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Conformational Study of Sequential Lys and Leu Based Polymers and Oligomers Using Vibrational and Electronic CD Spectra

VLADIMIR BAUMRUK,[1,*] DONGFANG HUO,[1] RINA K. DUKOR,[1,†] TIMOTHY A. KEIDERLING,[1,‡] DOMINIQUE LELIEVRE,[2] and ANDRÉ BRACK[2]

[1]Department of Chemistry, University of Illinois at Chicago, 845 W. Taylor St., Chicago, Illinois 60607, USA; and [2]Centre de Biophysique Moléculaire, CNRS, 45071 Orléans, Cedex 2, France

SYNOPSIS

Vibrational CD (VCD) and electronic CD (ECD) spectra of some sequential Lys and Leu based oligo- and polypeptides were studied as a function of added salt and (for ECD) as a function of concentration in aqueous solution. For these samples, the VCD spectra can only be measured at relatively high concentrations under which the well-known salt-induced transition to a β-sheet form can be observed for the KL based species, but only the end-state α-helical conformation is obvious for the LKKL based samples. ECD concentration dependence demonstrates that, at high concentration with no added or with added salt, LKKL based oligomers and polymers give α-helical spectra. These data provide evidence of aggregation induced secondary structure formation in an exceptionally simple peptide system. Similarly, the KL based oligomers and polymers give β-sheet like spectra at high concentration or at high salt. These systems further provide model systems under "normal" aqueous conditions that yield VCD band shapes that correlate to the major secondary structural types of polypeptides. They are in substantial agreement with those spectra obtained on homopolypeptides and on proteins, confirming the relative independence of the VCD technique from side-chain and solvent effects. © 1994 John Wiley & Sons, Inc.

INTRODUCTION

It is well established that polypeptides with alternating Lys and Leu residues, $(KL)_n$ type polymers, can be changed from a coil form to an apparently soluble β-sheet type structure by change of ionic strength from low to high added salt.[1,2] Similarly, it has been shown that from a similar peptide having an alternate relative ordering of these residues, $(LKKL)_n$, an α-helical form can be stabilized with respect to the coil form under these same high salt conditions.[3] Such systems that can have different conformational structures under different ionic strength conditions offer good model systems for determination of characteristic spectral band shapes and frequency patterns of those structures in a fairly "normal" aqueous environment such as might be appropriate for the study of protein samples. Obtaining "pure conformer" spectra in this manner can facilitate development of theoretical models for explaining these spectral features and facilitating their utilization in peptide structural studies.

Several recent studies have also pointed out that increased stability of helices, in particular, can be derived from interhelix interactions.[4] Such designs have often emphasized the amphipathic helix, which leads to helical bundle formation.[5] In such cases, it might be expected that increasing peptide concentration would be a structure-forming perturbation.

Spectroscopic techniques have long been used for study of peptides and proteins to determine aspects of the polymeric conformation, with an emphasis on secondary structure. Besides the conventional tech-

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* Permanent address: Institute of Physics, Charles University, Ke Karlovu 5, 121 16 Prague 2, Czech Republic.

† Present address: Amoco Technology Co., Bioinformatics, P. O. Box 3011, Naperville, IL 60566.

‡ To whom correspondence should be addressed.

niques such as UV electronic CD (ECD) and ir spectroscopy, vibrational CD (VCD) has developed as a technique that combines the sensitivity to molecular detail that is characteristic of vibrational spectroscopy in the ir with the sensitivity to stereochemistry that is typical of a measurement of optical activity such as ECD.[6-10] VCD is the measurement of the differential absorbance of left- and right-circularly polarized ir radiation by fundamental vibrational modes of a molecule that is typically studied in solution.

Polypeptides and oligopeptides provide an excellent field of application for VCD studies as well as provide models for the various secondary structural types that can be found in proteins.[6,10] We and others have demonstrated that VCD has the ability to differentiate between the common secondary structures that polypeptides assume in solution[6,10-13] and even to distinguish among structures that are not easily separated by ir or ECD.[14] In particular, VCD has a much shorter length dependence in terms of developing a characteristic band shape and normalized intensity for a peptide oligomer or segment than does conventional ECD.[15-17] We have also shown that VCD has an enhanced sensitivity to conformation in proteins via an empirical comparison to ECD and ir results[18] and statistical analysis of the resulting band shapes in terms of secondary structures.[19,20] This probably results from that short-range length dependence and certainly is evidenced in the distinctive, substantial nature of the β-sheet contribution to the protein VCD band shape. By contrast, the ECD band shape of proteins is dominated by the α-helical contribution.[20] Thus the Lys, Leu based sequential peptide systems provide a nice test case for "calibrating" this newer peptide spectroscopy on relatively well-defined secondary structures for virtually the same peptide in a solvent environment much like that used for protein studies.

EXPERIMENTAL

Sequential poly(LKKL)^3 and poly(KL)^{1,2} have been synthesized by polycondensation of the corresponding protected peptide p-nitrophenyl esters in the presence of a stoichiometric amount of 1-hydroxybenzotriazole. Lysyl amino side groups were protected with benzyloxycarbonyl groups compatible with the o-nitrophenylsulphenyl groups used for α-amino group protection. After removal of the lysine side-chain protecting groups, the free polypeptides were dialyzed exhaustively against dilute aqueous HCl and recovered as hydrochloride salts by lyophilization. Ac-(Leu-Lys-Lys-Leu)$_5$-NHEt and Ac-(Lys-Leu)$_{10}$-NHEt have been prepared by solid-phase synthesis using a Boc/Pam resin strategy. Acetylation was carried out on the resin with acetic anhydride/triethylamine in dimethylformamide. Cleavage of the acetylated protected peptide from resin was achieved with ethylamine in methanol to afford the C-terminal ethylamide. After removal by HBr/trifluoroacetic acid (TFA) of the 2-chlorobenzyloxycarbonyl groups protecting the lysine side chains, the free peptides were purified by high performance liquid chromatography on a C18 Lichrospher (Merck) column using an acetonitrile–water–0.2% TFA gradient system. The pure peptides were recovered as trifluoroacetate salts by lyophilization.

Samples for VCD and ir spectroscopic study were deuterated twice following our standard procedure[18] before final D_2O solutions at a concentration of 20–45 mg/mL were prepared. To monitor salt effects, NaCl was added to a final concentration of 0.1 M for the oligomers and 0.1 M to 0.3 M for the polymers.

VCD and ir absorption spectra were measured on the dispersive instrument at the University of Illinois at Chicago, the design and use of which has been previously described in detail in the literature.[7,8] Aqueous samples were placed between two CaF_2 windows separated with a 0.05 mm teflon spacer. Unfortunately, the KL based samples under high salt conditions tended to form a gel at the concentrations needed for VCD studies. Spectra were recorded at $\sim$ 10 cm^{-1} resolution and averaged over 4–6 scans, each of which was collected with 10 s time constant. Baseline correction was accomplished by subtraction of the VCD spectrum of a sample of poly(D,L-lysine) or N-methyl acetamide (required for those samples evidencing high β-sheet components) or of poly(D,L-Lys, D,L-Leu) in D_2O whose peak absorbance for the amide I′ band was matched to that of the sample. For these experiments, baseline scans were relatively featureless.

For further characterization, Fourier transform ir (FTIR) spectra were collected on the same samples at 4 cm^{-1} resolution over the whole middle ir region on a Digilab FTS-60 spectrometer using a TGS detector. These spectra were corrected for background absorbance and resolution enhanced by Fourier self-deconvolution using standard Digilab software. Similarly, ECD spectra were obtained with a Jasco J-600 instrument in the range of 190–260 nm, to provide a basis for comparison with previously reported results on these peptides. Samples for ECD study were dissolved in double-distilled water or in 0.1 M NaCl at various concentrations over the 0.7–15 mg/mL range and were placed be-

tween two quartz windows separated by either 15, 25, or 50 μm teflon spacers. For the measurements at the lowest concentrations, a standard cylindrical quartz cell with a 0.1 mm path length was used. ECD spectra were averaged over 3 consecutive scans, collected with a time constant of 2 s and were corrected with a baseline obtained by scanning the ECD of just the solvent in the same cell.

RESULTS

Measurement of the ECD spectra of dilute LKKL and KL based samples gave data consistent with that obtained in the previous studies.[1-3] However, the VCD spectra of the higher concentration samples indicated that even with no added salt, substantial structural transformation had taken place. In Figure 1a are shown the VCD and ir absorption spectra in the amide I' region for the D_2O solution of the LKKL polymer without added salt. The VCD spectrum of the polymer with 0.15M added salt (Figure 1b) is very similar but has slightly higher overall intensity and a weaker negative lower frequency lobe at $\sim$ 1630 cm^{-1}. (All ir spectra presented here are normalized to A_{max} = 1.0 for the amide I' band to aid comparison of spectra. Thus the VCD scales in the figures read $\Delta A/A$ directly for the peak position.) The peak absorbance at 1642 cm^{-1} coincides roughly with the lower energy zero crossing of the VCD in both cases. The band shape and intensity observed are typical of an N-deuterated, highly α-helical polypeptide or protein.[21]

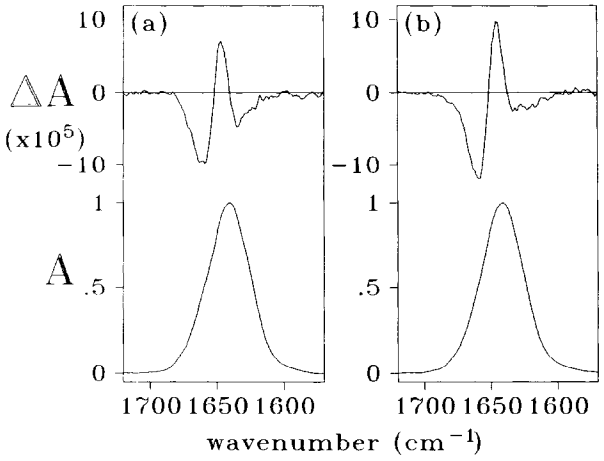

Figure 1. Dispersive ir absorption and VCD spectra for poly(LKKL) at $\sim$ 23 mg/mL in D_2O with no added salt (a) and $\sim$ 22 mg/mL in 0.15M NaCl (b), both at $\sim$ 10 cm^{-1} resolution. Spectra have been normalized to have a peak absorbance of the amide I' band of A_{max} = 1.0 so that the VCD scale reads directly in $\Delta A/A$ at A_{max}. Actual experimental conditions gave rise to absorbances of $\sim$ 0.3–0.5. The VCD spectra are the result of averaging 4 scans each obtained with a 10 s time constant. The baseline for correcting the VCD was obtained from identical scans of the poly(D,L-lysine) solution whose peak absorbance in the amide I' region matched that of the sample.

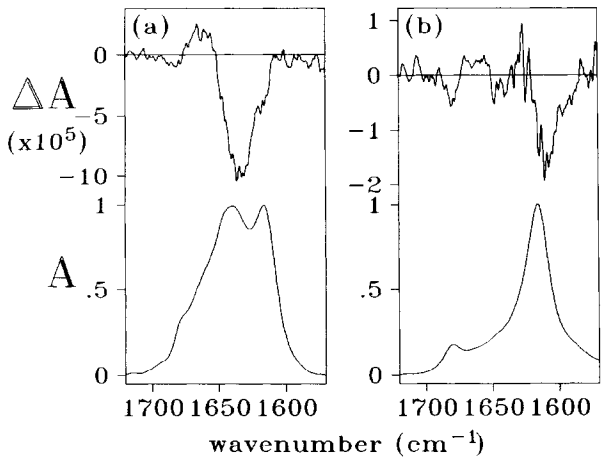

Figure 2. Same as Figure 1 for poly(KL). For a baseline correction VCD scans of poly(D,L-Lys, D,L-Leu) and N-methyl acetamide were used for a sample at (a) $\sim$ 23 mg/mL and with no added salt and (b) $\sim$ 22 mg/mL but high salt (0.3M), respectively. Note the different scale used for plotting the VCD spectra in (a) and (b). Spectra have been normalized to have a peak absorbance of the amide I' band of A_{max} = 1.0. This normalization does not give optimal proportions between the two VCD spectra. A better comparison might be based on normalization to the area of the amide I' band.

In Figure 2 are shown our ir absorption and VCD spectra in the amide I' region for D_2O solutions of the KL polymer with no added salt (Figure 2a) and 0.3M NaCl (Figure 2b). It is clear that under conditions of no added salt a mixed conformation exists as is evidenced by the sharp absorption features at 1616 and 1680 cm^{-1} and the distortion of the nearly typical "random coil" VCD band shape with negative low- and high-frequency components that are indicative of antiparallel β-sheet formation. If care is taken in correcting for VCD baseline artifacts in the 0.3M NaCl, high salt spectrum, two negative VCD bands, which are correlated to the sharp antiparallel β-sheet ir bands, dominate the spectra (Figure 2b). These absorption features often are indicative of aggregation[22] and sometimes of gel formation, both of which can be the source of large VCD artifacts. These negative features are in good agreement with

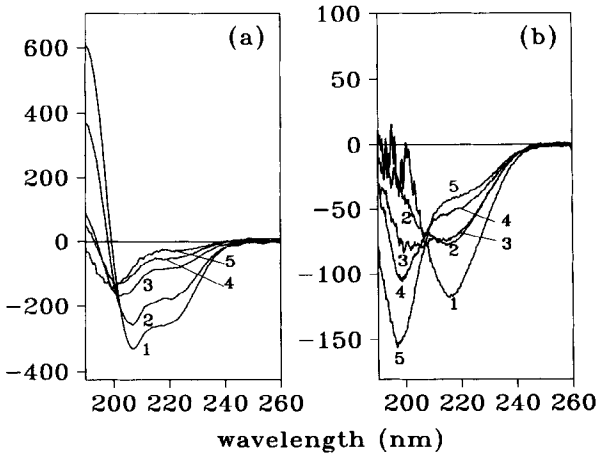

Figure 3. ECD concentration dependence for (a) poly(LKKL) (sample concentration was 11.85, 5.93, 2.96, 1.48, and 0.74 mg/mL for spectra 1–5, respectively), and (b) poly(KL) (sample concentration was 22.4, 14.9, 11.2, 5.6, and 1.6 mg/mL for spectra 1–5, respectively) with no added salt. High absorbance of the two most concentrated poly(KL) samples prevented adequate measurement of the corresponding ECD spectra below 200 nm. All spectra were normalized to a constant concentration–path-length product.

previous measures of VCD for antiparallel β-sheets.[11,12,23]

After obtaining these results, it was clear that, while a change of salt concentration affected a change in the KL spectrum, the high polypeptide concentrations used for VCD may have caused it and the LKKL spectrum to evidence features at low salt that were expected only under high salt conditions. Thus we remeasured the ECD spectra of both polymers as a function of concentration. In Figure 3a are shown the ECD spectra of the LKKL polymer and in Figure 3b of the KL polymer as a function of polymer concentration. It should be noted that use of short pathlength cells is required for these measurements. Since the nominal thickness of the spacers used provides only an estimate of the actual path length obtained, even after path-length corrections, the relative intensities can be in some error. However, the ECD shapes show a consistent pattern and provide a secure basis for interpretation. In both cases, in agreement with previous results obtained[2,3] for these polypeptides under dilute conditions with no added salt, the polymers are in what is commonly referred to as a "random coil" conformation,[17] which is indicated by the negative ECD at 200 nm. Increasing polymer concentration or increasing salt concentration results in the same effect: a conformational transition to an ordered form is found, α-helical for the LKKL and β-sheet for the KL, as indicated by the ECD patterns found, which are typical of those ordered forms. Adding salt to the concentrated solution results in no change of the ECD, indicating that the final ordered state for high polypeptide concentration is achieved independently of salt effects. On the other hand, adding salt to the dilute species results in the same spectral and conformational transformations that were previously reported.[1-3] (The spectra for the higher concentration samples in Figure 3b are unreliable below 200 nm due to optical density effects.)

To explore this conformationally sensitive equilibrium further, we carried out a parallel set of experiments on the oligopeptides $(LKKL)_5$, $(KL)_{10}$, and $(KL)_8$. For the LKKL sample, a VCD band shape of virtually the same form as but of lower intensity than that seen for the polymer (Figure 1) was obtained for the oligomer (Figure 4). A large absorbance band is present at 1673 cm^{-1} corresponding to the lysine side-chain CF_3COO-counterions, which are a residual of the synthesis.[24] This spurious absorption appears to have no deleterious effects on the VCD spectrum. ECD studies of the concentration dependence for the $(LKKL)_5$ oligomer conformation showed the same sort of behavior

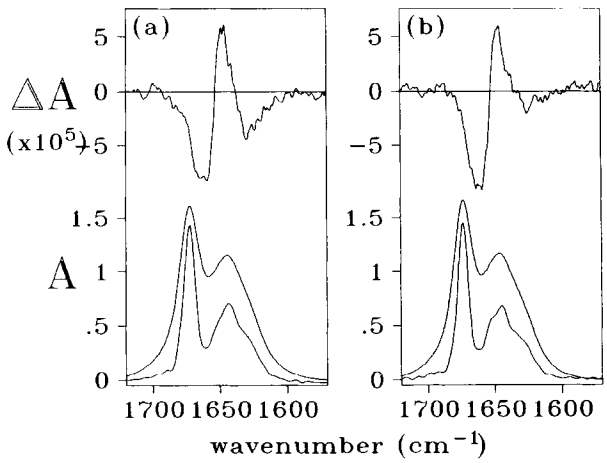

Figure 4. Dispersive VCD, FTIR absorbance, and deconvolved FTIR spectra (top to bottom) of the oligomer $(LKKL)_5$ at ~ 25 mg/mL in D_2O with no added salt (a) and at ~ 23 mg/mL in 0.1 *M* NaCl (b). The peak at 1673 cm^{-1} is due to the presence of CF_3COO^- counterions to the lysine side chains. Absorbance spectra were fit and the lower frequency component was used for normalization. Fourier self-deconvolution used a resolution enhancement factor of 2.0, bandwidth of 14 cm^{-1}, and Bessel apodization.

as seen for the polymer. At low concentrations, a random-coil-like spectrum was seen, and at high concentrations, a transformation to the α-helical type spectrum occurred (Figure 5a). However, the spectral change with concentration is sharper for the oligomer, and occurs at a higher concentration, indicating a more concerted transformation. Again, errors in path-length normalization may obscure some detail of the transformation. On increase of concentration, for the oligomer in 0.1 *M* salt solution, some increase in the ellipticity was seen so that substantially higher values are eventually obtained under conditions of high salt and high concentration than with just concentration or salt. This is a different behavior from that of the polymer.

Turning to the KL oligomers, the VCD does not precisely follow a consistent pattern with the polymeric result. It appears from the ir spectra that the sample under VCD concentration conditions has a high component of sheet and little coil, even without added salt (Figure 6). The characteristic low-frequency mode of the antiparallel β-sheet at 1616 cm^{-1} is prominent in the ir absorbance spectrum of $(KL)_8$, but the higher frequency mode at 1683 cm^{-1} is obscured by the TFA absorbance band [deconvolution allows its facile identification (Figure 6b)]. For both peptide transitions, a couplet VCD pattern (a negative couplet corresponding to the high-frequency absorbance band and a less intense positive one corresponding to the low-frequency feature) is seen that

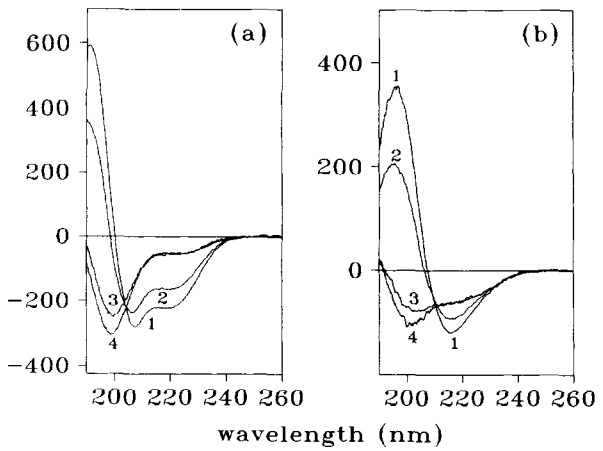

Figure 5. ECD concentration dependence for (a) $(LKKL)_5$ (sample concentration was 14.2, 7.1, 3.55, and 1.28 mg/mL for spectra 1–4, respectively), and (b) $(KL)_{10}$ (sample concentration was 14.2, 7.1, 3.55, and 1.28 mg/mL for spectra 1–4, respectively) with no added salt. Spectra were normalized to a constant concentration–path-length product.

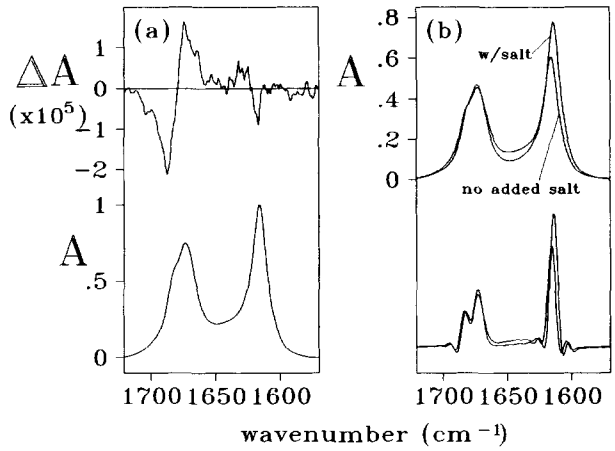

Figure 6. (a) Dispersive ir absorbance and VCD spectra of the oligomer $(KL)_8$ at ~ 25 mg/mL in D_2O with no added salt. For a baseline correction, VCD scans of a mixture of TFA and N-methyl acetamide were used for which the absorbance matched that of the sample. VCD spectra were normalized for A_{max} = 1.0 of the low frequency absorption band at 1616 cm^{-1}. (b) FTIR absorbance and Fourier self-deconvolution spectra of the same $(KL)_8$ oligomer sample with no added salt and at ~ 23 mg/mL in 0.1 *M* NaCl. Deconvolution parameters and 1673 cm^{-1} peak due to CF_3COO^-, as in Figure 4.

is not quite like the characteristic two negative band VCD pattern observed for other β-sheets,[11,12,19,23] or even that in Figure 2 for the KL polymer. The relative VCD intensity of the high- and low-frequency bands is reversed for this oligomer as compared to previous polymer results. To avoid any possible artifact problem, a mixture of TFA and N-methyl acetamide (closely matching the sample absorbance) was used to obtain the VCD baseline. Although great care was taken, a gel-like consistency resulted for the high salt samples and gave rise to artifacts in the VCD spectra whose elimination proved to be difficult. This was true for both the $(KL)_8$ and $(KL)_{10}$ oligomers.

In terms of the ECD (Figure 5b), a rather sharp change is seen with increasing concentration from a spectrum typical of a random coil conformation to one typical of a β-sheet conformation. In the $(KL)_{10}$ case, the transition occurs at somewhat lower concentration than for the $(LKKL)_5$ oligomer and appears to be stable with regard to added salt.

Analysis of the FTIR spectra, obtained at higher resolution and S/N, using second-derivative and Fourier self-deconvolution methods, gave evidence for multiple bands in the amide I′ region for the LKKL oligomers at low (no added) and high salt

conditions (Figure 4). The major deconvolved feature shifted from 1642 to 1645 cm^{-1} with added salt, but the shape was relatively consistent. This central frequency position is typical of an α-helical polypeptide that maintains a fairly uniform (high persistent length) structure in D_2O.[11-13,25] A major absorbance shoulder at 1630 cm^{-1} (1632 cm^{-1} with salt) correlates to the low frequency negative VCD band, but it did not significantly decrease in intensity with added salt as did the VCD band. The same sort of central frequency was seen for the polymer but the low-frequency shoulder was higher in frequency and the high-frequency shoulder was stronger in intensity than for the oligomer.

For the $(KL)_{10}$ samples, the 1616 cm^{-1} feature is clearly dominant, and deconvolution allows a clear separation of the 1683 cm^{-1} peptide peak from the 1673 cm^{-1} TFA peak (Figure 6b). The two peptide features are fully consistent with antiparallel β-strand features assigned in the polypeptide literature.[25] Addition of salt caused a small downshift and an increase in intensity for the low-frequency absorption feature (Figure 6b).

DISCUSSION

As is clear from the forgoing results, we have demonstrated by using VCD, ir, and ECD spectra that the effect of salt on the conformation of sequential peptides based on LKKL and KL can be largely duplicated by increased concentration. Increasing concentration in these systems does increase the ionic strength, since at pH 7 the Lys side chains are protonated, making the effective salt concentration approximately 0.06 *M* for the peptide alone under VCD sample concentrations. But probably a more important factor is the effect of peptide–peptide interaction as enhanced by the high concentration. Our observation that both the polymers and the oligomers, each with very different counterions and different length distributions, behave so similarly argues strongly in favor of this latter interpretation. It would seem that in the KL case, the dominant interaction is straightforward interchain hydrogen bonding to form an antiparallel β-sheet. In the LKKL case, the interaction is possibly similar to that seen in coiled-coil twisted helices[26] or to the helix stabilization present in helical bundle protein models.[4,5]

Considering just our ir data, it may be noted that the amide I′ frequencies reported here differ from those normally taken as typical for α-helices and even for β-sheets in globular proteins.[27-30] In the β-sheet case, the low frequencies for KL are consistent with aggregate formation as proposed by Mantsch and Surewicz.[22,27] In support of this, the VCD spectral patterns seen here for KL polymer are very similar to those we and others have seen for aggregating peptides and proteins (Refs. 11, 12, and 23; also M. Urbanova and T. A. Keiderling, unpublished results). The oligomer VCD results are unusual and may be related to the intermediate conformations seen in the poly(lysine) VCD.[11]

In the α-helix case, the relatively low amide I′ frequencies observed for LKKL are typical of α-helices formed from synthetic polypeptides in D_2O, which typically have charged side chains.[11-13,25] They are somewhat low compared to amide I′ frequencies seen for globular proteins in D_2O.[22,27-30] While it might seem that partial deuteration could account for these differences, we feel that explanation cannot account for the low frequencies seen here. Such a large shift to low frequency has been attributed to helix length effects,[29] but the similarity of the absorbance frequencies for the oligomers and the polymers presented in this study argues against that interpretation and suggests that ionic conditions may be a more important factor. The shifts for the main feature at 1642 cm^{-1} on just increasing ionic strength argue that these frequencies are sensitive to the peptide environment. As seen in our earlier study[31] of $(Met_2\text{-}Leu)_n$ oligomers, the VCD band shape characteristic of the α-helical conformation is maintained for relatively short helices. The comparison of polymer and oligomer VCD obtained here for the LKKL samples further confirms this short-range aspect of peptide VCD. It is this characteristic band shape that gives us confidence in assigning these frequencies to α-helical segments. However, the relative decrease in intensity of the lower frequency negative VCD component on salt addition suggests that this particular feature might originate in some "coil" aspect of the polymer conformation. The negative sign and broad width of the VCD feature centered around 1630–1640 cm^{-1} are consistent with such an interpretation.[17] Previous assignments[32] of amide I′ frequencies at $\sim$ 1640 cm^{-1} to a 3_{10}-helix contribution clearly do not apply here and, by extension, should probably not be applied to extended peptides in D_2O.

Finally, it can be noted that an initial goal of the study was reached in that the sequential Lys-Leu system did give rise to characteristic VCD band shapes for a peptide in the α-helix, β-sheet, and (by elimination of the β-sheet contribution for the KL mixed conformation cases) the random coil forms in aqueous solution conditions comparable to those

used for protein VCD studies. These VCD band shapes are fully consistent with the VCD band shapes seen for these conformations in nonaqueous environments and in proteins, if effects of N—H deuteration are taken into account. Thus these data further confirm the stability of the amide I′ VCD band shape and its independence from influences of the side chain, transition frequency, and to some extent, solvation environment. On the other hand, the absorption frequencies, which are often used to determine secondary structure using FTIR data alone,[27] are again demonstrated to be ambiguous for this purpose since they are subject to substantial change due to the environment and the nature of the side chains involved in the structure.[28]

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