Circular dichroism of oriented α-helices. II. Electric field oriented polypeptides

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A transition moment polarized parallel to the helical axis, as predicted to occur in an exciton split component (near 208 nm) of the peptide $\pi\pi^*$ transition, can be exploited to study the structural orientation of the helical sections in proteins. In order to test this important feature of the exciton theory for helical polymers, many investigators have measured the polarization of electric field oriented polypeptides. The qualitative support for the theory provided by early experiments was recently overshadowed by a more extensive measurement [Yamaoka *et al.*, J. Am. Chem. Soc. **108**, 4619 (1986)] which showed the polarization of the 208 nm band not to be parallel to the orientation axis of the polypeptides. This finding is confirmed in this paper by our circular dichroism measurement of electric field oriented poly-γ-benzyl-glutamate in ethylene dichloride and in dioxane. Thus the exciton theory appears to be contradicted, as similarly concluded by Yamaoka *et al.*, if the polypeptides are rod-like and rigid. However, our theoretical study of the bending effect on long polypeptides shows that our experiment is in fact consistent with the prediction of the exciton theory, which was unambiguously proven recently in paper I by using short membrane peptides [Olah and Huang, J. Chem. Phys. **89**, 2531 (1988)].

I. INTRODUCTION

Ultraviolet circular dichroism (UVCD) has been used widely as a diagnostic tool for the analyses of the secondary structures of proteins.[1] Although theoretical computations of the CD spectra are well developed,[2] there are few distinct and definitive theoretical predictions with which one could test the theory. One exception is Moffitt's prediction of exciton splitting in the amide $\pi\pi^*$ transition of α-helical polypeptides. The observed negative CD band of α-helical polypeptides at wavelength ~208 nm and the positive band at ~192 nm have been assigned to be these exciton split components. The theory predicts that the 208 nm band is polarized parallel to the helical axis of the polypeptide, while the 192 nm band is a composite of several perpendicularly polarized components (see the details in Ref. 2). This theoretical description and the qualitative support for it from early polarization measurements[4–6] were very important for the development of the CD theory for biopolymers. The band at 208 nm has also been used to study the orientation of α-helical sections in membrane proteins according to the theory.[7–9] Therefore the recent report by Yamaoka *et al.*[10] that the electric linear dichroism of poly-γ-methyl-glutamate (PMLG) contradicts the exciton theory has to be taken seriously. We have performed two experiments to measure the orientation effect of α-helices on CD, one with a short membrane peptide, alamethicin, oriented in defect-free lipid multibilayers and another with a long polypeptide, poly-γ-benzyl-glutamate (PBLG), oriented by electric field. We found that the result of the experiment with alamethicin is consistent with the exciton theory (paper I[11]), while the result of PBLG is, like that of Yamaoka *et al.*,[10] in apparent disagreement with Moffitt's prediction. We believe that the exciton theory is correct and the apparent disparity between our two experiments can be understood if we take into account the flexibility of long α-helical polypeptides. In this paper we will describe our experiment with PBLG and discuss the result with a calculation of the effect of flexibility on the polarization of a long α-helix. We will show that our electric dichroism experiment is consistent with the exciton theory; however, we also note that the result of Yamaoka *et al.* can not be explained in a similar fashion.

PBLG was chosen for study because it is perhaps the best characterized polypeptide. It is soluble in a large number of solvents in the α-helical form and its electric properties have been measured extensively.[12] In general an α-helix carries a net dipole moment pointing from the C terminus to the N terminus. This is primarily the vectorial sum of the peptide residue moments arranged on the helical backbone. However, polar side chains, such as those of PBLG, may also contribute to the total moment. In this case the net dipole moment of a polypeptide depends on the dielectric constant of the solvent. In a solvent of high dielectric constant, the interaction between a side chain moment and the backbone moment is screened by the dipoles of the solvent molecules; consequently the side chains tend to be randomly oriented and have a small or no contribution to the total moment. As the screening effect decreases with the dielectric constant, the side chains tend to orient antiparallel to the backbone moment due to the dipole–dipole interactions. Hence the total moment of a polypeptide decreases with the dielectric constant of the solvent. In order to test the consistency of our measurements with the solvent effect, we used one solvent of high dielectric constant ($\epsilon = 10$), i.e., ethylene dichloride (EDC), and one of low dielectric constant ($\epsilon = 2.2$), i.e., dioxane. (They were also chosen for having good UV transmission down to 202 nm and low electric conductivities.) The dipole moment per residue of PBLG was found to be

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dependent on the degree of polymerization (DP). In dioxane, the measured value is 0.33–0.38 e Å for DP = 18 and 0.27–0.29 e Å for DP = 870–1000[13,14]; in EDC, it is about 0.7 e Å for DP = 200–900.[12] (Unfortunately, the uncertainties in the dipole values are often as large as 10%.)

Let E be the applied electric field, μ the magnitude of a dipole, k_B the Boltzmann constant, T the temperature, and the ratio $w = \mu E/k_B T$. In order to align a dipole, w has to be substantially larger than one. Since, in practice, it is difficult to maintain a dc field of more than, say 50 kV/cm across a sample, it is necessary to use long polypeptides, typically with DP $\gtrsim$ 1000.

It was shown by Tinoco and Hammerle[15] that in an anisotropic medium, if the direction of propagation of the probing light $\hat{\mathbf{k}}$ is along an optic axis of the medium, the circular dichroism can be expressed as $\langle \hat{\mathbf{k}}\cdot\mathbf{G}\cdot\hat{\mathbf{k}} \rangle$, where $\mathbf{G}$, a second-rank tensor, depends only on the properties of the molecules, and does not depend explicitly on the vectors specifying the electromagnetic field. $\langle\ \rangle$ indicates an average over the appropriate distribution of molecular orientations. In our sample, the optic axis is the direction of E. With $\hat{\mathbf{k}}$ in the direction of E and α being the angle between a dipole and $\hat{\mathbf{k}}$, we have

$$\langle \hat{\mathbf{k}}\cdot\mathbf{G}\cdot\hat{\mathbf{k}} \rangle = G_{\parallel} \langle \cos^2 \alpha \rangle + G_{\perp} \langle \sin^2 \alpha \rangle, \tag{1}$$

where $G_{\parallel}$ is the average CD along the dipole (i.e., the CD component whose transition moment is perpendicular to the dipole) and $G_{\perp}$ is the average CD perpendicular to the dipole. Since the orientation of the dipole is governed by the Boltzmann factor $\exp(w \cos \alpha)$, the measured CD as a function of E is given by

$$\theta(E) = G_{\parallel} + 2\left(\frac{1}{w^2} - \frac{\coth w}{w}\right)(G_{\parallel} - G_{\perp}). \tag{2}$$

We note that this formula implicitly assumes that all polymers have the same magnitude of dipole moment or that the dispersion of it is negligible. Dividing Eq. (2) by $\theta(0)$, the CD in the absence of E, one obtains a function

$$\frac{\theta(E)}{\theta(0)} = 3\left[x + 2\left(\frac{1}{w^2} - \frac{\coth w}{w}\right)(x-1)\right]/(x+2) \tag{3a}$$

$$= \frac{3x}{(x+2)} - \frac{6(x-1)}{w(x+2)} + O\left(\frac{1}{w^2}\right) \qquad \text{for } w \gg 1 \tag{3b}$$

depending only on two unknown parameters $x = G_{\parallel}/G_{\perp}$ and μ. The purpose of this experiment is to determine x to test the exciton theory of CD, particularly at wavelength $\sim$208 nm. μ will be compared with the values previously determined by dielectric measurements as a test of consistency. If the polypeptide is rod-like and rigid, by symmetry its dipole must be in the direction of the helical axis, as assumed in all previous investigations. Then, according to the exciton theory, one would expect x to be zero for the 208 nm band.

CD of electric field oriented polypeptides was first measured by Tinoco,[16] but his measurement was limited to above 340 nm. Hoffman and Ullman[5] were the first to measure $\theta(E)$ in the far UV but could only cover the 224 nm $n\pi^*$ band. Mandel and Holzwarth[6] extended the measurement down to 190 nm, but they obtained only 5% difference in CD between oriented and unoriented samples at 208 nm, which, we believe, is insufficient for a definitive determination of the polarization of the 208 nm band. Yamaoka *et al.*[10] measured the linear dichroism of electric field oriented PMLG (M_w = 135 000, DP ~ 950) in hexafluoro-2-propanol (HFP) with a field strength up to 26.5 kV/cm. They concluded that the electric transition moment of the 208 nm band is not in the direction of the helical axis (which is assumed to be the direction of the dipole), but rather in a direction pointing 51° away from the axis.

II. EXPERIMENT

A. Materials

Poly-γ-benzyl-L-glutamate (PBLG) (Lot #85F-5020, M_w = 260 000) was purchased from Sigma Chemical Co., St. Louis, MO. PBLG was used without further purification. 0.1% (w/v) stock solutions of PBLG in 1,2 dichloroethane (EDC) and dioxane were prepared. Stock solutions older than 5–7 days were always discarded and fresh stock solutions were used in order to avoid possible problems with polymer aggregation.

1,2 dichloroethane (EDC) (Lot #07916TP) was purchased from Aldrich Chemical Co., Milwaukee, WI. EDC was spectroscopic grade. EDC was dried by refluxing over P_2O_5, and fractionally distilled. The conductance of the dried EDC was such that the current through the samples was never higher than ~5 μA for the highest applied voltages.

Dioxane (Lot #CB368) was purchased from EM Science, Inc., Cherry Hill, NJ. Dioxane was reagent grade. Further purification of this lot was unnecessary since far UV transmission was high enough and the conductivity (~5 μA max) was low enough for this experiment.

All solvents and stock solutions were stored in a N_2 purged desiccator lined with P_2O_5.

The circular dichroism was measured on a JASCO J500-A spectropolarimeter. 0.06% (w/v) ammonium (+)-10-camphorsulfonate/H_2O was used to calibrate the CD scale assuming a molar ellipticity of $[\theta]_{290.5\,\text{nm}} = +7879°$. The 586 nm peak of neodymium glass was used to calibrate the wavelength. Corning glass (#00-0211, 80 μm × 26 mm × 40 mm) was purchased from J. Melvin Freed Glass, Inc., Perkasie, PA. This glass was masked and etched with hydrofluoric acid into a U shape and used as a spacer which defined the path length of the sample. See Fig. 1 for clarification.

Fused silica plates (1 in. × 2 in. × 0.5 mm) were purchased from Optical Instruments, Inc., Houston, TX. In order to remove any possible stress in the plates, they were temperature annealed at 1150 °C for 6 h. The plates were slowly cooled at a rate of 10 °C/h down to 900 °C and then at a rate of 100 °C/h until room temperature was reached. Indium tin oxide (ITO) was then coated on one side of the plates by Thin-Films Technology, Inc., Buellton, CA. An e-beam deposit in vacuum was used which produced fairly stable coatings. Coatings prepared by sputtering were unstable and oxidized rapidly in air which resulted in a marked increase in

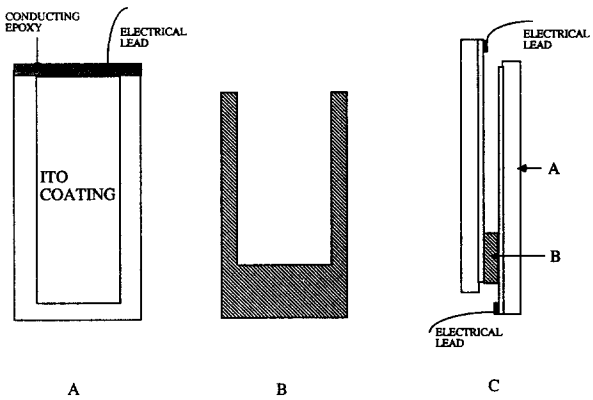

FIG. 1. Description of optical cell. Cell consists of three parts: two fused silica plates coated with ITO (A), and a 80 μm thick U shape glass spacer (B). C shows the side view of the middle cross section of the assembly. The assembly is held together by an aluminum case (not shown).

resistivity. The *e*-beam deposited plates, however, could be stored safely in air for a few days with no apparent oxidation problems. If the ITO coating does oxidize, then temperature annealing at 200–600 °C for 30–45 s usually alleviates the problem. Optimal thicknesses of deposit were 150–250 Å. ITO coatings thicker than 250 Å resulted in very poor UV transmission, while coatings thinner than 150 Å were not stable and resulted in electrical problems. Resistivity of ITO was between 0.5–2 kΩ/□. Wires were attached to the ITO by using conducting epoxy.

A Kepco model BHK 2000-0.1(M) voltage supply was purchased from Kepco, Inc., Flushing, NY. An electronic switch/gate was made and used to trigger the voltage applied to the sample for a predefined time interval. This time interval was begun approximately 0.25 s before the start of the CD scan and finished with the scan. Scan lengths were either 16 or 32 s. The conductivity of our samples was such that joule heating was not a serious problem for these time intervals.

B. Methods

A simple schematic of the optical cell is shown in Fig. 1. An aluminum holder was used to clamp the glass pieces of the optical cell together. The pieces included two 1 in. × 1 in. × 0.5 mm fused silica plates with ITO deposited on a single side and a 80 μm thick U-shaped glass spacer. About 1/16 in. wide strip of the ITO coating was removed from the edges of the plates (except on the side to which the wire was attached with epoxy) to avoid any possible electrical breakdown by ensuring no contact with the aluminum holder. Screws on the holder could be adjusted so that no nonuniform stress would be induced in the fused silica while at the same time affording enough pressure on the plates to seal them against the glass spacer. A Pasteur pipet was used to fill the cell. Since the top of the cell was left open and the glass seal was not perfect, the cell required frequent cleaning and refilling. The cell was easily cleaned by blowing the solution out with a stream of clean nitrogen, rinsing with pure solvent, and blowing dry again.

CD measurements using EDC as a solvent were performed as follows. The wavelength range of the measurement was from 202–255 nm so as to cover the ~224 nm and ~208 nm bands. (The wavelength range below 202 nm was impossible to measure due to the high absorbance of EDC.) This range was divided up into five separate regions: 202–215 nm, 212–225 nm, 222–235 nm, 232–245 nm, and 242–255 nm. The spectrum was divided up into regions so that high E fields applied to the sample could be kept as short as possible and thus avoid heating the sample. The scan rate was set so that each region could be scanned in ~16 s. (We actually measured the temperature increase for our system by placing a temperature monitor in the sample. Heat dissipation was good enough so that ~10 μA of current through the sample would raise the temperature only ~2–3 °C in 60 s.) For each wavelength region, a single scan set included five scans in the following order: a base line scan (EDC alone), 0 V scan (sample), applied voltage scan (sample), 0 V scan (sample), and a base line scan. Only if the two base line scans coincided and the two 0 V scans coincided was the applied voltage scan considered good. Also, the current through the sample was recorded by measuring the voltage drop across a 1 MΩ resistor in series with the sample. Therefore, only if the current through the sample was below 5 μA did we trust the applied voltage scan. For each applied voltage, the number of applied voltage scans averaged for each region was: 202–215 nm, 16 scans; 212–225 nm, 10 scans; 222–235 nm, 8 scans; 232–245 nm, 4 scans; 242–255 nm, 2 scans. The full spectra (see Fig. 2) were constructed by subtracting the appropriate base line and piecing together the five regions for each applied voltage scan. The 0 V files for all scan sets, with the appropriate base line subtracted, were averaged and pieced together to give the 0 V spectra shown in Fig. 2.

The CD of PBLG in dioxane was not broken up into regions as was done for PBLG in EDC. Instead, the scan rate was increased so that the scan time for the full wavelength range 202–255 nm was ~32 s. We felt justified in increasing the scan time because (1) a check at a particular wavelength (224 nm) using scan times $\lesssim$ 1 s showed the same orientation effect on the CD as the value measured from the averaged full spectra, and (2) we did make a temperature mea-

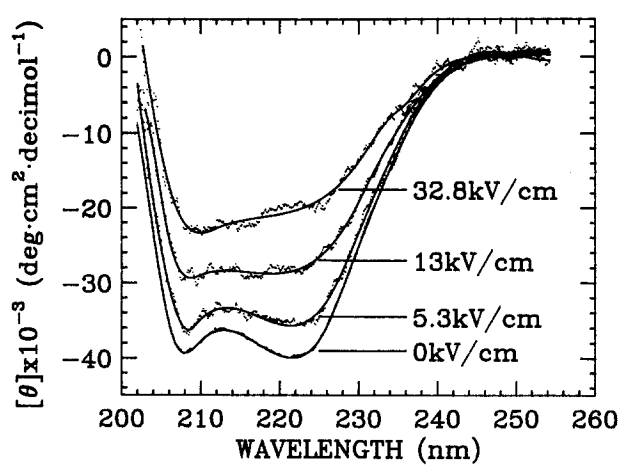

FIG. 2. Circular dichroism of PBLG in EDC at four different electric field strengths. The background (CD of pure solvent) has been subtracted. The solid lines are ten knot spline fits.

surement which showed that the joule heating would increase the temperature no more than ~1 °C for the highest field applied. However, the scan set (i.e., in order, base line, 0 V, applied voltage, 0 V, base line) was done as similarly done for PBLG in EDC. The spectrum in Fig. 3 was the result of averaging 24 applied voltage scans. The 0 V scan was, therefore, the average of 48 scans since two 0 V scans were made per scan set.

Assuming the applied voltage to be uniform, the E field for the sample was determined by dividing the applied voltage by the cell path length. Twelve different E fields were applied to PBLG in EDC: 0, 3.8, 5.3, 6.6, 9.2, 13.0, 19.7, 26.2, 32.8, 39.4, 45.9, 59.0 kV/cm. Ten different E fields were applied to PBLG in dioxane: 0, 9.8, 16.4, 21.0, 26.2, 32.8, 49.2, 59.0, 72.2, 92.0 kV/cm.

III. DATA ANALYSIS

Figures 2 and 3 show the CD spectra of PBLG at different field strengths (for clarity, only four are shown). The zero-field spectra are that of typical α-helices in solution.[1] The amplitudes at 208 and 224 nm are in agreement with previously reported values. Ideally the next step is to decompose each spectrum into a superposition of individual bands. However, as was pointed out in paper I, this procedure does not produce a unique result. Under the circumstances, it seems more reasonable to assume that the spectrum near 208 nm is dominated by the 208 nm band and that the amplitude (or the rotational strength) of the band is proportional to the spectral value at the peak.

We now ask if $G_{\|}$ at 208 nm vanishes. The amplitude at 208 nm (normalized by the zero-field value) is plotted as a function of $1/E$ in Figs. 4 and 5. If $G_{\|}$ were zero, the extrapolation of the data to $1/E = 0$ would go through the origin—this is clearly not the case in either figure. Good fit between the data and Eq. (3a) indicates that we can indeed regard each sample as a collection of independent dipoles. It also indicates that the dispersion of the magnitude of dipole moment is sufficiently small. The dipole moments determined from our data, 0.60 ± 0.04 e Å per residue in EDC and 0.22 ± 0.02 e Å per residue from dioxane, are consistent with the previously measured values by dielectric methods in both solvents (see Introduction). Thus we believe that the consistency of our data is well established.

Our results $G_{\|}/G_{\perp} = 0.27 \pm 0.06$ in EDC and $G_{\|}/G_{\perp} = 0.36 \pm 0.06$ in dioxane at wavelength 208 nm, support the finding of Yamaoka *et al.*,[10] i.e., the polarization of the 208 nm band does not coincide with the direction of the dipole. However, we do not think that this is necessarily a

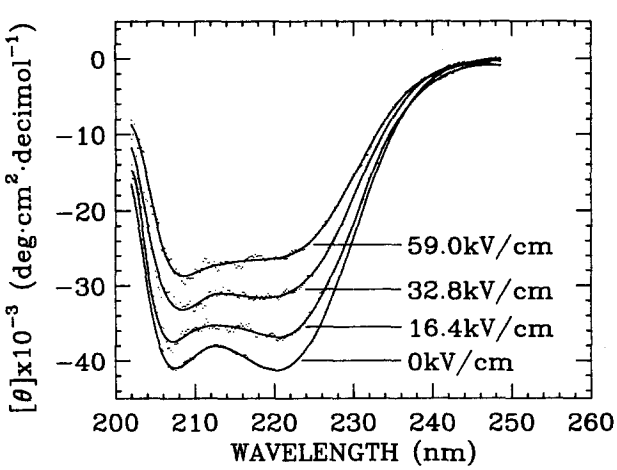

FIG. 3. Circular dichroism of PBLG in dioxane at four different electric field strengths. The background (CD of pure solvent) has been removed. The solid lines are ten knot spline fits.

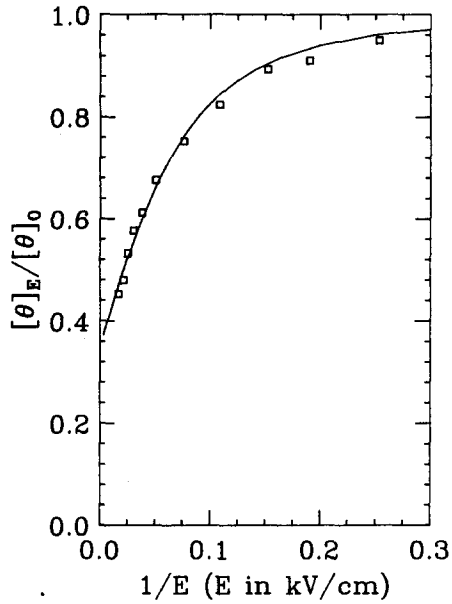

FIG. 4. Circular dichroism at 208 nm of PBLG in EDC, normalized by the zero field value, vs $1/E$. The solid curve is the least-squares fit of Eq. (3a) to the data. The parameters of the best fit are $G_{\|}/G_{\perp} = 0.27 \pm 0.06$, $\mu = 714 \pm 45$ e Å.

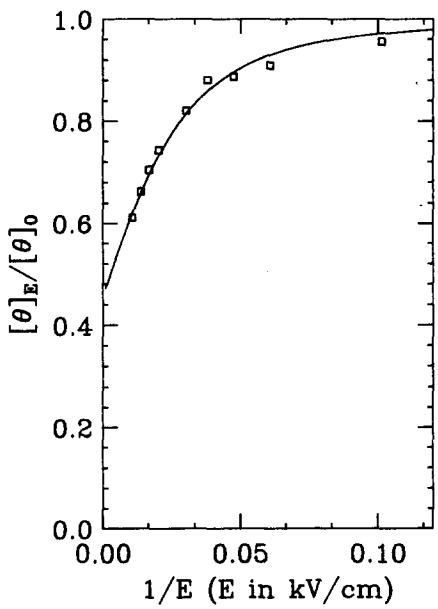

FIG. 5. Circular dichroism at 208 nm of PBLG in dioxane, normalized by the zero field value, vs $1/E$. The solid curve is the least-squares fit of Eq. (3a) to the data. The parameters of the best fit are $G_{\|}/G_{\perp} = 0.36 \pm 0.06$, $\mu = 259 \pm 22$ e Å.

contradiction to the exciton theory, because an α-helix of DP $\gtrsim$ 1000 is probably flexible. There are at least three independent pieces of experimental evidence for this: (1) dielectric measurements of PBLG showed that the dipole moment per residue decreases with increasing DP[12,13,17]; (2) light scattering and hydrodynamic studies showed that the pitch per residue (the length of the projection of a monomeric unit on the helical axis) of PBLG and other polypeptides is a decreasing function of the molecular weight[18]; (3) bending α-helices are often seen in protein crystals.[19] Therefore we shall now consider a flexible α-helix and calculate its CD according to the polarizations predicted by the exciton theory.

IV. POLARIZATION OF FLEXIBLE α-HELIX

An α-helix is often considered to be rod-like, because it is apparently rather stiff. The evidence of flexibility mentioned above also indicates that the possible curvature in a helix is in general small at each point. Thus we may consider a long helix as a macroscopic linear system with axial symmetry. The change in the free energy due to a small curvature can be written as[20]

$$\Delta F=\frac{1}{2}\int_0^L a\left(\frac{\partial \mathbf{t}(s)}{\partial s}\right)^2 ds, \tag{4}$$

where s is the contour length of the polymer, varying from 0 to the total length L, $\mathbf{t}(s)$ is a unit vector along the tangent at s, and a is the elastic constant. For a given shape configuration, the dipole of the polymer is proportional to the end-to-end vector

$$\mathbf{R}=\int_0^L \mathbf{t}(s)ds. \tag{5}$$

For example, in EDC the dipole moment of the PBLG we used (DP = 1190) is 0.60 e Å per residue, about 85% of the maximum dipole for a straight helix ($\sim$0.7 e Å per residue).[12] That means R is about 85% of L (since the side-chain effect in reducing the total dipole moment is small in EDC). The width of the R distribution $\langle(\Delta\mathbf{R})^2\rangle^{1/2}/L$ is about 1.5% based on the simple random walk distribution.

In an electric field, the free energy also includes the dipolar energy. This energy is significant for the consideration of the total dipole moment and its orientation, but not for the shape configurations for a given R. As we have seen, the variations of the magnitude of the total dipole are small. Therefore, to a good approximation, the statistical average over the shape configurations and the average over the (dipolar) orientations can be carried out separately. The statistical mechanical problem of free energy (4) has been solved by Saito, Takahashi, and Yunoki.[21] The Green function expressing the probability of finding $\mathbf{t}(s)$ at s for a given $\mathbf{t}'(s')$ at s' is given by

$$G[\mathbf{t}(s)|\mathbf{t}'(s')]=\sum_l e^{-l(l+1)D(s-s')}\sum_m Y_{lm}(\mathbf{t})\,Y_{lm}(\mathbf{t}'), \tag{6}$$

where Y_{lm}'s are spherical harmonics and $D=k_BT/2a$.

It is an experimental fact that the CD spectrum of a whole protein is the superposition of the spectra of its segments.[1] Theoretical studies[22,23] showed that the spectrum of a helix varies insignificantly once DP is above 10, except for the overall amplitude which is proportional to DP. This is proven experimentally in the case of alamethicin which has a helical section of about ten amino acids and exhibits a typical solution CD characteristic of long α-helical polypeptides; furthermore, its CD obeys the exciton theory as we showed in paper I. Accordingly, we may calculate the absorption coefficient or the rotational strength of a long α-helical polypeptide as the linear superposition of the contributions of small straight segments. Imagine a long polymer being divided into short segments ($i=1,2,\ldots$) of equal length h. Let us be concerned only with the 208 nm band. Thus each segment is characterized by an absorbance parallel to its axis, denoted by $a_\parallel h$, and a corresponding perpendicular rotational strength $g_\perp h$. Let θ_i be the angle between the ith segment and $\mathbf{R}$. Then $A_\perp$, the total absorbance perpendicular to $\mathbf{R}$, and $A_\parallel$, the total absorbance parallel to $\mathbf{R}$, are given by

$$A_\perp=a_\parallel h\left\langle\sum_i \sin^2\theta_i\right\rangle,\quad A_\parallel=a_\parallel h\left\langle\sum_i\cos^2\theta_i\right\rangle. \tag{7}$$

Similarly, $G_\parallel$ and $G_\perp$, the corresponding total rotational strength parallel and perpendicular to $\mathbf{R}$, respectively, are given by

$$G_\parallel=g_\perp h\left\langle\sum_i \sin^2\theta_i\right\rangle,\quad G_\perp=g_\perp h\left\langle\sum_i\cos^2\theta_i\right\rangle. \tag{8}$$

The smallness of the length of the segments allows us to replace the sums with integrals:

$$h\left\langle\sum_i\cos^2\theta_i\right\rangle=\left\langle\int_0^L ds[\mathbf{t}(s)\cdot(\mathbf{R}/R)]^2\right\rangle, \tag{9a}$$

$$h\left\langle\sum_i\sin^2\theta_i\right\rangle=\left\langle\int_0^L ds[\mathbf{t}(s)\times(\mathbf{R}/R)]^2\right\rangle = L-h\left\langle\sum_i\cos^2\theta_i\right\rangle. \tag{9b}$$

Finally, since the dispersion of R is, as mentioned above, relatively small, we approximate the integral (9a) as follows

$$h\left\langle\sum_i\cos^2\theta_i\right\rangle\approx\frac{1}{\langle R^2\rangle}\left\langle\int_0^L ds[\mathbf{t}(s)\cdot\mathbf{R}]^2\right\rangle. \tag{10}$$

The mean-square average of $\mathbf{R}$ is already known[20]

$$\langle R^2\rangle=\int_0^L\int_0^L ds_1\,ds_2\langle\mathbf{t}(s_1)\cdot\mathbf{t}(s_2)\rangle = \frac{1}{2D^2}(2DL-1+e^{-2DL}). \tag{11}$$

The integral in Eq. (10) by the Green function (6) is somewhat tedious but straightforward. The result is

$$h\left\langle\sum_i\cos^2\theta_i\right\rangle=\frac{1}{\langle R^2\rangle}\frac{1}{D^3}\left[\frac{1}{3}(DL)^2+\frac{7}{18}(DL)-\frac{13}{27}+\frac{1}{2}(1+DL)e^{-2DL}-\frac{1}{54}e^{-6DL}\right] \tag{12a}$$

$$=\frac{L^3}{\langle R^2\rangle}\left\{1-\frac{4}{3}DL+\frac{7}{5}(DL)^2+O[(DL)^3]\right\};\quad \text{for } DL\ll 1. \tag{12b}$$

TABLE I. Characteristics of PBLG ($M_w = 260\,000$; $T = 300$ K).

Solvent	$\frac{G_\parallel}{G_\perp}$ at 208 nm (measured)	Elastic constant a (calculated)	$\langle R^2\rangle^{1/2}/L$ (calculated)	Dipole at high DP/dipole at low DP (to be compared with $\langle R^2\rangle^{1/2}/L$)
EDC	0.27	1.2×10^3 (kcal/mol)Å	0.87	0.86
Dioxane	0.36	8.3×10^2 (kcal/mol)Å	0.83	0.80

In the limit $a\to\infty$ or $DL\to0$ (rigid rod), we have $\langle R^2\rangle\to L^2$ and the quantity (12b) becomes L as expected. By the combination of Eqs. (7), (8), (9), and (12a), we see that the ratio $G_\parallel/G_\perp$ ($=A_\perp/A_\parallel$), like $\langle R^2\rangle/L^2$, depends only on one parameter DL.

In EDC, the measured value 0.27 for $G_\parallel/G_\perp$ of the 208 nm band gives $DL = 0.46$. With $L = 1780$ Å, $T = 300$ K, one obtains $a = 8.0\times10^{-11}$ erg Å or 1.2×10^3 (kcal/mol)Å. In dioxane, we have $G_\parallel/G_\perp = 0.36$, $DL = 0.64$ and $a = 5.8\times10^{-11}$ erg Å or 8.3×10^2 (kcal/mol)Å. We can then calculate the value of $\langle R^2\rangle^{1/2}/L$ by Eq. (11): 0.87 in EDC and 0.83 in dioxane. A crude estimate of $\langle R^2\rangle^{1/2}/L$ may be given by the ratio of the dipole moments: in EDC, where the contribution of the side chains to the dipole moment is small, we use our measured value over the maximum value of a straight helix $\sim0.60/0.7 = 0.86$; in dioxane, the long polymer value over the short polymer value $0.28/0.35 = 0.80$. For clarity, these results are summarized in Table I.

V. DISCUSSION

Until the recent report by Yamaoka *et al.* (YUK),[10] the proof of the exciton theory was largely based upon the early polarization measurements of long polypeptides.[4–6] YUKs experiment and ours clearly contradict those early papers by showing the polarization of the 208 nm band not to be parallel to the dipole orientation of a long α-helix. We believe that the rigid rod assumption applied to long α-helical polypeptides in solution is probably wrong. The net dipole of a flexible helix is in the direction of the end-to-end vector **R**. Transition moments locally parallel to a flexible helix in general give rise to a total moment neither parallel nor perpendicular to **R**. Thus, long polypeptides are not an ideal system for testing the exciton theory.

Our quantitative analysis is handicapped by the difficulty or, in fact, the impossibility of decomposing the spectra uniquely into individual bands (see paper I). Rather than choosing a nonunique decomposition, we prefer to assume that the spectral value at 208 nm is proportional to the area of the 208 nm band. This amounts to making two assumptions: (1) the band dominates the spectrum at 208 nm, and (2) its width is independent of the applied field and orientation. The results are therefore susceptible to possible errors due to these assumptions. Our elastic constants or persistence lengths (defined as $1/2D$ which is 1940 Å in EDC and 1390 Å in dioxane) seem to be a factor of 1.5 to 2 higher than the values, 700–1400 Å, obtained by light scattering.[24] However, the DP dependence of the dipole moment per residue is in favor of our values as shown in Table I. Overall our data appears to be at least qualitatively consistent with the exciton theory of a flexible α-helix.

The result of YUK, however, does not seem to fit this model. According to the exciton theory of flexible helices, the measured angle Ψ between the net transition moment of 208 nm and the net dipole moment is given by $\langle\cos^2\Psi\rangle = A_\parallel/(a_\parallel L)$ [Eq. (7)]. For PMLG in HFP, Ψ is deduced by YUK from their electric linear dichroism measurement to be 50.6°. This would give, from Eq. (12a), $DL = 7.6$ and, from Eq. (11), $\langle R^2\rangle^{1/2}/L = 0.35$. These values of very soft PMLG (in HFP) are not consistent with the dipole moment per residue of 0.5 *e* Å as measured by Ueda.[25] Given the proof of the exciton theory in paper I, unless there are large uncertainties in YUKs spectral decompositions (five Gaussian components from 187 to 250 nm) or there are complications in the electric properties of polypeptide solutions that we are not aware of, we cannot explain their result.

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