Vibrational circular-dichroism spectroscopy of homologous cyclic peptides designed to fold into β helices of opposite chirality

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Cyclic β-helical peptides have been developed as model structured biomolecules for examining peptide adsorption and conformation on surfaces. As a key prerequisite to circular-dichroism (CD) analysis of these model peptides on surfaces, their conformations and the corresponding vibrational spectra in the 1400–1800 cm^{-1} range were analyzed by vibrational circular-dichroism (VCD) spectroscopy in solution. The two model peptides ("β Leu and β Val") were examined in chloroform, where they each fold into a homogeneous well-defined antiparallel double-stranded β-helical species, as determined previously by NMR and electronic CD spectroscopy. Because the β-helical conformations of β Leu and β Val are well characterized, the VCD spectra of these peptides can be unambiguously correlated with their structures. In addition, these two β-helical peptides differ from one another in two key respects that make them uniquely advantageous for CD analysis—first, while their backbone conformations are topologically similar, β Leu and β Val form helices of opposite chiralities; second, the two peptides differ in their sequences, i.e., composition of the side chains attached to the backbone. The observed VCD spectra for β Leu and β Val are roughly mirror images of each other, indicating that the VCD features are dominated by the chirality and conformation of the peptide backbone rather than by the peptide sequence. Accordingly, spectra similarly characteristic of peptide secondary structure can be expected for peptides designed to be structural analogs of β Leu and β Val while incorporating a variety of side chains for studies of surface adsorption from organic and aqueous solvents. © *2011 American Vacuum Society.*
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I. INTRODUCTION

The interdependence of form and function in biomolecules is a fundamental principle of biology. For biointerphases, understanding this interdependence is crucial for elucidating and controlling conformational and functional changes in proteins during and after adsorption to a surface. Protein adsorption is involved in a number of technologically and commercially significant processes; however, the large size of the adsorbing proteins coupled with the heterogeneity of many real-life surfaces makes direct spectroscopic investigation of such biointerphases prohibitively difficult.[1–3]

A promising alternative strategy is using model peptides to reduce the complexity of the problem.[4–8] For spectral data to be interpretable and informative in the broader context of protein structures, however, peptide model systems must be chosen carefully to embody both the *level* of structure (primary, secondary, tertiary, etc.) and *type* of structure (helix, sheet, turn, etc.) that one wishes to investigate. A model system that we analyzed in this study, for example, is β helices—helices composed of alternating D- and L-α-amino acids that are stabilized by β-sheet hydrogen bonding.

The conformation and chirality of peptides and proteins in solution are typically determined by NMR and circular-dichroism (CD) spectroscopies.[9–14] For extending the conformational analysis to small peptides in biointerphases, we are particularly interested in optical spectroscopies because they can be employed either *ex situ* or *in situ* to obtain information—potentially in real time—about molecular conformation, chirality, and orientation.[15–20] Electronic CD (ECD), which uses circularly polarized light in the UV range and in the simplest cases provides information about the relative orientation of backbone amide chromophores, is emerging as a promising tool for investigating peptide and protein conformation on surfaces.[19–27] While ECD is a powerful technique for characterizing the overall conformation of chi-

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ral biomolecules, ECD relies on transition moments that are delocalized over large portions of the molecule, resulting in broad spectral features, for which a detailed correlation with peptide conformation can be ambiguous.

A complementary technique, vibrational CD (VCD), uses circularly polarized IR light and provides spectra with detailed "fingerprints" analogous to standard IR spectra. VCD measurements in the amide I and II regions have been widely used to study conformations of small peptides, including small cyclic peptides and turns[28–32] that resemble the peptides analyzed in this work. These measurements can be challenging because VCD bands tend to be weaker than IR absorbance bands by a factor of 10^4–10^5; however, the vibrational transitions probed by VCD are typically more localized to specific chromophores or normal modes and are sensitive to dipole coupling of nearby residues. As a consequence, VCD spectra can be more conformationally informative than ECD. Calculations at the level appropriate for assignments and interpretation of VCD spectra of medium and large peptides are computationally demanding.[29] Even predictions for two-amino-acid β turns vary depending on method, basis set, and choice of solvent model.[31] Therefore, in this work we aim to explore how reference data from model peptides having well-defined conformations can be used to assist in empirical interpretation of results for larger, complex, and heterogeneous peptides.

Knowing the conformation of a peptide in solution is a prerequisite for elucidating conformational changes that occur upon adsorption; thus, superficially β helices may seem an unlikely model system. For example, the archetypal β-helical peptide, gramicidin D (gD), not only is chemically inhomogeneous but also forms a complex mixture of double-stranded (ds) and single-stranded (ss) species in solution; in addition, ds forms can have either parallel ($\uparrow\uparrow$) or antiparallel ($\uparrow\downarrow$) orientation of the two strands.[33] In earlier work, we solved the problem of conformational promiscuity in β helices by using turn sequences to cyclize the peptides. Conceptually, we designed "β Val" to contain two nine-residue strands of alternating D-,L-α-amino acids. To constrain the nine-residue strands into an antiparallel conformation, we joined them with two copies of the reverse β turn, D-Pro-Gly. We further constrained the molecule by joining the N and C termini of the two nine-residue D-,L-strands forming a cyclic D-,L-peptide. The peptide was designed to adopt a right-handed ds-antiparallel conformation having approximately 5.6 residues per turn (a right-handed $\uparrow\downarrow\beta^{5.6}$ helix, β Val in Fig. 1).[14] We confirmed this conformation in solution using NMR spectroscopy and measured ECD spectra of the constrained right-handed β Val helix. We then designed and synthesized a second ds-antiparallel peptide ("β Leu" in Fig. 1) composed of Leu amino acids, which at equivalent positions in the sequence (numbers in Fig. 1, top) are opposite in chirality to the Val amino acids of the original β Val helix. Even though the two peptides have different sequences, the features in their ECD spectra are approximately equal in magnitude and opposite in sign, indicating that, as expected, peptide β Leu is left handed (i.e., a left-handed $\uparrow\downarrow\beta^{5.6}$ helix)

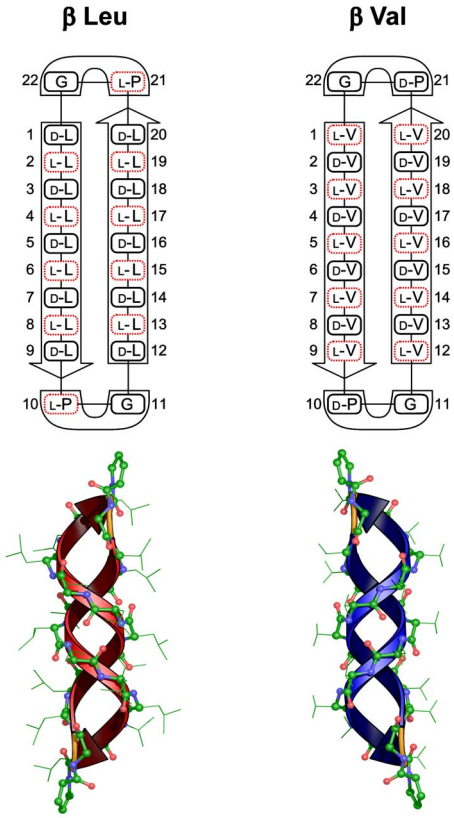

FIG. 1. (Color online) Two cyclic peptides designed to fold into β helices of opposite chirality. Top: sequence and numbering scheme for β Leu and β Val displayed in a flat β-hairpin representation; solid line emphasize D residues and glycines and dashed lines emphasize L residues. Bottom: the solution structures of β Leu and β Val in chloroform (Ref. 13).

while simultaneously confirming that the ECD spectra of these peptides are dominated by their chirality and secondary structure.[13]

Here, we examine the conformation of β Val and β Leu by VCD spectroscopy to obtain spectral signatures having finer structure than those observed in ECD spectra. We begin by validating the assumption that structurally homologous model peptides result in correspondingly similar, distinct, and uniform vibrational spectra. We then quantitatively examine the features observed in VCD and IR spectra for the two peptides to investigate the effect of peptide sequence and secondary structure on VCD spectra, which generally can contain features characteristic of both peptide backbone and side chains.

II. MATERIALS AND METHODS

Peptides were synthesized and purified as previously described.[13] VCD experiments were recorded at 295 K using a BioTools Chiral IR Dual-PEM VCD Spectrometer at 8 cm^{-1} resolution. Solutions were prepared by dissolving pure lyophilized peptide in spectroscopic grade chloroform for a final concentration of approximately 10 mM. Samples were held in a demountable liquid cell with CaF_2 windows and a fixed path length of 100 μm. Spectra were collected for 12 h, averaged over all scans, and corrected for back-

ground by taking the ratio to a blank spectrum of pure chloroform. The IR and VCD spectra measured for β Val and β Leu were analyzed in the region of 1480–1750 cm^{-1} by fitting them to a minimum number of Lorentzian bands using SigmaPlot 10 (Systat) as described in the text.

III. RESULTS

Adapting solution spectroscopic techniques for analysis of model structured peptides on surfaces can be achieved through a series of model studies, each designed to validate the measurement for a system that incorporates increasingly more realistic features.[4–8,24,25,34] This work is the first step in such a series of studies designed with the ultimate goal of performing structurally sensitive optical and vibrational spectroscopy measurements at realistic biointerphases between structured water-soluble peptides and model surfaces in aqueous solutions. As in many other studies of biointerphases, the experimental constraints for designing the appropriate initial set of experiments are inherited from the current status of both the model system and the analytical technique. In our case, the initial IR and VCD measurements in the amide I and II regions can be facilitated and disambiguated by simplifying two experimental parameters: using organic rather than aqueous solutions and analyzing chemically and structurally uniform model peptides. Serendipitously, the same simplifications are suggested by constraints on design and synthesis of the model β-helical peptides, as chemically uniform cyclic helical peptides are most readily synthesized in nonpolar solvents from amino acids that have hydrophobic side chains (Val and Leu). Complementary IR and VCD measurements of β Val and β Leu model peptides in a nonpolar organic solvent, therefore, serve as the first essential step toward the ultimate goal of analyzing more realistic peptide-based biointerphases by providing both an initial validation of our experimental methodology and critical reference vibrational spectra for future studies. Finally, we note that results obtained in chloroform are directly relevant to *nonpolar biological environments* such as the interior of a lipid membrane.

Solutions of β Val and β Leu were prepared as described in Sec. II. Although both peptides are freely soluble and fold into the desired ds $\uparrow\downarrow\beta^{5.6}$ helices in both trifluoroethanol and chloroform, we used the latter due to its greater IR transparency in the spectral region of interest (approximately 1400–1800 cm^{-1}). To achieve high signal to noise in VCD spectra, we used approximately 10 mM peptide concentrations, which are higher than those required for simple IR absorbance measurements. Figures 2(a) and 2(b) show the VCD and IR absorption spectra, respectively, for β Val and β Leu in the amide I region (C=O stretch) and amide II region (which are predominantly bands due to C–N stretching vibrations coupled to the N–H bending). The IR spectra [Fig. 2(b)] are essentially identical to those that we measured previously for these peptides;[14] in addition, the two spectra are very similar to each other, indicating that the IR features in the amide I and II regions primarily originate from common structural elements of the peptides. The IR spectra also resemble those of gD acquired in dioxane, where the left-handed $\uparrow\downarrow\beta^{5.6}$-helical dimer predominates;[35] in particular, the largest peak in the amide I region appears at approximately 1635 cm^{-1} for all three peptides. For comparison, Naik and Krimm calculated a value of 1636 cm^{-1} for the amide I band of an idealized $\uparrow\downarrow\beta^{5.6}$ helix.[36,37]

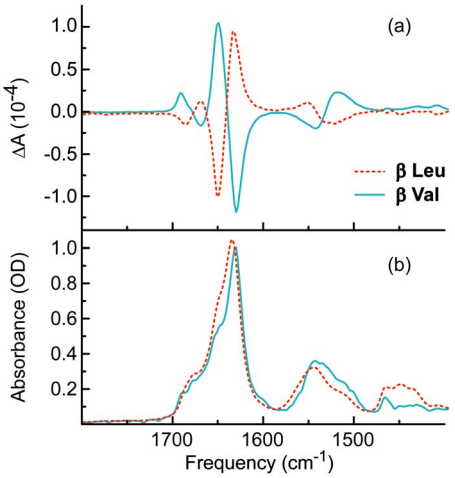

FIG. 2. (Color online) Complementary vibrational spectra of β Leu and β Val. Overlayed VCD (a) and IR absorption (b) spectra are shown for β Leu (dotted line) and β Val (solid line).

The VCD spectra for the two peptides exhibit clear and distinct features of both positive and negative polarities, validating our rationale for analyzing model peptides having well-defined structures. The dominant feature of the VCD spectrum of β Leu is a positive VCD couplet, with a negative VCD component near 1650 cm^{-1} and a positive VCD component near 1632 cm^{-1}, which are close to the respective peak positions observed for gD in dioxane.[35] The VCD bands in the amide II region for β Leu also resemble those of gD in dioxane,[35] with a weaker negative VCD couplet having a positive VCD component near 1550 cm^{-1} and a negative VCD component near 1520 cm^{-1}. Unlike gD, however, β Leu also shows a small positive VCD couplet at higher frequency, with a negative component near 1690 cm^{-1} and a positive component near 1670 cm^{-1}. The VCD spectrum of β Val is nearly equal in magnitude yet opposite in sign to that of β Leu, indicating that the two spectra originate from structures that are almost identical (in agreement with IR data), albeit of opposite handedness.

IV. DISCUSSION

Previous VCD studies of β-helical peptides have focused exclusively on gD, a mixture of linear pentadecapeptides composed of alternating D- and L-α-amino acids.[35,38–40] Gramicidin D comprises gramicidins A, B, and C, which vary slightly in their sequence but are nearly functionally equivalent. This chemical heterogeneity, together with the aforementioned tendency of gD to exist in solution as a mixture of species (ss, ds, parallel, antiparallel, etc.), has led experimentalists to vary the solvent to favor the relative population of different species in solution. For example,

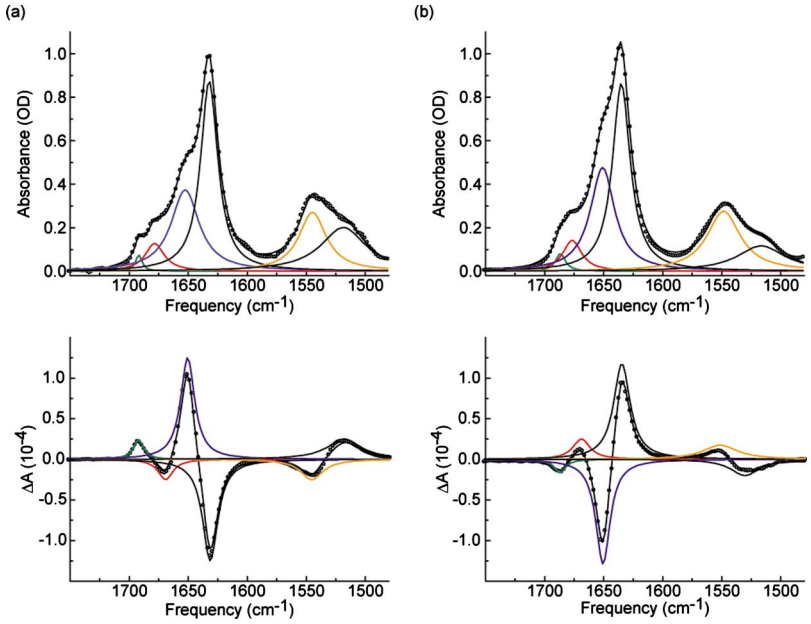

FIG. 3. (Color online) Fitting of vibrational spectra of β Leu and β Val. Complementary vibrational absorption (top traces) and circular-dichroism spectra (bottom traces) are shown for β Val (a) and β Leu (b). The measured spectra are shown as points; the composite fit spectra and individual bands are indicated by black and colored lines, respectively. Spectral parameters determined for the bands are provided in Table I.

Zhao and Polavarapu characterized gD in dioxane, CD_3OD, ethanol, propanol, and $CHCl_3$, with the latter containing trace ethanol as a stabilizer ($CHCl_3$/EtOH).[35] In each case, a positive VCD couplet was observed in the amide I region with a negative VCD component near 1650 cm^{-1} and a positive VCD component near 1632 cm^{-1}, similar to the VCD spectrum of β Leu in Fig. 2(a). We have shown previously by NMR that β Leu folds in chloroform solution to give a single species—a left-handed $\uparrow\downarrow\beta^{5.6}$ helix;[14] thus, one might naively conclude that the VCD spectra reported by Zhao and Polavarapu are characteristic of the left-handed $\uparrow\downarrow\beta^{5.6}$-helical form of gD. However, NMR studies by other groups have shown that, while the $\uparrow\downarrow\beta^{5.6}$-helical form of gD does predominate in dioxane,[41,42] the peptide forms a complex mixture of species in both methanol,[33] ethanol,[43] and propanol.[33] Clearly, in terms of structural homogeneity β Leu is a superior model of a $\uparrow\downarrow\beta^{5.6}$ helix than gD, and the VCD spectrum of β Leu can be more reliably attributed to the $\uparrow\downarrow\beta^{5.6}$-helical structure.

The VCD spectrum of β Val is nearly a mirror image of that of β Leu, verifying that the two peptide helices have opposite handedness. Furthermore, the similarity of the shape and magnitude of the VCD spectra of these peptides in amide I and II regions demonstrates that they primarily depend on the conformation and chirality of the peptide backbone. For the peptides examined in this study, therefore, the VCD features appear to be insensitive to differences in amino acid composition even though any such spectral contributions might be expected to be enhanced by our choice of uniform peptide sequences as models.

The apparent lack of sensitivity of the VCD spectra to differences in the identity and rotamers of the hydrophobic side chains is an encouraging indication for our plans to use structurally analogous peptides in surface adsorption experiments. We note, however, that both Val and Leu side chains used in this study can affect vibrational spectral features in the amide I and II regions indirectly, i.e., through their influence on the backbone conformation.[30] Water-soluble variants of the original model peptides necessarily include hydrophilic side chains,[44] which may contain chromophores that contribute directly to the vibrational spectra in the amide I and II regions. For hydrophilic peptides designed to fold into the same $\beta^{5.6}$-helical conformation,[44] the important question that we plan to address in future studies is whether the chiral VCD signatures of helical backbones (Fig. 3) will be perturbed by hydrophilic side chains. Ultimately, understanding the VCD signatures of hydrophilic peptides is important for investigating practical processes involving surface adsorption of peptides and proteins. This study of β Val and β Leu is the first step toward quantitatively elucidating VCD spectra for moderate-size structured peptides by taking advantage of the chemical uniformity and structural simplicity of model hydrophobic β helices.

We note that a qualitative comparison of our data to published VCD spectra of gD in presence of cations[39,40] indicates that resolving the ambiguity in correlating β-helical structural motifs to VCD spectral signatures will require additional model systems with well-defined structures. For example, in 4.8 mM Ca^{2+} in CD_3OD solution, where the left-handed $\uparrow\uparrow\beta^{5.7}$ helix predominates,[45] the salient feature of the VCD spectrum of gramicidin D is a positive VCD couplet in the amide I region.[40] This observation, together with our observation here that VCD spectrum of the left-handed $\uparrow\downarrow\beta^{5.6}$-helical peptide β Leu is also dominated by a positive couplet in the amide I region, might lead the reader to conclude that a strong positive VCD couplet in the amide I re-

TABLE I. Spectral parameters for bands from Lorentzian fits to IR and VCD spectra of β Val and β Leu.

	IR				VCD			
	β Leu		β Val		β Leu		β Val	
	Int.[a]	ν (w) [b]	Int.[a]	ν (w) [b]	Int.[a]	ν (w) [b]	Int.[a]	ν (w) [b]
Band 1	0.08	1686.6 (5.1)	0.08	1691.9 (3.3)	−0.16	1687.0 (5.9)	0.24	1692.0 (5.1)
Band 2	0.14	1676.6 (9.5)	0.13	1678.4 (9.7)	0.25	1668.8 (8.7)	−0.25	1669.2 (7.5)
Band 3	0.48	1650.9 (12.5)	0.37	1652.6 (14.4)	−1.29	1650.6 (7.4)	1.25	1650.5 (7.2)
Band 4	0.86	1635.0 (9.1)	0.87	1632.2 (8.8)	1.18	1634.5 (8.5)	−1.25	1631.3 (7.8)
Band 5	0.28	1548.4 (17.2)	0.27	1544.7 (14.1)	0.17	1551.3 (17.9)	−0.25	1545.3 (11.6)
Band 6	0.12	1516.6 (24.6)	0.20	1518.6 (23.2)	−0.20	1530.5 (14.5)	0.25	1517.9 (17.5)

[a]Intensities for VCD bands are multiplied by 10^4.
[b]Frequency and width, ν (w), are expressed in cm^{-1}.

gion is characteristic of left-handed ds β helices, regardless of the orientation the two strands. However, Zhao and Polavarapu also found that the VCD spectrum of gD in CD_3OD containing 40 mM Cs^+—conditions under which the right-handed $\uparrow\downarrow\beta^{7.2}$ helix predominates—shows no prominent VCD couplet in the amide I region,[40] suggesting that properties of a β helix other than its handedness—for example, the number of residues per turn—also exhibit a strong influence on the VCD spectra of these peptides.

We analyzed the IR and VCD spectra of β Val and β Leu more quantitatively to further demonstrate that the bands in the amide I region are very similar for the two peptides except for the inverted sign of the VCD intensity, which reflects their mirror-image conformation and chirality. The normal modes associated with the bands in IR and VCD spectra are the same, therefore, vibrational bands that are resolved with little or no overlap should have the same band center frequencies and widths while differing only in the VCD and IR intensities, a situation that is commonly observed for smaller molecules such as α pinene.[46] In the present study, however, the molecules of interest are peptides composed of 22 residues, each with overlapping and dipole-coupled contributions to each type of observed band (that is, amide I and amide II). Nevertheless, we found that the observed spectra of β Val and β Leu can be well represented as a sum of fewer than 22 bands.

We analyzed the IR and VCD spectra to determine band frequencies, widths, and intensities by fitting to six Lorentzian bands (the same procedure was used separately for each peptide). We initially attempted to fit both the VCD and IR spectra to bands with the same frequencies and widths for each peptide, only allowing their intensities to vary, as one would expect for a simpler system without overlapping bands from several modes. Achieving a satisfactory fit with this approach proved impossible, especially near 1670 cm^{-1}. Instead, we fit the IR and VCD spectra sequentially with the frequencies and widths of VCD bands constrained to be within a few cm^{-1} of the values initially determined for the IR bands. The results for the bands determined in these sequential fits are listed in Table I and the calculated bands and composite spectra are shown with the data in Fig. 3. We note that the six bands shown in Fig. 3 were the minimal number required to fit both sets of IR and VCD data.[47,48] The center frequencies and widths determined for the intense bands in the IR and VCD spectra for each peptide are very similar to one another, with the notable exceptions of band 2 for both peptides and of band 6 for β Leu. Among the overlapping and coupled modes in the amide I and amide II regions, the strongest bands are different in the VCD and IR spectra. The differences in the observed prominent VCD and IR bands depend on details of the secondary backbone structure of the peptides. Since each peptide folds into a unique species in chloroform, we can confidently ascribe the features in each spectrum to a single peptide species rather than to contributions from a mixture of species (as is the case, for example, with gD). The peptides we have synthesized and the spectra we have measured for them are potentially useful for identifying how subtle factors in the peptide structure affect the spectra, which might be accomplished with calculations of the spectra based on the known structures.

The color coding in Fig. 3 highlights that all the spectral components identified in the fits undergo polarity changes in VCD spectra, as would be expected for features associated with mirror-image structural elements. In the amide I region, the strongest band in the IR spectrum is near 1630 cm^{-1}. On the high frequency side of the strongest band, three other bands appear with intensities decreasing with increasing frequency. The two weak bands near 1690 and 1675 cm^{-1} may correspond to the β turns, the amide I parallel component for an antiparallel β sheet, or both. In the amide II region, there are at least two discernible bands between 1520 and 1550 cm^{-1}.

Finally, the quantitative comparison of the IR and VCD spectra in Fig. 3 and Table I provides some perspective on the feasibility of using VCD spectroscopy for analyzing biointerphases. The intensities of VCD peaks in these solution measurements are a factor 10^4 lower than the intensities of the corresponding IR peaks. While ECD on surfaces is well established,[19,20,22,25–27,49] direct VCD measurements of peptides adsorbed on surfaces thus will be challenging and will require us to explore sensitivity enhancement strategies. Examples include increasing the absorption on surfaces, for instance, by using stacked or multilayer samples or adsorption on microparticles or nanoparticles, and we note that Bieri *et*

al.[50] and Yao *et al.*[51] recently exploited the high surface area of metal nanoparticle suspensions to measure VCD spectra of adsorbed small molecules. Specific immobilization of the structured peptides that promotes their common orientation will be another important approach for enhancing the VCD signal from a given surface density of peptides. Other potential enhancement strategies include using surface-enhanced infrared absorbing substrates[52,53] or surface-specific methods such as sum frequency generation (SFG).[4–6,18] The close correspondence between the VCD and IR peaks in Fig. 3 also suggests that the solution VCD spectra will be useful as references for elucidating the features observed in other structurally sensitive vibrational spectroscopies such as SFG.[4–6,18]

V. CONCLUSIONS

Beginning with β-helical peptides, the structures of which had been previously well characterized, we were able to unambiguously correlate the VCD spectra of these peptides with their structures. In particular, by using model peptides that have homogeneous rather than promiscuous folded conformations under appropriate solvent conditions, we obtained complementary IR absorbance and VCD spectra that we could fit using a set of only six vibrational bands for two moderate-sized (22 residues) cyclic peptides. Quantitative analysis of the six spectral components identified for β Leu and β Val—which have opposite chirality and different primary structures of alternating L- and D-amino acids—demonstrates that the VCD spectra of these two peptides are nearly mirror symmetric. The bands prominent in the IR and VCD are different for these peptides with well-defined and determined structures, a fact which is a unique opportunity to better understand how subtle differences in VCD and IR relate to and correlate with peptide conformation. This near reversal of polarity in VCD spectra shows that the amide I and II regions for β Leu and β Val are dominated by spectral signatures associated with the chirality and secondary structure of these peptides and that these side chains have little or no effect on the VCD spectra. We are currently working to establish the generality of this observation by preparing water-soluble variants of β Leu and β Val that will require a greater diversity of side chains than those comprising the original two peptides.

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furthermore, that for one protein examined, α-chymotrypsin, which comprises mostly β sheet and β turn secondary structures, the VCD spectrum has six identifiable VCD peaks that are similar in frequency and relative intensity to the six VCD peaks of β Val and β Leu that we modeled in the present study.
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