

Helix-sheet interconversion in a synthetic pore lining peptide derived from a designed ion channel

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We have designed a four-helix protein that is expected to tetramerize in the membrane to form an ion channel with a structurally well defined pore. A synthetic peptide corresponding to the channel lining helix facilitates ion transport across liposomal membranes and largely helical in membranes. Detailed circular dichroism studies of the peptide in methanol, water and methanol-water mixtures reveal that it is helical in methanol, β -structured in 97.5% water and a combination of these two structures at intermediate compositions of methanol and water. A fluorescence resonance energy transfer study of the peptide shows that the peptide is monomeric in methanol but undergoes extensive anti-parallel aggregation in aqueous solution.

THE three-dimensional structure of a protein is dictated by the sequence of its constituent amino acids, but it has not thus far been possible to develop reliable algorithms leading from a primary sequence to its three-dimensional structure. One approach to this problem has been to design proteins that would adopt a specified structure. We have designed a four-helix protein, SYNCHAN, that should form non-selective, ungated pores in membranes (Seth *et al.* in preparation). Faces of helices that interact extensively in the bacteriorhodopsin (bR) structure¹ have been used to design specific helix-helix interactions while the channel liner is an amphipathic helix based loosely on the alamethicin-zervamicin family of fungal peptide antibiotics². SYNCHAN has been designed to tetramerize in the membrane, each subunit contributing a helical segment to line an aqueous pore. The pore thus formed would be stabilized by helix-helix interactions within and between subunits. Thus, segments corresponding to each of the helices should partition effectively into membranes and interact with each other in a stereospecific manner. In addition, the channel-lining segment should be able to self-associate in a manner resulting in transmembrane ion channels.

The protein, SYNCHAN, should serve as a model for *Shaker* type potassium channels which are also tetramers^{3,4}, each of whose subunits has six putative transmembrane segments⁵⁻⁷. Details of the design will be

presented elsewhere (Seth *et al.*, in preparation). Briefly, helix-helix interactions have been designed based on interacting faces of helices in the bR structure¹. Figure 1 *a* presents a cross-sectional view of the channel, where helix 3 is the channel liner. Faces of helix 3 interacting with helices 2 and 4 have been based on the bR structure while the surface exposed to the aqueous pore is based loosely on the alamethicin-zervamicin family². The sequence of SYNCHAN is presented in Figure 1 *b*. Despite the reliance on bR for the design of interacting faces of helices, the overall sequence bears less than 25% homology to bR. We have synthesized a peptide, PCH3, corresponding to the channel lining segment (helix 3) by solid phase synthesis on a semi-automatic solid phase peptide synthesizer (LKB Biochem-Biolynx 4175). A part of the peptide was acetylated at its N-terminus, using acetic anhydride⁸ before cleavage from the resin. The peptide was checked for purity by analytical HPLC (Shimadzu SPD6A) on a C18 reversed phase

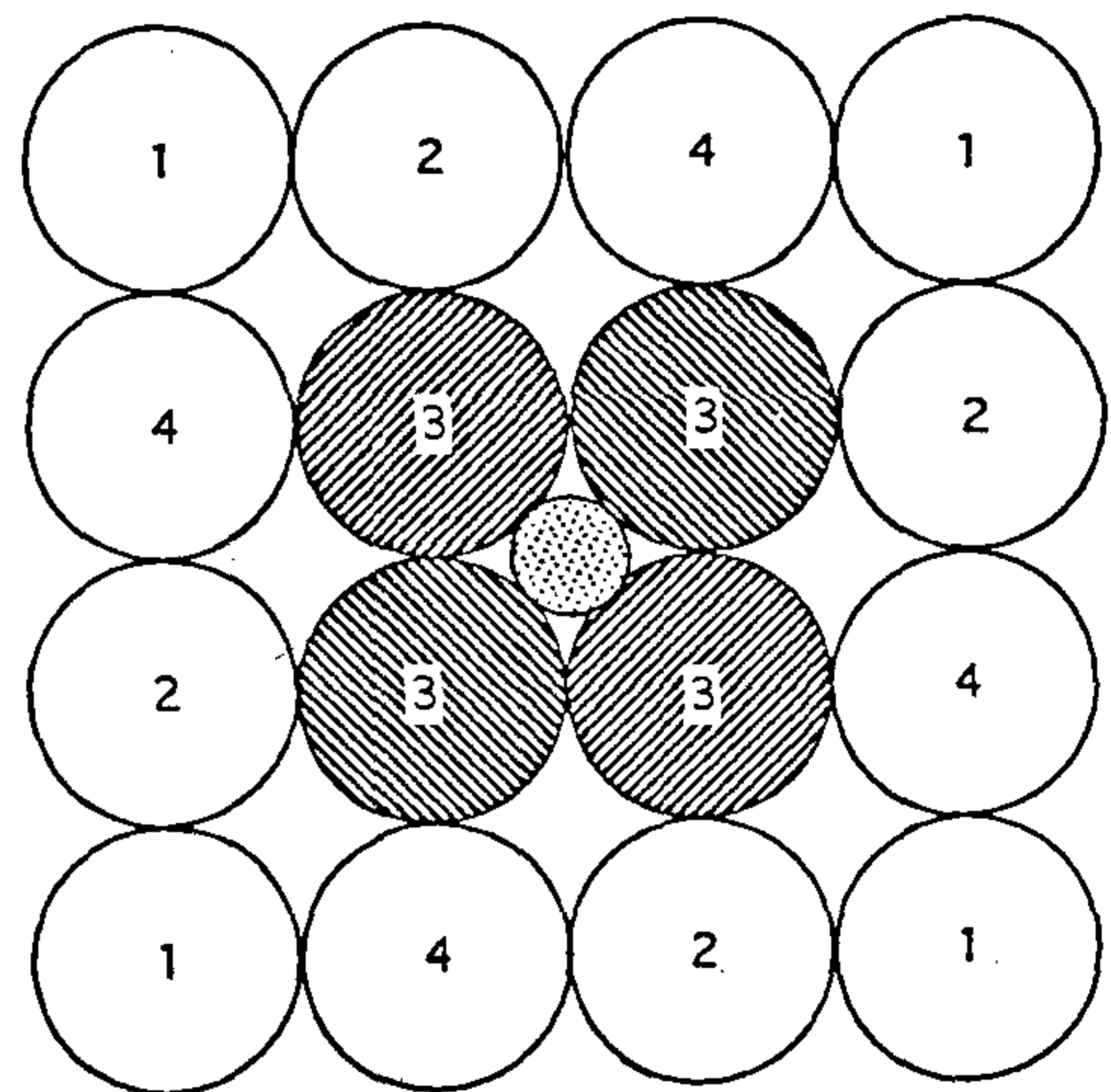


Figure 1 *a*. The synthetic channel is designed to be a tetramer of a protein with 4 transmembrane helices, one of which (no. 3 here) lines the channel. The cross section of the assembled tetramer is shown, with the channel liner hatched. The central pore is also indicated in this panel.

Amino Acid Sequence of The Designed Protein

Intracellular Segment	Membrane Spanning Segment	Extracellular Segment
	#1	
NH ₂ -MAQHIIHHIHRSR ◊	◊ TILYLVTAAIFIGFGLVFAITT ◊	◊ NVDSQLY ◊
	#2	
	◊ FFRVFWLLAYTALLYLLVLYF ◊	
	#3	
◊ KGDQGYRA ◊	◊ YCFALQFAPSUITFFVLLTLW ◊	◊ EGDSQLY ◊
	#4	
	◊ AVETLLFLGAALMILGAFVDAV ◊	
◊ KSLTDV-COOH		

Figure 1 *b*. The sequence of the protein SYNCHAN.

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Vydac column. Two-dimensional NMR studies (400 MHz) of the peptide confirmed its composition and sequence.

Transport of calcium across liposomal membrane was assayed as described in Nagaraj *et al.*⁹. Influx of calcium from the suspending medium was detected by an increase in the fluorescence of chlortetracycline (CTC) entrapped in liposomes. A rise in the intensity of CTC fluorescence on adding the ionophore requires that CTC and Ca^{++} are in separate compartments prior to the addition of the ionophore. The observed rise on adding a known calcium ionophore, X537A, thus serves to establish that the liposomes are intact (Figure 2a). Introduction of AcPCH3 from a methanolic stock at $3.75 \mu\text{M}$ results in a rise in CTC fluorescence comparable to that seen with $20 \mu\text{M}$ X537A demonstrating the efficacy of the peptide in mediating ion transport (Figure 2b). The kinetics of the rise in fluorescence intensity reflect the kinetics of channel formation rather than ion transport as the latter is very rapid. Interestingly, the efficiency of ion transport decreases dramatically if the peptide is delivered from a stock in methanol and water (1:1 v/v). In order to find out why this is so, we have investigated the structure of the peptide in methanol-water mixture of varying composition by circular dichroism (CD) spectroscopy.

The CD spectrum of the peptide dissolved in methanol exhibits minima at 224 and 209 nm characteristic of an α -helical conformation (Figure 3). On the other hand, the spectrum of the peptide diluted into HEPES buffer from a methanolic stock has a single minimum at 215 nm characteristic of β -structure (Figure 3)¹⁰. Addition of a methanolic stock solution to 1,2-dimyristoyl-*sn*-glycero-3-phosphatidylcholine (DMPC) liposomes results in a largely helical structure although CD band intensities are lower than those in methanol (Figure 3). It should be noted that CD spectra of small peptides containing aromatic residues reflect the algebraic sum of contributions from the peptide bonds and from the aromatic residues^{11,12}. Consequently, estimates of fractional helicity from these spectra should be viewed with caution. We have not, therefore, used curve deconvolution techniques to further analyse the spectra. However, for a given peptide, alteration of CD spectra on going from one solvent to another should reflect changes in its secondary structure.

The spectrum of the peptide in mixed methanol/water systems depends on the solvent composition. Instead of using curve deconvolution programs, we have plotted the ratio $[\theta]_{222} / [\theta]_{215}$ against percentage of water in the mixed solvent system (Figure 3, inset). A decreasing

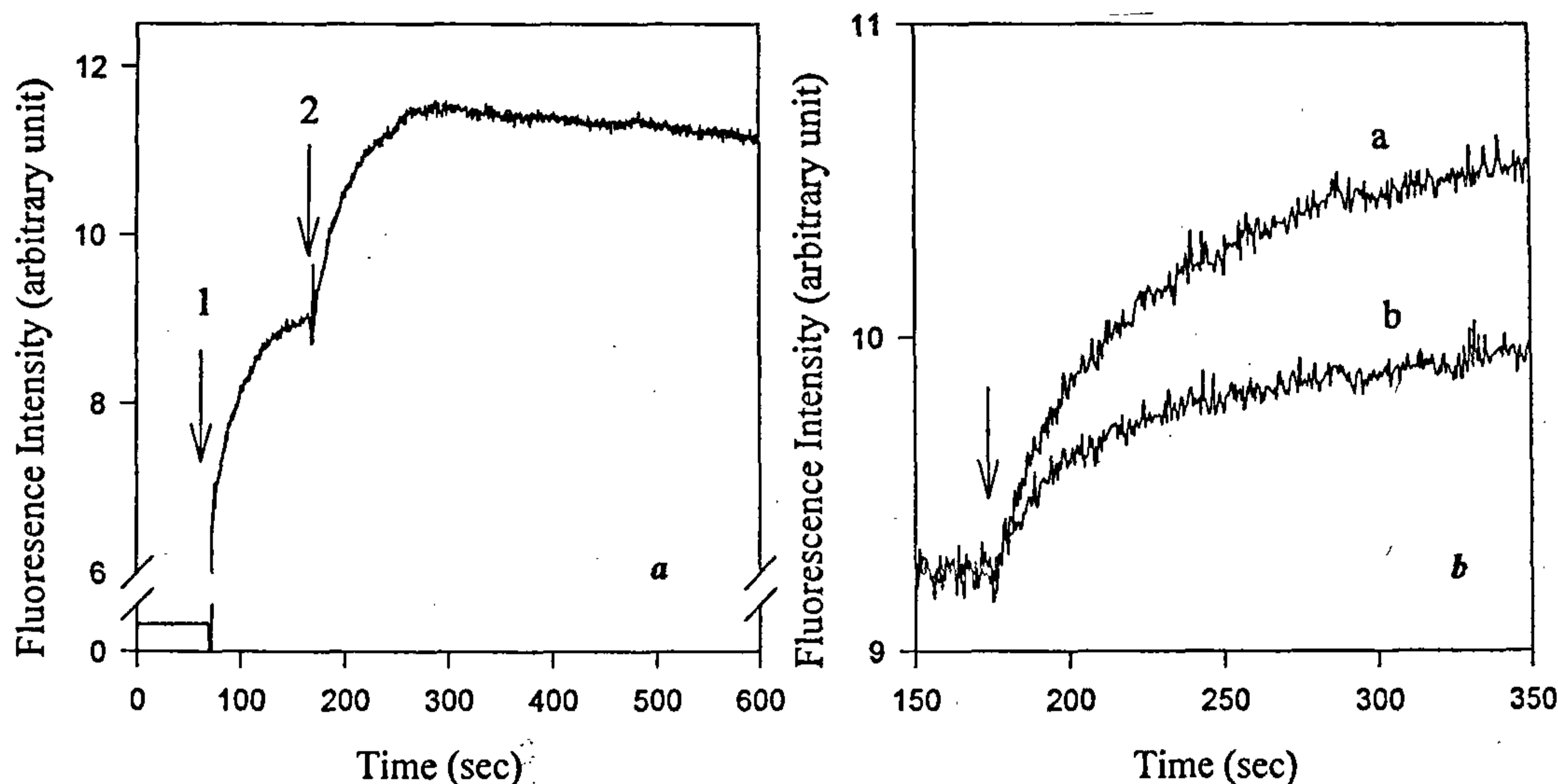


Figure 2. Ion transport across liposomal membranes. Ion transport was assayed fluorimetrically on a Spex Fluorolog 2 as described in ref. 9. $100 \mu\text{l}$ of the liposome suspension (15 mg/ml) was taken in a $3 \mu\text{l}$ fluorescence cuvette along with $10 \mu\text{l}$ of 2.3 mM CTC solution in deionized water. After incubation for 2–3 min, the mixture was diluted with 1.8 ml of buffer and stirred continuously while monitoring at $\lambda_{\text{ex}} = 400 \text{ nm}$ and $\lambda_{\text{em}} = 530 \text{ nm}$. $50 \mu\text{l}$ of 40 mM CaCl_2 solution was added after collecting a baseline. Test samples were delivered through an injection port as a methanolic or aqueous solution after a steady state had been achieved. An influx of calcium into the liposomes mediated by the injected sample can be detected as an increase in the intensity of CTC fluorescence. *a*, Ionophore activity of the calcium ionophore, lasalocid (X537A). CaCl_2 (1 mM) is added at the point indicated by arrow #1; arrow #2 indicates the addition of the ionophore ($20 \mu\text{M}$). *b*, Ion transport activity of (a) AcPCH3 ($3.75 \mu\text{M}$) from a methanolic stock and (b) AcPCH3 ($3.75 \mu\text{M}$) from a stock in methanol/water mixture (1:1 v/v). Addition of peptide is indicated by the arrow.

ratio ($[\theta]_{222}/[\theta]_{215}$) indicates increase of fractional β -structure of the peptide as concentration of water is increased. Addition of methanolic stock into HEPES (final concentration 97.5% water : 2.5% methanol) results in β -structures. Forty-eight hours after mixing, band intensities for the β -like spectrum decrease ~40% (data not shown). The half-time for interconversion is clearly greater than 48 h. In order to investigate whether this was due to the formation of large, insoluble aggregates, the sample was spun at 100,000 g for 1 h. No changes in absorption spectra were seen between the supernatant so obtained and the starting material, indicating that the aggregates must be small and soluble.

Ac-YC(Acm)-FALQFAPSLITFTVFLTLW (AcPCH3) is intrinsically fluorescent with a tyrosine at its N-terminus and a tryptophan at its C-terminus. We have also prepared a dansylated derivative with the dansyl group attached to its N-terminus (PCH3-Dans). Excitation of tryptophan at 285 nm leads to either emission from tryptophan or resonance transfer of energy to the dansyl group and subsequent emission from that chromophore. The efficiency of this phenomenon falls off with the

sixth power of distance and is 50% efficient at a distance of 23.0 Å (R_0) for the Trp-Dans pair^{13,14}.

$$R = R_0(E^{-1} - 1)^{1/6}, \quad (1)$$

$$E = \text{Transfer efficiency} = (1 - F_{DA}/F_D), \quad (2)$$

where F_D and F_{DA} are the emission intensities of

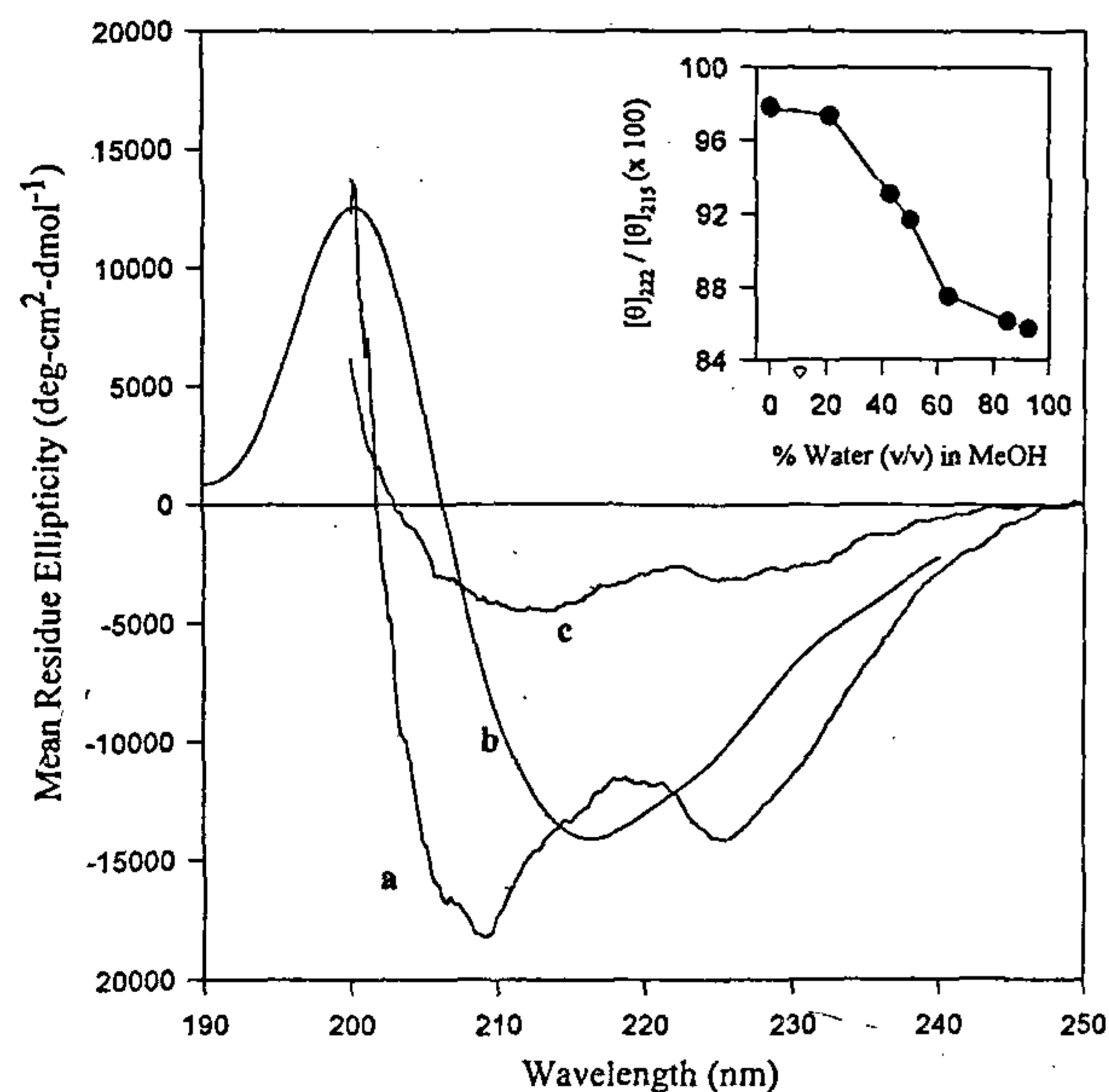


Figure 3. Far-UV CD spectra of Ac-PCH3 at 25°C. CD spectra were recorded on a Jasco720A spectropolarimeter after the instrument was calibrated with (+)-10-camphorsulfonic acid. 1 cm pathlength cells were used and spectra scanned at 50 nm/min in steps of 0.025 nm. 32–64 scans were averaged for each spectrum. Band intensities are represented as mean residue ellipticity ($[\theta]_M$). *a*, 50 μ l methanolic solution of the peptide added into 1.95 ml methanol; *b*, 50 μ l methanolic solution added into 1.95 ml 1 mM HEPES buffer (pH 7.0); *c*, 50 μ l methanolic solution added into 1.95 ml 1 mM HEPES buffer (pH 7.0) in presence of DMPC liposomes. Small unilamellar vesicles (SUV) were prepared following the procedure described in ref. 19 except that DMPC was used. *Inset*: $[\theta]_{222}/[\theta]_{215}$ is plotted against percentage of water in the mixed solvent system.

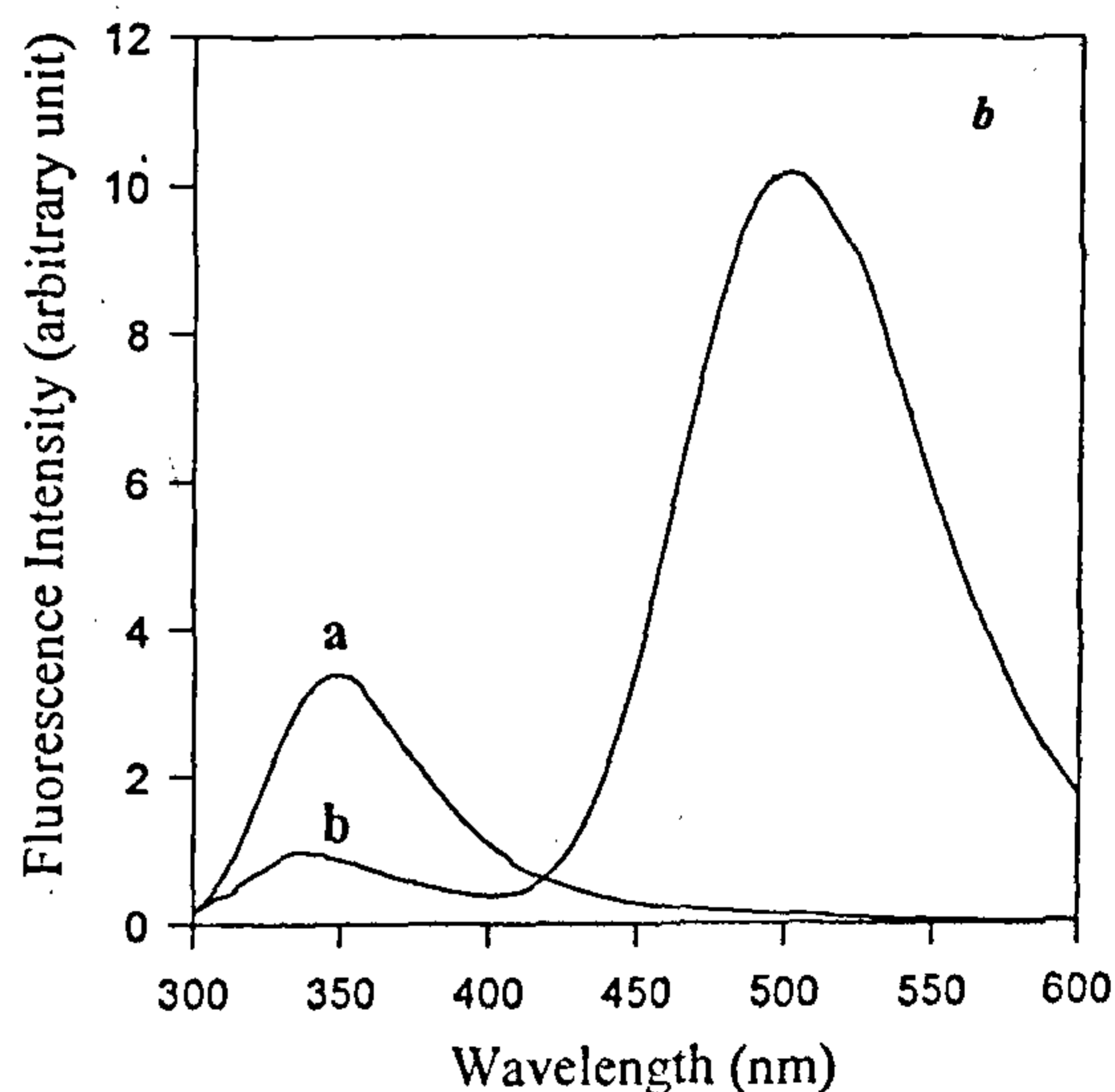
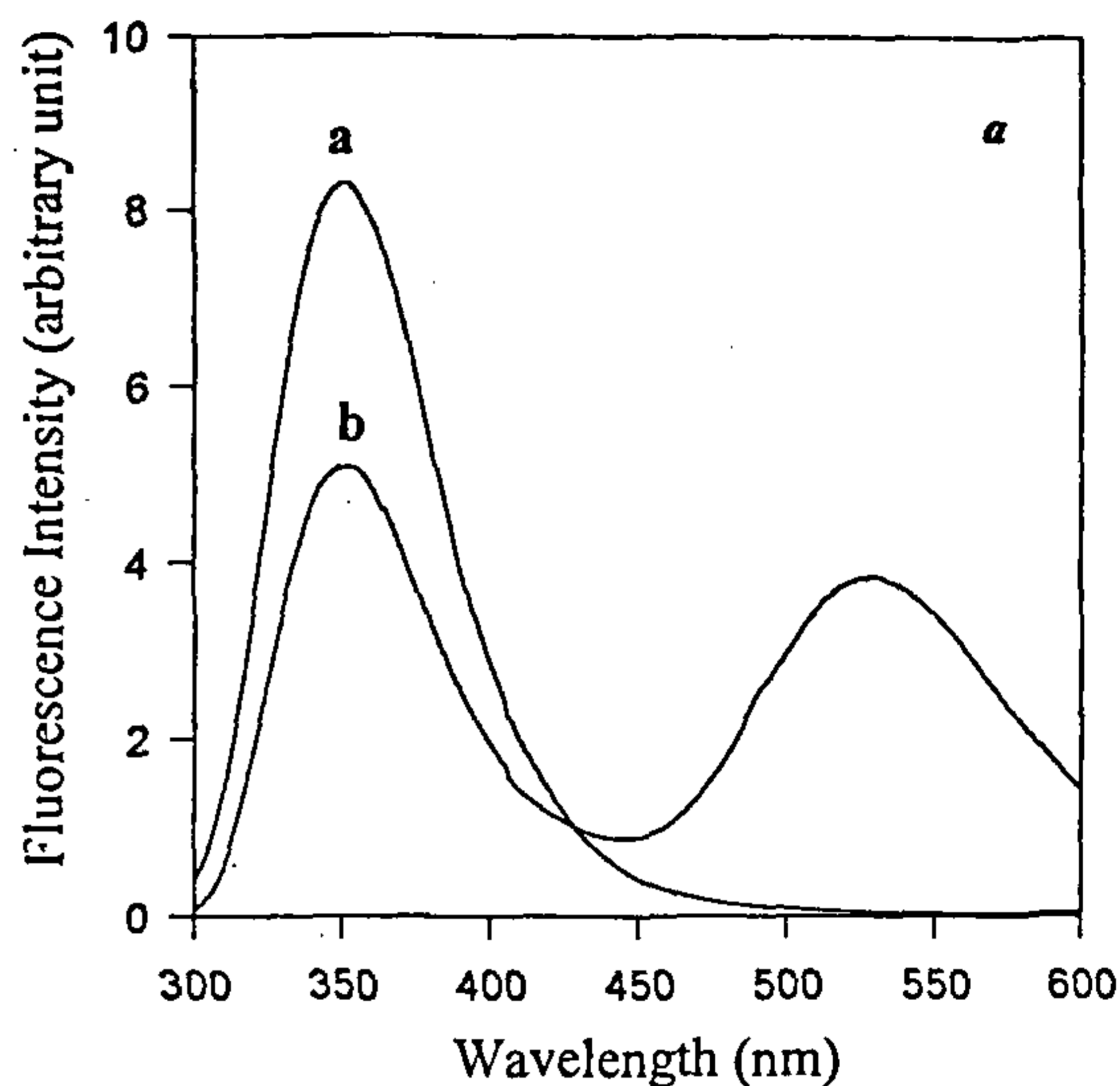


Figure 4. Fluorescence spectra were recorded on a Spex Fluorolog 2 with 1.8 nm excitation bandpass and 1.25 nm emission bandpass with a step size of 0.5 nm per point and an integration time of 1.0 s. Four scans were averaged for each spectrum. All spectra were recorded at 25°C, $\lambda_{ex} = 285$ nm and peptide concentration 3.75 μ M. *a*, Fluorescence emission spectra of AcPCH3 (*a*) and PCH3-Dans (*b*) in methanol. *b*, Fluorescence emission spectra of AcPCH3 (*a*) and PCH3-Dans (*b*) in HEPES buffer (pH 7.0).

tryptophan in absence and presence of the acceptor respectively.

In methanolic solution (Figure 4a), the presence of the dansyl group decreases the emission intensity of tryptophan by 39% for a peptide concentration of 3.75 μM with a concomitant increase in emission at the dansyl wavelengths. The distance corresponding to this efficiency of energy transfer is 24.8 Å which is less than the 33 Å predicted for a 22-residue α -helical peptide. If aggregated, interchromophore distances are likely to be equal to or less than 20 Å in helical, antiparallel aggregates. The dansyl emission at 530 nm indicates that the dansyl group is not shielded¹³, which in turn is indicative that the peptide is not aggregated. We therefore interpret this data as arising from largely monomeric and helical peptides with an average end to end distance of 24.8 Å.

The energy transfer efficiency in HEPES buffer (Figure 4b) at peptide concentration 3.75 μM is much higher (74%) indicative of an average interchromophore spacing of 19 Å. The dansyl emission at 500 nm clearly indicates that the peptide is aggregated¹⁵. The CD spectrum of the peptide under these conditions shows that the peptide is β -structured. We therefore conclude that the peptide forms stacked anti-parallel β -sheet structures in water. Energy transfer in liposomes is also high, close to 75% indicative of association of the peptide in the membrane phase (data not shown).

The conformation of AcPCH3 is dependent on its environment. Such solvent dependent as well as other types of conformational switching have been reported elsewhere¹⁶⁻¹⁸. In case of AcPCH3, addition of largely helical peptide from a methanolic stock to a liposome suspension results in largely helical peptide. This is likely to be due to insertion into the liposomal bilayer as the peptide in the suspending buffer is expected to take up β -structures (Figure 3) under these circumstances. The peptide is largely helical in the membranes (Figure 3) suggesting that channels made by AcPCH3 are made up of transmembrane helices.

In summary, the peptide AcPCH3 is α -helical in methanol and in membranes, and takes up β -structures when diluted into water from a methanolic stock. It mediates ion transport through liposomal membranes when a significant fraction of the peptide is helical. This sequence may provide a useful model for studying conformational switching in peptides.

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