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CD Studies of a Peptide Mimetic of L-Type Calcium Channels

Niels H. Andersen* and Robert B. Palmer

Dept. of Chemistry, University of Washington, Seattle, Washington 98195

Abstract: A synthetic peptide known to form functional transmembrane ion channels when templated to form a four-helix bundle has been examined in its non-templated form by circular dichroic spectroscopy. In aqueous fluoroalcohol media, the peptide is largely helical while in dodecylphosphocholine micelles, a β sheet motif seems to predominate.

Numerous biological processes are mediated by the movement of ions across a cellular membrane.[1,2] The regulation of and communication between cells as well as the electrical passage of neural and muscular impulses are controlled in this manner. The peptide sequence,

D P W N V F D F L I V I G S I I D V I L S E

is implicated as a key pore lining structure of voltage gated calcium channels. A tetrameric bundle of this sequence was prepared,[3] and has been shown to form functional ion channels with the pharmacological properties of the natural channel. The secondary structural characteristics of this peptide alone or in a templated bundle are, however, as yet unknown. For this reason, circular dichroism (CD) spectroscopic studies were performed to determine the propensity of this peptide to adopt the helical conformation presumed to be necessary for channel pore formation.

As an aid to interpretation, the CD spectra were reconstructed from a set of reference spectra derived in our laboratory (**Figure 1**). The curve for β sheet structure is essentially that of Brahms & Brahms[4] and the reference spectrum for helices in non-fluoroalcohol containing media was derived from the reports out of the Yang laboratory.[5] The reference spectrum (αH15) illustrated for an α helix in aqueous flouroalcohol (FA) corresponds to that expected for the central 15 residues of a helical 17 residue linear peptide. It is an extrapolation from the results of spectral deconvolutions based on experimental data for 7-12 residue helical domains in aqueous fluoroalcohol media. The αH15 reference spectrum shows the blue shift in the zero crossing point and narrower band width of the 207nm feature observed for aqueous fluoroalcohol media.[6]

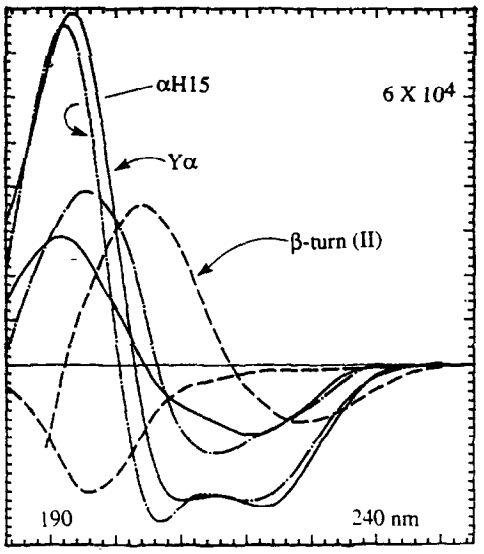

Figure 1. Reference CD spectra for secondary structures: the Yang helix (Yα) and type-I/III β-turn are shown as solid lines; the dashed line traces are β-turn (type II) and disorder; the dot-interrupted lines correspond to β-sheet and a 15-residue α-helix (αH15) in aqueous fluoroalcohol. The Yα, αH15 and β-T(II) traces are labelled in the figure.

Since the αH15 reference curve was derived from spectra of linear peptides at 298 K, it also incorporates the effects of end-fraying at this temperature and domain length. The disorder spectrum, which was similarly derived from fluoroalcohol titration studies assuming two-state behavior, corresponds to high temperature disorder.[7] Two other secondary structure signatures are included in **Figure 1**, type I/III and II β turns. These were obtained as difference spectra (the turn model less the corresponding length of a disordered peptide).[6] The 'type II spectrum' is very similar to that given by Brahms & Brahms.[4] The 'type I/III spectrum' is an almost perfect match to that derived by Perczel & Fasman[9,10] which they attribute to a type-I turn. Since the key ϕ/ψ values at the two corner residues of a β turn, fix the orientations of three carbonyl groups, the turn signatures were attributed to a three residue count for deriving residue-molar ellipticity values.

Multiple spectral reconstructions were performed to match both the curve shape and absolute intensities of each experimental spectrum with the further stipulation that the sum of the constituents had to be within the range 0.85-1.10 mole fraction. Secondary structure compositions are expressed as percentages (whether or not the sum of mole fractions was 1.00).

Experimental Results and Discussion

The conformation of IVS3, a calcium channel mimetic, was examined in aqueous fluoroalcohol solution and in a membrane-mimicking dodecyl-phosphocholine (DPC) micelle. The addition of fluoroalcohols (FAs), such as HFIP used in the present case, to water produces media in which intramolecular hydrogen-bonding is favored and thus increases secondary structuring.[12,13] For linear peptides, FAs generally increase helix population and extent. Most studies suggest that helix stop signals are retained even upon FA addition[13,14]; however, there are instances recorded in which α helical structure is induced in peptide sequences, isolated[14] or in denatured proteins[15], that are not α helices in the corresponding native protein. Thus, some caution is suggested; aqueous fluoroalcohol media cannot *a priori* be assumed to be an accurate mimic of physiologically relevant environments.

CD spectra of monomeric IVS3 in 50 volume percent HFIP at 270 and 300 K appear in **Figure 2-A**. The curve shape is clearly that associated with a largely α helical peptide. Since FA-induced increases in helicity plateau[6,16] at FA levels lower than that present in this solution, the observed curve shape and temperature gradient can be used to estimate the length of the helical domain. The $[\theta]_{191}/[\theta]_{220}$ ratio (-2.11) observed for IVS3 in 50% HFIP at 300 K is significantly smaller than that observed [-2.4±0.1, in our laboratory; similar results have also appeared in the literature -- *e.g.* Zhou *et al.*[17]] for 11-18 residue linear peptides in which the helical domain includes essentially the entire sequence or in the reference spectra (-2.52) in **Figure 1**. This suggests that non-helical residues (besides end-fraying) are present. The temperature gradient at the 220nm band was 80° residue-molar ellipticity/°C. Since the increasing value of $[\theta]_{220\text{-}222}$ upon cooling once a peptide has been titrated to its maximum helicity by fluoroalcohol addition is attributed to diminshed end-fraying at the lower temperature, the temperature gradient is expected to decrease as the length of the helical domain increases. We[18] and others[19] have observed values of 125±23°/°C for 14 residue helices. Our studies of C-peptide analogs in 20-40% aqueous HFIP[6] gave gradients of 207±30°/°C for 10-11 residue helical domains. Therefore, we used 15-18 residue helix reference spectra in our three component fitting of the 50% HFIP spectrum (see **Figure 2-C**). The secondary structure makeup derived was 75±8% α, 10±5% disorder and 15±5% β. The best fits were obtained using the αH15 reference in **Figure 1** and always required a 12-16% β component for optimal matches. The Yang helix gave significantly less good fits.

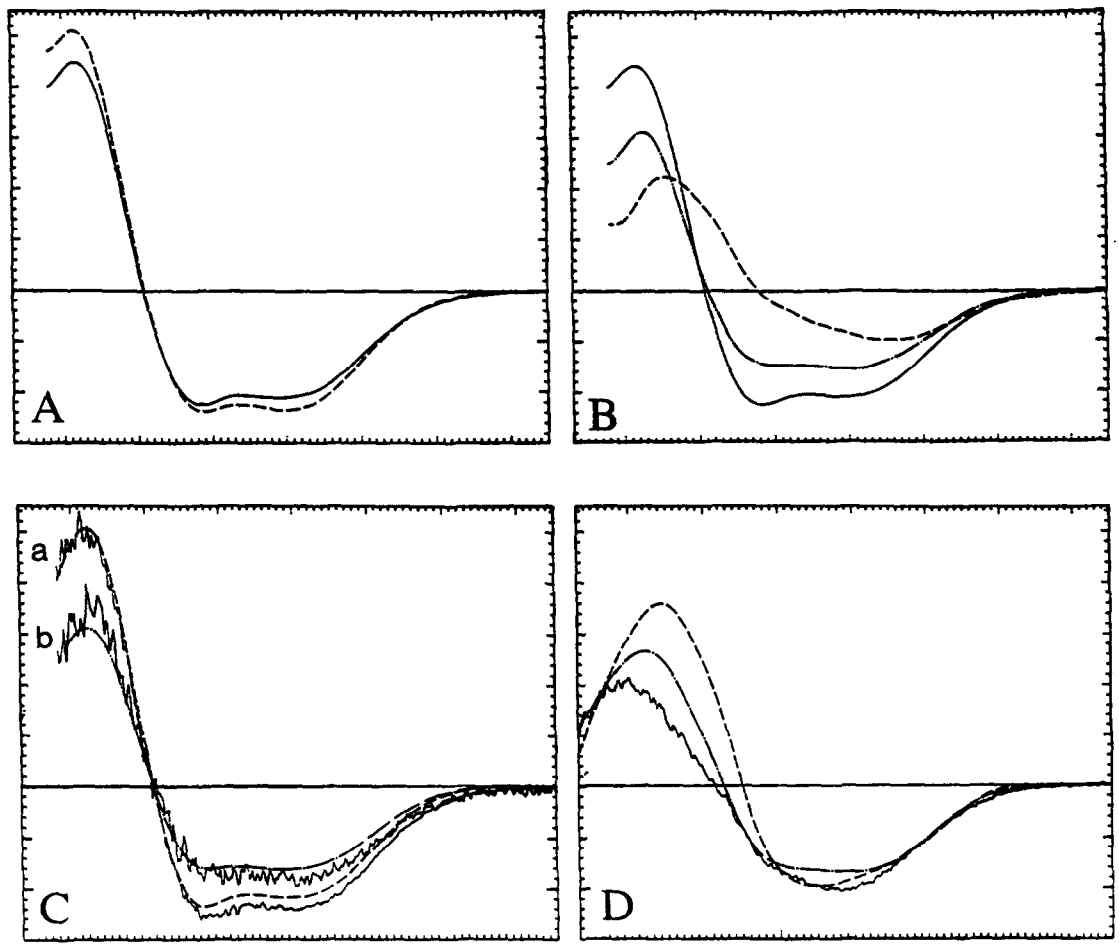

Figure 2. Experimental CD traces[20] of peptide IVS3 monomer and curve reconstructions from secondary structure reference curves. Each panels illustrates the 183 - 255 nm λ-range with a residue-molar ellipticity scale of -30,000 - +55,000°.

A) 29μM in 50% aqueous HFIP at 270 K (dashed line) and 300 K (solid line), smoothed data

B) Smoothed CD spectra recorded at several points on an aqueous HFIP titration at 300 K -- the 50% aqueous HFIP stock (29μM; solid line trace) was diluted with water to final HFIP concentrations (volume percent) of: 20% (dot-interrupted line), 10% (data not shown), 5% (dashed line) and 2% (data not shown). The red-shifted minimum at ≥10% HFIP suggests some β turn contribution.

C) The reconstruction of IVS3 CD spectra at 50 (**a**) and 20 (**b**) volume percent HFIP from secondary structure reference spectra. The unsmoothed traces are the experimental curve. The illustrated fit for the 50% HFIP spectrum corresponds to 68% αH15, 16% β-sheet, 11% disorder and 5% β-turn(II). At 20% HFIP, the illustrated fit is: 45% αH15, 22% β-sheet, 28% disorder and 5% β-turn (II).

D) The unsmoothed trace is the CD of 72μM IVS3 in the presence of a 40-fold excess of DPC at pH 6. The two constructed spectra represent: dashed line [18% Yα, 59% β-sheet and 23% disorder], dot-interrupted line [22% Yα, 17% β-sheet, 33% disorder and 28% β-turn (type-I/III, = 3_{10} turn)].

Figure 2-B shows CD traces recorded upon serial dilution of the 50% HFIP stock with increasing volumes of water. Helicity clearly decreases but the absence of an isodichroic indicates that this does not even vaguely approximate a standard two-state helix → disorder equilibrium. In order to fit the IVS3 spectrum at 20 vol-% HFIP, both the disorder and the anti-β components must be increased. The best fits (see trace **b** of **Figure 2-C**) suggest 40-50% helix, 20-25% anti-β and approximately 25% disorder with a small amount of type-II β turn improving the curve shape agreement. The spectra observed at HFIP levels below 20 vol-% suggest aggregation. At concentrations ≤ 10% HFIP, the CD curves cannot be reconstructed from standard reference spectra. The curves suggest the formation of aggregates too large for standard transmission spectroscopic measurements. All fits approximating these curves had a sum of component populations significantly less than 0.70.

The IVS3 monomer (at 72 μM) gives highly reproducible CD spectra (**Figure 4-D**) in the presence of a 40-fold excess of dodecylphosphocholine (DPC), presumably by the production of uniformly loaded mixed micelles. The resulting 'solutions' can be lyophillized and reconstituted with no observable change. The same spectrum (data not shown) results when a 4-fold molar quantity of the dihydropyridine calcium channel antagonist, nifedipine, is included in the mixture. Note the shift of the minimum to 217 rather than 221nm. Of the standard reference spectra, that observed most resembles the β sheet. However, a distinct shoulder at 207nm is retained suggesting some residual helical structuring.

The CD observed for IVS3 in the micellular system is difficult to deconvolute quantitatively. If fits are restricted to a three component reference library containing α helix, anti-β and 'disorder', the conclusion is that the β structure component increases to 60±8% (with *circa* 15% helix and 20% disorder). The Yang helix reference spectrum gives better matches than the aqueous fluoroalcohol helix; however, we do not view the fits as sufficiently accurate for deriving reliable secondary structure composition estimates. If the fit is expanded to include less well-characterized secondary structural types, constructed spectra containing the type-I-β-turn reference spectrum generated in our laboratory give improved, but still unsatisfactory fits. Typically, the sum of βI/III turns and helix contribution was about 60% and the β-sheet component dropped to 20%.

The latter observation raises an interesting question. Is it possible that this actually reflects a significant population of 3_{10} helix? *The CD signature of an authentic solution-state 3_{10} helix does not appear to have been reported.* In one case,[23] ESR studies of a spin labeled Ala-rich peptide was suggested as supportive of a 3_{10} conformation; the CD signature from 200-240nm was not notably different from that expected for an α helix. Manning & Woody[24] have calculated CD spectra for a variety of static helix models. The 'theoretical spectra' of 3_{10} helix models are readily distinguishable from both that derived for α helix models and that experimentally observed for type-I β turns. There are certainly some structural analogies which might suggest that 3_{10} helices and type-I and III β turns could have similar CD signatures. The 'theoretical' 3_{10} spectrum has a much reduced intensity at the 192nm maximum and a blue shift for the intense $n \rightarrow \pi^*$ band. Therefore, the possiblity that IVS3 forms some 3_{10} helical structure in a micellular environment, though speculative, cannot be dismissed.

Conclusions

The CD spectrum of monomeric IVS3 in 50% HFIP can be fully explained using reliable reference spectra for relatively long α helices in this medium. However, even under these helix-favoring conditions a small amount of β structure is detected and the helical state does not appear to display a $[\theta]_{220}$ value as large as would be expected for a continuous α helix of this length. In aqueous media at lower levels of added fluoroalcohol, β structuring (presumably an oligomer) and then aggregate formation are observed. The aggregate formation is presumably driven by hydrophobic association due to the high density of branched fatty sidechains. Other data collected in this laboratory support the notion that the $[\theta]_{220}$ value of a helical domain reflects the net sum of helical state populations over all of the residues of the domain. On that basis, the diminshed $[\theta]_{220}$ value for the IVS3 monomer can be taken as an indicator of a discontinuity in a residue by residue profile of otherwise high helical probabilities. The sequence includes a substantial number of residues (D,N,G,S) with reduced helix propagation constants;[23] of these, the Gly^{14}-Ser^{15} locus is the best candidate for the discontinuity. It will be very interesting to ascertain whether the diminished $[\theta]_{220}$ value is retained in the templated bundle derived from this sequence.

According to our preferred rationale for the CD spectra in mixed micelles with DPC, which should mimic the conditions used for patch clamp ion conductance measurements of ion channel formation, IVS3 displays rather low populations of standard α helical conformations. *(In the alternative, and more speculative, rationale higher populations of 3_{10} helices would be allowed).* At this point, we suggest that the low efficiency of channel formation[24] with monomeric IVS3 probably reflects the small population of sufficiently long α helices in the medium used for the measurements which thereby precludes self-assembly. Further, we find that a dihyropyridine calcium channel antagonist does *not* serve as a non-covalent template for assisted assembly. CD studies of covalently templated IVS3 will be required to resolve the questions concerning the peptide secondary structure present in this calcium channel mimic.

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20. All aqueous HFIP samples were prepared by dissolving weighed amounts (0.3-0.7 mg) of freshly lyophillized peptide directly into a solution of 50% HFIP in water containing approximately 1 volume percent glacial acetic acid followed by serial dilution. The aqueous buffers used for the dodecylphosphocholine (DPC) micelle studies were 5mM phosphate (pH 5.1-9.6). Mixed micelles were obtained by spreading a thin film of 0.13 mg of IVS3 over a glass centrifuge tube surface by dispersal in hexane followed by evaporation to dryness. To this film was added 0.6 mL of a DPC suspension (prepared from 1.81 mg DPC and 1.44 mL of phosphate buffer, pH 6.04). The resulting mixture was sonicated for 3 minutes and then allowed to stand at ambient temperature for 4.5 hours prior to spectroscopic examination. CD spectra were recorded using a JASCO model 720 spectropolarimeter as reported previously.[21]

Concentrations of IVS3 in solution were determined by UV spectrophotometry (using a CARY 3E spectrophotometer equipped with a GRID MFP/320s computer) assuming ε_{279}=5580 $cm^2/mmole$[22] for a peptide containing a single Trp residue. The UV assay was carried out directly on the mixed micelle 'solutions' used for CD studies. In the case of the less ideal solutions of IVS3 in aqueous hexafluoroisopropanol (HFIP), this option was unavailable. The concentration of the IVS3 stock solution (in 50% HFIP) was determined by difference UV spectroscopy as the sample did not give a spectrum with a flat baseline. The UV spectrum of a standard solution of a tryptophan-terminated tetrapeptide, GHKW, in the same medium was determined. Fractions of this spectrum were then subtracted from the spectrum of IVS3 until the UV band fine structure of the tryptophan was nulled. The concentration corresponding to that fraction was used as the estimate of the IVS3 stock concentration. Volumetric dilutions of the stock were performed for all other concentrations of HFIP and their concentrations calculated accordingly. Work in our laboratories has indicated that the mixing volume effect of water and HFIP is negligible.

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26. Grove and Montal (ref. 3 and subsequent studies) find that IVS3 monomer forms functional channels in extremely low yield under conditions which give efficient channel formation with the templated form of IVS3.

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