Ab Initio Simulations of the Vibrational Circular Dichroism of Coupled Peptides

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Abstract: The vibrational circular dichroism (VCD) of peptides in different conformations was simulated using the magnetic field perturbation (MFP) model for the model dipeptide $CH_3-CONH-CH_2-CONH-CH_3$. The geometry was optimized in the 4-31G basis set with the torsion angles constrained to mimic the α-helical, β-sheet, 3_{10}-helical, and polyproline II conformations. An effective mass was used for the appropriate H on each C_α position to simulate the local chirality of L-alanine residues. The normal modes and VCD spectra were calculated at the *ab initio* quantum mechanical level with the same basis set using analytical derivatives (CADPAC) and the MFP formulations of Stephens and co-workers. These calculations gave simulated VCD spectra that were quite comparable to experimental results with respect to the VCD sign pattern and relative intensities of both the amide I and II modes for polypeptides having these conformations. The MFP results are further shown to be superior to a similar calculation based only on dipole coupling, by comparison to experimental polypeptide spectra.

Introduction

A significant application of vibrational circular dichroism (VCD) for structural studies has developed in the area of peptide and protein conformational analysis.[1-9] The primary approach to analysis of these spectra, to date, has centered on empirical correlations of VCD spectra for systems of similar secondary structure. Each major secondary structural type yields a distinctive VCD band shape for the amide I and II bands ($C=O$ stretch and $C-N-H$ deformation plus $C-N$ stretch, respectively).[3-7] These systematic spectral patterns lead to facile qualitative classification of peptide conformations for molecules which have a uniform structure. For globular proteins with mixed secondary structures, a quantitative decomposition of the VCD band shape and a regression analysis based on the resulting components has been developed which yields reasonable predictions of the fractional contribution of the major secondary structure types to the total protein conformation.[2]

Ideally one would like to be able to predict the VCD band shape from a theoretical model based on the peptide geometry.

If such a calculational scheme were shown to be reliable, simulation of spectra based on various probable structures, determined by modeling, for example, could be used for structure determination. Schellman and co-workers[10] and, more recently, Diem and co-workers[9,11] have attempted to simulate oligopeptide VCD using the simple coupled oscillator or dipole coupling (DC) model.[12] While the DC model appeared to predict the α-helical amide I VCD correctly, it failed for the β-sheet amide I VCD band shape,[3] for the α-helical amide II modes,[7] and (as will be shown) for the polyproline II (Pro II) helical amide I VCD.[4]

On the other hand, the magnetic field perturbation (MFP) model as formulated by Stephens and co-workers[13] and implemented at the *ab initio* level has proven to be quite successful in reproducing the VCD of many small molecules.[14-16] Similar success has been evident in calculations using other *ab initio* quantum mechanical approaches to VCD simulation.[17] Part of the key to the success of all of these calculations is the use of *ab*

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initio quantum mechanical force fields to determine the proper relative ordering of transitions. Use of the MFP method has been previously restricted to small molecules due to the size of the basis set needed to obtain the most accurate level of calculation. However, tests in our laboratory with smaller basis sets indicate that the MFP-predicted spectra, particularly for the more intense features, are qualitatively the same as those obtained with the larger basis sets.[15] This observation makes it reasonable to consider *ab initio* MFP VCD simulation for larger molecules with correspondingly modified goals in terms of precision.

We have recently used the MFP model to delineate the applicability of the coupled oscillator model to strongly coupled, symmetrically equivalent local C–H and C–D stretching modes for a number of small molecules.[16] These calculations demonstrated that although the DC model works well for weakly coupled oscillators, it fails for oscillators nearby groups that are highly polarizable or have delocalized bonding. By contrast, in all cases studied for which experimental data were available, the MFP results were in excellent agreement with the data.

Since the peptide group is an extended π-system and since the peptide groups interact substantially in an oligo- or polypeptide, as evidenced by the well-established conformational sensitivity of their vibrational force fields and frequencies,[18] use of calculations at the *ab initio* MFP level may be needed for interpretation of peptide VCD. Furthermore, it is expected that such MFP-computed VCD will be a substantial improvement over DC model predictions for peptides.[9,10] Due to our computational constraints, an *ab initio* MFP calculation of the VCD of an extended peptide chain was not realistic, but modeling of an idealized molecule containing two peptide bonds (here termed the "dipeptide") was possible. Despite the molecular size constraint, the higher energy, amide-centered modes can, in fact, be calculated with some precision and are the sole focus of this report. Since the amide modes are only weakly perturbed by the side chains, these model calculations can be reasonably used to simulate spectra of any sequence and should yield the fundamental amide–amide interactions and their contribution to the VCD band-shape pattern for polypeptides. Previous studies have addressed *ab initio* conformational energy minimization and force field determinations for dipeptides,[19] but this is the first attempt to simulate their VCD with a high-level theory such as the *ab initio* MFP.

Even though restricted to the dipeptide level, this representation of near-neighbor amide interaction could eventually be grafted onto a larger VCD calculation that uses a simpler model, such as the DC, to represent longer range amide interactions for which the DC model is applicable.[16] First, however, it is necessary to establish that the MFP model can be successfully used for computation of the characteristic VCD band shapes corresponding to the major polypeptide secondary structure types. That can now be done, and the first such report is the topic of this paper.

Methods

Our computations were done in two stages. First the molecular geometry of the "glycine-like" model dipeptide, CH_3–$CONH$–CH_2–$CONH$–CH_3, was optimized by energy minimization in the 4-31G basis set using the Gaussian 88 programs[20] on an IBM 3090 computer. All calculations on the dipeptide were limited to the standard 4-31G basis set by our computer constraints. [Some test calculations on smaller molecules (see below) used alternative, larger basis sets.] The minimi-

Table I. Geometry Constraints and SCF Energies of the Dipeptide in the Constrained Conformations

conformation	torsions, deg			E(SCF), a.u.
	ω	ϕ	ψ	
α-helix	180	−57	−47	−453.1421
β-sheet	180	−119	113	−453.1475
3_{10}-helix	180	−60	−30	−453.1441
Pro II	180	−78	149	−453.1470

zations were constrained to give conformations with ϕ,ψ,ω torsional angles (listed in Table I) appropriate to segments of the α-helical, β-sheet, 3_{10}-helical, and Pro II secondary structures. Then a Cartesian-based force field was calculated and the atomic polar and axial tensors[13] for computing the MFP VCD were evaluated, with the same basis set, using CADPAC version 4.0[21] on an Ardent Titan computer with a P3 processor, 32 MB of memory, and about 1.2 GB of available disk space. Although CADPAC generates dipole (*D*) and rotational (*R*) strengths directly, the normal modes were recalculated in internal coordinates with the same force field but different masses to evaluate the effects of isotopic modification. Then those normal modes and the *ab initio* atomic polar and axial tensors computed with CADPAC were transferred to a separate program for recalculation of the isotopic *R* and *D* values in the distributed origin gauge with origins at the nuclei.[13]

Our approach is based on the assumptions that the vibrational spectra are determined primarily by short-range interactions and that the *ab initio* force field calculation in an intermediate-sized basis set can be used to obtain a reasonable prediction of the order of the vibrational modes. [It can be noted that previous reports of dipeptide *ab initio* force fields have utilized even smaller basis sets, and the results there have been successfully compared to experimental spectra.[19]] Here we discuss only the high-frequency amide A, I, and II modes of interest in current VCD analyses. These should be unaffected by the constraint to a fixed conformation because they are very weakly coupled to and well separated from the low-energy torsional modes. Similarly, since we have shown that VCD of the characteristic amide modes is independent of the nature of the side chains,[1-7] using effective masses to simulate the side chains is a reasonable approximation that gives these calculations a general applicability.

To test the reliability of using a 4-31G basis set for determination of the vibrational and dipolar characteristics of the amide group, a series of calculations with different basis sets were carried out following the above methods on a single peptide, *N*-methylacetamide, in the *trans* conformation (termed the "amide" here). The 4-31G, 6-31G**, and DZP basis sets, as standardly supplied with the CADPAC program,[21] were used to optimize the geometry and calculate the force fields, normal modes, and transition dipole strengths. As will be reported in detail separately,[22] the dipole strength changes <25% and the direction of the dipole moment varies <14° for these modes is calculated with the three different basis sets. Frequency variation with basis set change was substantial but not large in a relative sense (amide I ~5%). The absolute values obtained were ~15% higher than those found experimentally, as is to be expected using SCF-level force fields. Since detailed force field computations are already available for this molecule,[23] extensive discussion is unwarranted here.

With specific application to this study, a test of rotational strength sensitivity to basis set variation was carried out by selective isotopic substitution of the amide. In these tests, although the rotational strengths did vary with basis set, all the computed "intrinsic amide VCD" magnitudes were small. The predicted $\Delta A/A$ values were all $\leq 10^{-5}$ (or just detectable) for the amide A, I, and II modes.[22] Our primary goal was to compute the conformationally dependent VCD band-shape changes for the amide I and II modes, which generally have relatively intense VCD. On the basis of the results for this single-amide test case,[22] in those cases where the predicted rotational strengths in our calculations for the dipeptide are

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Table II. *Ab Initio* MFP Results for the Highest Amide Modes of the Dipeptide[a]

mode	glycine-like				alanine-like				N-deuterated			
	ν	$\Delta\nu$	D	R	ν	$\Delta\nu$	D	R	ν	$\Delta\nu$	D	R
					α-Helix							
Ao	3411	−17	38	2.1	3411	−17	38	2.4	2488	12	43	−1.4
Ai	3393		45	−5.8	3393		46	−5.8	2500		46	−1.2
Ii	1655	12	763	−220	1652	12	788	−212	1641	10	905	−328
Io	1643		220	199	1640		190	191	1632		246	309
IIo	1498	−12	610	16	1489	−14	722	101	1389	−30	798	96
IIi	1485		536	−51	1475		644	−198	1359		892	−214
					β-Sheet							
Ao	3391	−2	72	6	3391	−2	72	6	2487	−2	74	1.7
Ai	3389		36	0	3389		36	0	2485		37	0.4
Ii	1631	13	105	−52	1628	14	68	−42	1612	10	106	−79
Io	1618		940	−12	1614		958	−13	1602		1249	25
IIo	1522	−26	621	63	1508	−20	833	141	1416	−22	522	40
IIi	1495		626	−5	1487		803	−142	1393		1137	−40
					3_{10}-Helix							
Ao	3424	−25	42	6	3424	−25	42	6	2510	−20	47	0.8
Ai	3399		48	−6	3398		49	−6	2493		49	−1.6
Ii	1647	15	518	−26	1645	14	541	−45	1633	11	601	−62
Io	1632		518	6	1631		485	13	1622		609	22
IIo	1502	−17	652	30	1494	−19	775	48	1397	−37	825	49
IIi	1484		549	−112	1475		644	−186	1360		893	−194
					Pro II							
Ao	3409	−6	58	−2	3409	−6	58	−2				
Ai	3403		47	6	3403		48	6				
Ii	1641	10	268	240	1640	12	286	208	1631	11	610	76
Io	1631		691	−287	1627		664	−272	1620		727	−124
IIi	1503	13	593	−19	1495	13	627	−19	1433	13	128	−5
IIo	1490		684	18	1483		81	−25	1420		215	24

[a] Modes are indicated by their major contribution as o, out of phase, and i, in phase, combinations of the amide A, I, and II local oscillators. Column definitions: ν (cm⁻¹), vibrational frequency; $\Delta\nu$ (cm⁻¹), splitting between i and o modes; D (10⁻⁴ D), dipole strength; R (10⁻⁸ D), rotatory strength.

large, reflecting experiment, it is unlikely that they will have been adversely affected by the limited, 4-31G basis set we have used.

Dipeptide Calculations and Results

In separate geometry optimizations run for each of the dipeptide conformers, all coordinates were varied except the six ϕ,ψ,ω torsion angles of the main peptide chain, which were fixed to those values shown in Table I.[24] The optimized SCF energies obtained for these four constrained conformations are also included in Table I. For the α-helical and 3_{10}-helical conformations, $3N−6$ positive vibrational frequencies were determined. However, the β-sheet and polyproline II conformations had one and two negative frequencies, respectively.

The force field, atomic polar, and axial tensors and the vibrational spectra were computed with the 4-31G basis set using the CADPAC program for the "glycine-like" dipeptide (formula as given in previous section). For presentation here, the frequencies were all scaled by a factor of 0.874 to align them better with the experimentally observed frequencies. Such scaling is consistent with the usual experience with SCF force fields and is the simplest correction that can improve frequency prediction without altering the *ab initio* computed frequency ordering that we sought. These results are given for the amide A, I, and II modes (each having two components, in and out of phase) in columns 2–5 of Table II. The column $\Delta\nu$ indicates the splitting of that amide mode as predicted by the *ab initio* force field. $\Delta\nu$ is a clear indication of the relative ordering of the vibrational states and can be compared to results obtained from dipolar splitting alone that are commonly used as a basis for DC computations.[9]

Overlap with several C–H deformation modes is expected to complicate the amide IR and VCD in such a glycine-like

molecule.[18,25] Therefore, simulation of a chain of chiral L-alanine residues was accomplished by calculating the VCD of CH³⁰H¹⁵H–CONH–CH¹⁵H–CONH–CH³⁰H¹⁵H. This structure was derived by assigning a mass of 15 to the three hydrogens that would correspond to the side-chain positions in an oligomer of L-amino acid residues and a mass of 30 to the two hydrogens that would correspond to the positions where the main peptide chain would continue in the polymer, as illustrated for the α-helical and β-sheet conformations in Figure 1. [The larger terminal mass was chosen to reduce mixing with the amide modes and to represent the effect of the massive polymeric chain.] Normal mode frequencies as well as dipole and rotational strengths for this alanine-like dipeptide were computed for the amide A, I, and II modes as tabulated in columns 6–9 of Table II. It is important to note that the magnitudes of the rotational strengths for most of the modes in Table II are much larger (by a factor of 4–20 for the amide I) than those found in our test calculations noted above for a single amide.[22] This difference confirms that amide coupling generally leads to much larger VCD intensity than do intrinsic amide chirality effects. Given this, the magnitudes as well as the signs of R that we have computed are important because the predicted dipeptide VCD is more reliable for those bands with large rotational strengths. The alanine-like dipeptide VCD spectra predicted for the four peptide conformers simulated are illustrated in Figure 2. The amide I VCD results in these computations closely reflect those found in the glycine-like computations (compare columns 5 and 9 in Table II). As shown in Figure 2, the α-helical and Pro II helical conformations have intense but oppositely signed VCD couplets[26] predicted for the dipeptide amide I. On the other hand, the β-sheet and 3_{10}-helix are predicted to have weak net negative amide I VCD.

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(26) In the CD field it is common to refer to spectra with a derivative shape indicating at least two underlying transitions as a "couplet". By convention, a positive couplet is defined as having its positive component to lower frequency.
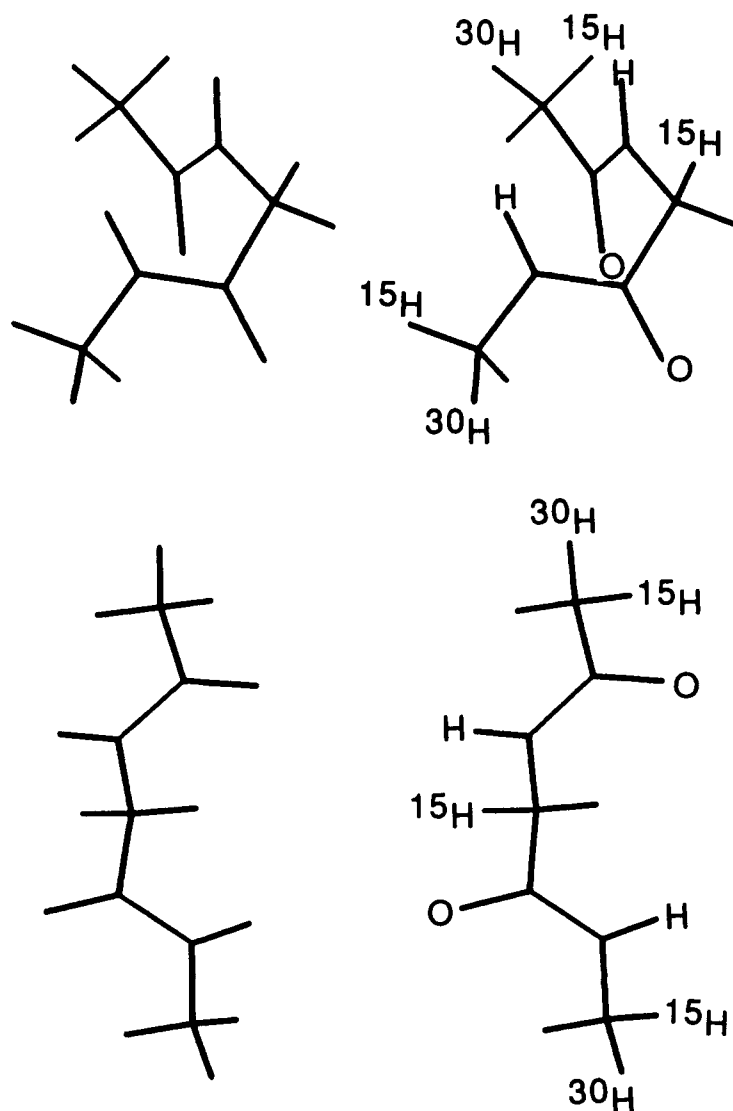
Figure 1. Stereoplots of the alanine-like dipeptide in (top) the α-helical conformation and (bottom) the β-sheet conformation where [15]H indicates the mass 15 substitutions for the alanine side chain and [30]H indicates the mass 30 substitutions for the continuation of the peptide chain.

The nature of the amide II VCD becomes clear with mass alteration to the alanine-like form, since interference and mixing with other internal coordinates is then reduced and made more realistic. The α-helical amide II VCD is predicted to be strongly negative while the β-sheet amide II VCD is predicted to be a strong negative couplet. The 3_{10}-helix is also predicted to have a strong negative amide II, like the α-helix, but in the 3_{10} case the amide II intensity is much larger than the amide I VCD. By contrast, the Pro II helix is predicted to have a weak amide II VCD, much smaller than its amide I VCD.

Overall, the *ab initio* MFP calculations at the 4-31G level predict the amide I, II region to have a clear pattern of conformationally sensitive sign and intensity patterns, just based on interactions within a dipeptide molecule. The predicted VCD for the amide III region is complex due to mixing of the amide modes with C_α–H deformations.[25] Nonetheless, it can be noted that the amide III VCD signals for the α-helical and 3_{10}-helical conformations are predicted to be dominantly positive, while those predicted for the β-sheet and Pro II structures are less clear. In a real peptide molecule, –C–H deformations arising from the side chains would probably obscure these predicted patterns.

N-deuteration effects were also calculated for the alanine-like dipeptide molecules as listed in Table II, columns 10–13. To simulate the Pro II conformation, a mass of 15 was used for the H(N) to simulate the tertiary amide vibrational characteristics of the proline residue.

Part of the variation observed in the simulated VCD spectra arises from the dependence of the force field on secondary structure.[18] Relative changes in frequency patterns can certainly arise for the isotopic substitutions used to simulate the spectra in Figure 2. Inspection of Table II for the different substitutions shows that, after the "alanine" substitution, the amide A parameters are virtually unchanged from the "glycine" result, which follows from our earlier assertion regarding the small influence of modes far in frequency from the amide modes of interest. On the other hand, the alanine-like amide I frequencies are lower than the glycine-like ones by 1–5 cm^{-1}, and the amide II frequencies are lower by 8–21 cm^{-1}, since those modes are closer in frequency to modes which involve motion of atoms altered by the mass substitution. Amide II intensities (D and R values) are the most sensitive to this substitution, even to the extent of an amide II sign pattern change in the Pro II simulation. Other than for the Pro II structure, which was simulated as two tertiary amides, N-deuteration leaves the amide I and II mode VCD qualitatively the same for the alanine-like computation.

Discussion

Dipeptide Simulation. The results of our MFP calculations have a satisfying stability with regard to the isotopic substitutions made and evidence substantial magnitudes (Table II) in terms of rotational strength as compared to those of the single-amide test calculations.[22] On the basis of our experience with com-
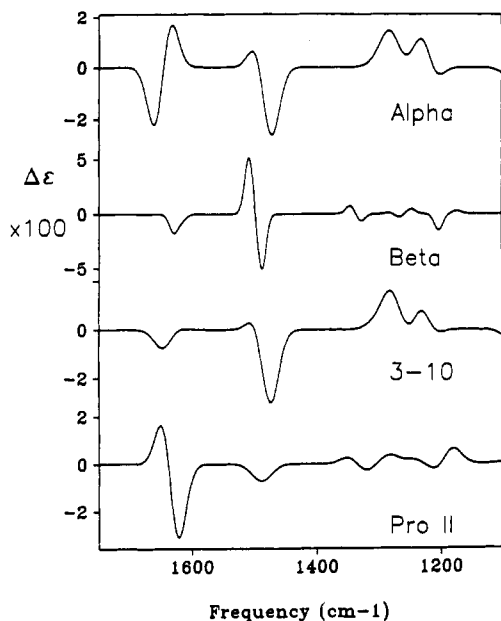
Figure 2. Simulated VCD spectra in the amide I, II, and III regions from MFP calculations with wave functions derived for the "L-alanine-like" dipeptide using pseudomasses on C_α as optimized with a 4-31G basis set and torsional angles constrained to those in Table I for the α-helical, β-sheet, 3_{10}-helical, and polyproline II (top to bottom) conformations.
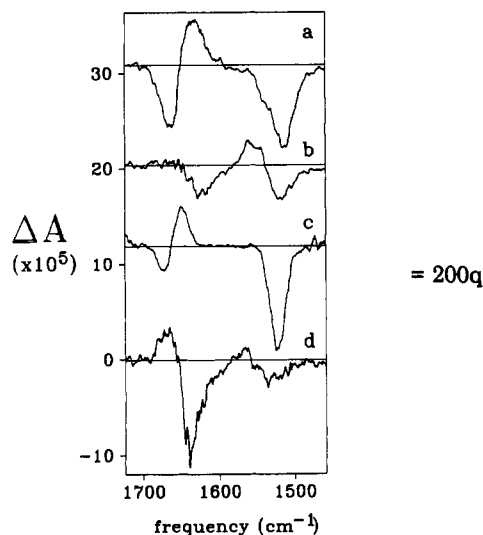

Figure 3. Experimental VCD spectra for (a) albumin in H_2O, a highly α-helical protein; (b) concanavalin A in H_2O, a highly β-sheet protein; (c) $(Aib)_2Leu(Aib)_5$ in $CDCl_3$, a 3_{10}-helical oligopeptide; and (d) poly-L-lysine in H_2O, a "random-coil" polypeptide that is locally like left-handed helical poly-L-proline II.

putations of VCD for small molecules, one can have some confidence in the reliability of prediction for bands computed to have large-magnitude D or R (absorbance or VCD) values. This is particularly true with regard to basis set effects. However, if these calculations were to exist in isolation, they would do little good for furthering the understanding of peptide VCD. Fortunately, that is not the case, as there now exists a large body of experimental data for the VCD of oligo- and polypeptides[1] and these calculations exhibit remarkable agreement with those results. To aid in such a comparison, example experimental polypeptide (or protein) VCD data are illustrated in Figure 3 for comparison to the predictions in Figure 2. The VCD data in Figure 3 are not unique but are typical of peptides and proteins having the dominant secondary structural types indicated.[1-7]

First let us consider the α-helix. All the calculations we have done give predicted VCD spectra in good qualitative agreement

with experiment: a strong positive VCD couplet[26] for the amide I and a negative VCD for the amide II shifted to the lower energy side of the absorbance maximum.[6,7] Also in accord with experiment, the negative amide II VCD is here predicted to be maintained upon deuteration (Table II). The predicted amide I VCD couplet is slightly negatively biased, as is usually seen in experiments.[6,7,27] but the calculations are not so precise that one should put much stock in that observation. The amide A mode is calculated to have a weak, negative couplet VCD, which is in agreement with results on α-helices in nonaqueous solution, but it is calculated to be biased strongly negative as opposed to the observed positively biased VCD.[6] At this stage, one must regard the amide A theoretical results as being unreliable. Since hydrogen bonding is not included in the model calculation, such an error is understandable but perhaps not truly explained. Finally, the theoretical spectrum in Figure 2 indicates a significant positive VCD in the amide III region, which does agree with experimental results for the α-helical poly-γ-benzyl-L-glutamate.[27]

The β-sheet computations are also in good agreement with experimental results. The weak negative amide I VCD reflects what has been seen experimentally in a number of antiparallel β-sheet examples.[3,28] In addition, the MFP calculations predict a strong amide II negative couplet[26] VCD, again in good agreement with experimental data on models and on proteins.[7] Good models for parallel β-sheets are difficult to find, but protein results[2] indicate that they should at least have negative VCD at the lower frequency end of the amide I range (~ 1630 cm^{-1}) and negative couplet VCD in the amide II region (Figure 3), which is consistent with these MFP calculations.

One of the most satisfying results was the predicted VCD for the 3_{10}-helix. Our model peptide work[5] has established that 3_{10}-helical oligomers yield much weaker VCD of the same sign pattern for the amide I mode and about the same band shape and intensity VCD for the amide A and II modes, as compared to the case of the α-helix. Our computations reflect this observation very well. In Figure 2, the weak positive couplet nature of the predicted 3_{10}-helix VCD is not clear, but the Table II values (columns 5, 9, and 13) confirm it. Since the predicted magnitudes are so small for this mode, it is possible that effects other than these near-neighbor interactions will prove to be significant for modeling the VCD of a longer 3_{10} chain. We have found that other, proposed 3_{10}-helical geometries[24] give different but still weak amide I VCD while still yielding the same strong amide II VCD. This indicates that these computational results can be taken to be a prediction of weak amide I intensity but should not be viewed as a reliable determination of the specific 3_{10}-helical amide I VCD band shape.

The Pro II calculations were done with two goals in mind. Experimentally, polyproline II, a left-handed helix of *trans* peptide bonds, has a strong negative VCD couplet[26] for the amide I mode, which is well matched by the calculated result in Figure 2.[4,29] In addition, the Pro II conformation is believed to be a good model for a significant component of the conformation traditionally identified as "random coil", which, on the local scale, must consist of a significantly ordered structure for many polypeptide systems.[4,30] In this respect, the "random coil" amide I VCD is again well predicted, as it must be from our previous experimental correlation. The calculated amide II VCD is inconclusive, but its small magnitude is in agreement with the small-magnitude VCD measured for model coil systems (Figure 3).[7] Our efforts

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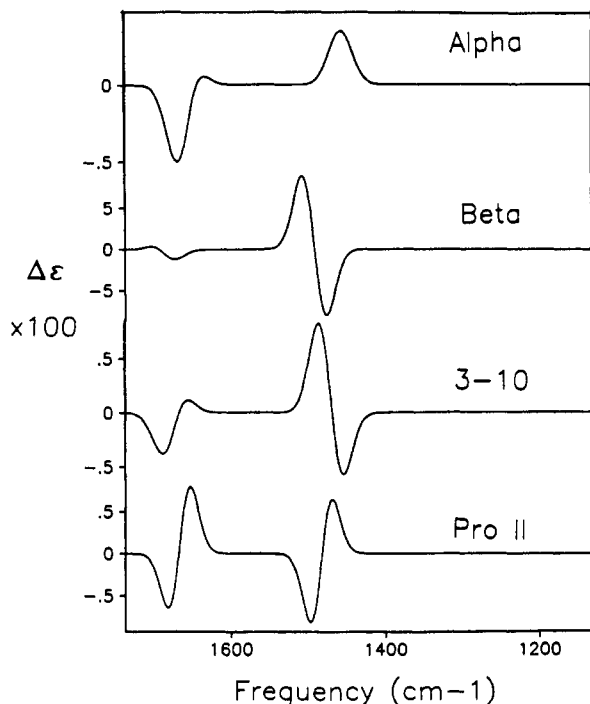
Figure 4. Comparison of the amide I and II VCD predicted with the dipole coupling model for the dipeptide in the α-helical, β-sheet, 3$_{10}$-helical, and Pro II conformations (top to bottom). Note scale changes for the β-sheet.

to model the Pro I (right-handed helix of *cis* peptides) VCD was not as successful, in that while the intensity of the amide I mode was predicted to be low, which is consistent with experiment,[29] the sign pattern was predicted incorrectly.[22]

Comparison to Dipole Coupling Calculations. To compare the MFP computational method with the previously used simpler and much cheaper (computationally) coupled oscillator approaches to the modeling of VCD spectra, the result of a parallel set of dipole coupling (DC) calculations[9–12] is presented in Figure 4. Coupling of only the two amide I and two amide II dipoles was considered. Average frequencies and dipole strengths taken from our single-amide test calculations were used as a basis for the DC calculations. The results of these and other simple model calculations will be discussed separately in detail.[22]

In summary, as evident from comparison of the spectral simulations based on the DC predictions in Figure 4 with the

experimental results in Figure 3, the DC computations fail to reproduce adequately the amide I mode VCD for the β-sheet and Pro II conformations and the amide II VCD for the α-helix, 3$_{10}$-helix, and Pro II helix. They do give good amide II VCD predictions for the β-sheet. However, the amide I VCD predictions for the Pro II helices have the incorrect sign. On this basis alone it is clear that the MFP predictions are superior representations of the oligo- and polypeptide VCD for standard peptide conformations and that the DC predictions are substantially lacking by comparison based on dipeptide computations.

Conclusion

The *ab initio* MFP calculations described here, despite their confinement to just a dipeptide model compound, did better at predicting experimental VCD than have any other models used to date. Given the success of the dipeptide MFP calculations in replicating the observed oligopeptide VCD band shapes for several conformations, one might ask, what is the next step? Our MFP-based calculations only include 1–2 amide–amide interactions, which can be thought of as the intrinsic nearest-neighbor helix term. Their success provides theoretical support to our empirically based proposal,[1] supported by studies of the length dependence of peptide VCD,[4,5] that VCD has a substantial contribution from short-range effects. The uniformly successful comparison of these predicted VCD spectra, necessarily computed considering only near-neighbor interactions, with experimental data from much longer oligomers and polymers strongly implies that the VCD is dominated by near-neighbor interactions. Extension of these computationally intensive MFP calculations for practical use on polymeric systems may be realized by combining these MFP, pairwise interactions with DC-type computation of longer range interactions. Longer range interactions would presumably lead to weak contributions to the VCD and force field for most structures, but their number may make the total effect significant for extended-chain molecules. This effect will have more importance for those modes which have an MFP-determined, weak near-neighbor contribution to the VCD. We are now pursuing such a hybrid approach to calculation of the VCD for more complex structures utilizing this MFP contribution.