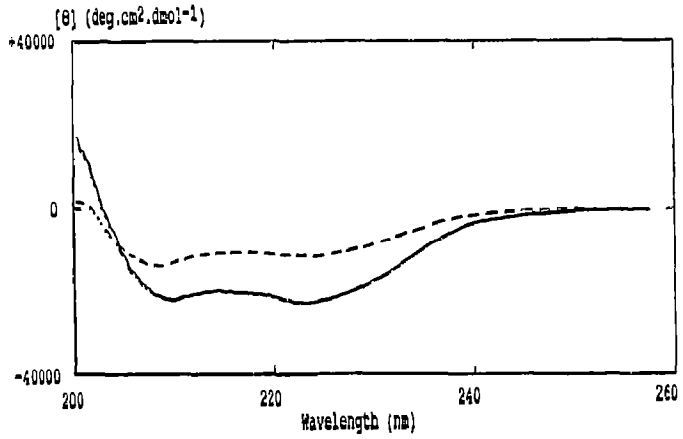

Fig. 1. Far UV CD spectra of M2 peptide incorporated into liposomes. Spectra were recorded at 20°C in a cell of path length 0.02 cm (—) DOPC liposomes; (- - -) DOPG liposomes. The liposomal peptide concentrations were 0.16 and 0.27 mg/ml, respectively.

Table I

Values of molar ellipticities at 225 nm for M2 peptide incorporated into liposomes

Sample	Ellipticity at 225 nm (deg·cm²·dmol⁻¹)
M2 peptide in DOPC liposomes (20°C)	−22,260
M2 peptide in DOPC liposomes (37°C)	−21,660
M2 peptide in DOPC liposomes (20°C) plus amantadine (4 μg/ml)	−21,970
M2 peptide in DOPC liposomes (20°C) plus amantadine (12 μg/ml)	−23,190
M2 peptide in DOPG liposomes (20°C)	−11,430
M2 peptide in 0.4% (w/v) SDS	−22,840
M2 peptide in 50% (v/v) TFE	−24,440
M2 peptide in buffer (20°C)	−700

Molar ellipticity values were calculated from the observed CD spectra using a value of 110 for the mean residue weight. The error in the ellipticity values is estimated to be ± 5%.

The M2 peptide incorporated into DOPG liposomes showed a considerably reduced CD spectrum (Fig. 1 and Table I). Analysis of this spectrum by the CONTIN procedure [17] yielded unacceptably high errors of estimates of secondary structure; the methods of Chang et al. [18] and of Siegel et al. [19] yielded values of 41 and 47% α-helix, respectively, in accord with the reduced values of the ellipticity at 225 nm (Table I). These experiments demonstrate a relationship between bilayer composition and the secondary structure of the peptide. Epand et al. [23] have demonstrated that the helical content of salmon calcitonin is dependent on the percentage of phosphatidylglycerol; altering the charge state of the membrane alters the helical content of the hormone. A similar phenomenon may also be found with peptide toxins where, for example, cardiotoxin and melittin are able to modulate lipid surface curvature and polymorphism in a lipid-specific manner [24].

In summary, the transmembrane domain of M2 adopts an α-helical conformation in DOPC liposomes, and this structure is not affected by the presence of the drug, amandatine. In view of recent developments in the study of M2 protein, these results answer the questions raised regarding M2 in bilayers [9,14,22], and help to explain its structural mechanism.

Acknowledgements: These measurements were carried out on the Scottish Universities CD Facility which is supported by the SERC.

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