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Structural Disorder of the CD3 ζ Transmembrane Domain Studied with 2D IR Spectroscopy and Molecular Dynamics Simulations

Prabuddha Mukherjee[†], Itamar Kass[‡], Isaiah T. Arkin[‡], and Martin T. Zanni^{*,†}

[†]Department of Chemistry, University of Wisconsin – Madison, Madison, Wisconsin 53706

[‡]The Alexander Silberman Institute of Life Sciences, Department of Biological Chemistry, The Hebrew University, Givat-Ram, Jerusalem 91904, Israel

Abstract

In a recently reported study [Mukherjee, et al. *Proc. Natl. Acad. Sci. U.S.A.* **2006**, *103*, 3528], we used 2D IR spectroscopy and 1-¹³C=18O isotope labeling to measure the vibrational dynamics of 11 amide I modes in the CD3 ζ transmembrane domain. We found that the homogeneous line widths and population relaxation times were all nearly identical, but that the amount of inhomogeneous broadening correlated with the position of the amide group inside the membrane. In this study, we use molecular dynamics simulations to investigate the structural and dynamical origins of these experimental observations. We use two models to convert the simulations to frequency trajectories from which the mean frequencies, standard deviations, frequency correlation functions, and 2D IR spectra are calculated. Model 1 correlates the hydrogen-bond length to the amide I frequency, whereas model 2 uses an ab initio-based electrostatic model. We find that the structural distributions of the peptidic groups and their environment are reflected in the vibrational dynamics of the amide I modes. Environmental forces from the water and lipid headgroups partially denature the helices, shifting the infrared frequencies and creating larger inhomogeneous distributions for residues near the ends. The least inhomogeneously broadened residues are those located in the middle of the membrane where environmental electrostatic forces are weakest and the helices are most ordered. Comparison of the simulations to experiment confirms that the amide I modes near the C-terminal are larger than at the N-terminal because of the asymmetric structure of the peptide bundle in the membrane. The comparison also reveals that residues at a kink in the α -helices have broader line widths than more helical parts of the peptide because the peptide backbone at the kink exhibits a larger amount of structural disorder. Taken together, the simulations and experiments reveal that infrared line shapes are sensitive probes of membrane protein structural and environmental heterogeneity.

Introduction

Many types of spectroscopies exist that can probe protein dynamics and their environments. Two important techniques are NMR and electron spin resonance (ESR) spectroscopies that rely on spin couplings and relaxation rates.^{1–3} In membrane systems, examples include ¹⁹F nuclear spins whose relaxation rates depend on oxygen concentration inside the membrane, and the electron spin labels whose relaxation rates scale with solvent exposure.^{2,4} Many years of hard work have gone into learning about the nuclear and electronic spin dynamics that make these techniques so useful. A much younger technique is two-dimensional infrared (2D IR) spectroscopy that is also now being used to study protein dynamics and environments.^{5–20} Like NMR and ESR, 2D IR spectroscopy can be used to probe structures through couplings

and line widths, although it is vibrational couplings and infrared line widths that are probed instead of spins. The structural sensitivity of vibrational spectroscopy is not as good as NMR, but it has the advantage that the couplings can be measured on a picosecond time scale²¹ and the line widths probe dynamics down to a few femtoseconds.^{11,15,22–26} Thus, structural motions measured with NMR and ESR will appear as a static distribution of states in a 2D IR spectrum, whereas femtosecond/picosecond motions such as those accompanying chemical reactions, hydrogen-bond breaking, and rapid structural changes will appear dynamic. Fast dynamics such as these might be important at the active site of enzymes^{27,28} or the transfer of protons in ion channels,²⁹ for instance. On the other hand, static inhomogeneity could be used as a diagnostic for measuring structural distributions or environments such as is done in the NMR and ESR measurements mentioned above. In a recent paper, we reported large spectral variations of the 2D IR line widths in the transmembrane domain of the CD3 ζ protein, similar in spirit to what might be observed in an ESR line width experiment. Yet unlike ESR (or NMR), the exact coupling mechanisms and environmental fluctuations that give rise to these observed infrared line widths are not well understood. In this paper, we use molecular dynamics to simulate the 2D IR observables and better understand how the vibrational dynamics scale with the protein structure and the membrane environment.

In two recent articles,^{11,12} we reported the vibrational dynamics of 11 residues along the transmembrane segment of the CD3 ζ protein measured using $1\text{-}^{13}\text{C}=^{18}\text{O}$ isotope labeling and 2D IR spectroscopy. The CD3 ζ peptide forms a tetrameric bundle in the membrane that is narrower on one end than the other because the helices are kinked at residue Leu-39.^{30,31} The bundle structure is shown in Figure 1a along with an illustration of a kinked helix in Figure 1b. We found in our previous work that both the structure of the peptide bundle and the nature of its environment were reflected in the amide I vibrational dynamics.^{11,12} The amide I mode is mostly created by the carbonyl stretch of the peptide backbone,³² and the experimental amide I line widths that we previously measured using 2D IR spectroscopy are shown in Figure 1c. One might expect that the vibrational dynamics of residues near the ends of the peptide would be different from those in the middle because the environment varies so drastically across the width of the membrane. In fact, the experimental 2D IR diagonal line width is 25% narrower for the amide I band of residues near the hydrophobic interior of the membrane than for residues near the surface. The funnel shape of the bundle structure appears as an asymmetric trend in the data with larger line widths at the C-terminal because the C-terminal end of the peptide more directly approaches the membrane surface. These variations in line width appear to be mostly caused by changes in inhomogeneous broadening, because the homogeneous lifetime and population relaxation times are nearly invariant with residue location in the membrane (not shown).¹² Thus, the line shapes must reflect the structural disorder of the peptide and/or its environment. One feature that was not satisfactorily accounted for in our previous study was the increased amount of inhomogeneity between residues Leu-39 and Val-44 near the center of the peptide. We suggested that this feature in the line width data was caused by a pocket of water molecules trapped inside the bundle.¹² This hypothesis is certainly feasible within the accuracy of the bundle structure because the structure was derived from data that lacked distance constraints between the helices.^{30,31} Further exploring the origin of this feature is one goal of this Article.

Shown in Figure 1d are the experimental center frequencies of 11 isotope labeled amide I bands taken from the data of previous publications that are compiled here for the first time.^{12,30,31} The average frequency is highest in the middle of the peptide and lowest at the ends. The data are also slightly asymmetric, with a $\sim 2\text{ cm}^{-1}$ lower frequency at the C-terminal than the N-terminal, although this difference lies within the error bars of the frequency measurements. Residues between Leu-39 and Val-44 have lower frequencies than do their neighbors, creating a feature similar to the trend in the inhomogeneous line widths. Although similar, the fact that the two measurements are not the same indicates that the frequency reports on different

structural and environmental details than do the line widths. The overriding aim of this study is to improve our understanding of the forces and dynamics that create the measured frequencies and line shapes.

To interpret the observed frequencies and line widths, a model is needed that links the vibrational dynamics to the peptide structure and its environment. Several models have been developed to relate vibrational frequencies to structure. The simplest model is a correlation between frequency and hydrogen-bond length. C–H and O–H bonds in crystals follow a nearly linear correlation over a wide range of hydrogen-bond lengths.^{33,34} A linear relationship between amide I frequency and hydrogen-bond length has been used to simulate infrared spectra, including 2D IR spectra.^{5,35–37} While simple, a hydrogen-bond model does not include frequency shifts that might be caused by environmental effects such as electrostatics. Even when hydrogen bonding is absent, environmental electric fields can shift vibrational frequencies. As a result, more sophisticated approaches that combine ab initio and molecular dynamics simulations are also being developed. From ab initio calculations of hundreds of small water/peptide clusters, researchers have correlated the electric field and potential of the cluster with the frequency of the peptide bond using the smallest peptide linkage *N*-methylacetamide.^{17,38–45} Using these correlations, a molecular dynamics trajectory can then be converted into a frequency trajectory from which the dynamic line width is calculated. Frequency correlation functions calculated in this manner reproduce photon echo experiments extremely well. These ab initio molecular dynamics methods even give good correlation functions for *N*-methylacetamide in relatively nonpolar solvents such as CDCl₃.⁴³

In the Article that follows, we examine our data from the perspective of these two models. First, we use a simple correlation between hydrogen-bond length and amide I frequency to convert a molecular dynamics simulation of the equilibrated membrane structure (Figure 1a) to frequency trajectories for each of the hydrogen-bonded amide I groups in the peptide and ultimately the 2D IR observables. We refer to this hydrogen-bond correlation as model 1. Second, we calculate the frequency trajectories again, this time using the ab initio derived correlation parameters between electric field and amide I frequency by Schmidt et al.,⁴² which we refer to as model 2. Both methods provide physical explanations for the trends observed in the experimental line widths, although neither adequately reproduces the experimental frequencies. Taken together, models 1 and 2 allow the frequency fluctuations to be related to the structural dynamics of the peptide backbone and environment. While neither model is quantitatively correct, it is clear that both electrostatic interactions of the environment and hydrogen-bond dynamics of the peptide itself contribute to the vibrational dephasing. Thus, the experiments are probing both the structural distribution of the protein and its environment.

Methods

The peptide structure used in this Article was developed by Arkin and co-workers using site-specific isotope labeling and attenuated total reflection Fourier transform infrared spectroscopy (ATR-FTIR).^{30,31} The transmembrane segment of the human CD3 ζ transmembrane domain spans residues Leu-31 to Leu-51 of the 163 residue protein. Using 1-¹³C=¹⁸O isotope labeling, Arkin and co-workers measured the infrared dichroism of 11 amide I bands along the 28–54 residue segment, which has the sequence DPKLG YLLDGILFIYGVILTALFLRVK.³⁰ In this study, we refer to the amide I modes according to the residue containing the carbonyl bond. The measurements were carried out on aligned bilayers of dimyristoylphosphocholine (DMPC), so that the dichroism is related to the tilt of the amide I transition dipole relative to the membrane normal. The tilts are consistent with a tetramer transmembrane helical bundle. The specific details of the resulting structure are given elsewhere,^{30,31} but the main feature of the model is that the helices are kinked at residue Leu-39, with the N-terminal tilted 18° to the membrane normal and the C-terminal portion at 9° (Figure 1b). The kink is the reason that the

bundle forms a funnel-like structure that is narrower at the C-end than the N-terminal end. The tetramer structural model was generated in vacuo using a truncated peptide from Leu-31 to Leu-51 with an acetyl N-terminal cap (called residue 30) and an NHCH₃ group for a C-terminal cap. To solvate the peptide bundle, the bundle was inserted into a hole that was created in a pre-equilibrated lipid bilayer consisting of 128 DMPC molecules and 3687 molecules of simple point charge (SPC) water (Berendsen et al.)⁴⁶ following the protocol of Faraldo-Gomez et al.⁴⁷ K⁺ counterions were introduced to neutralize the negative charges of the protein, which replaced water molecules corresponding to the lowest Coulombic energy of the ions. The resulting peptide/membrane system was equilibrated following the procedure below.

The molecular dynamics (MD) simulations reported here used the GROMACS MD simulation package (version 3.2.1).^{48,49} The extended version of the GROMOS87⁵⁰ force field parameters was used for the peptide and those of Berger et al.⁵¹ for the DMPC lipids. Hydrogen atoms along the hydrophobic chain of the lipid molecules were not included in the simulations; that is, a united atom method was adopted here. The bonds were constrained with the LINCS⁵² algorithm, and the temperature was set to 300 K. A Nose–Hoover^{53,54} temperature bath with a coupling constant of 3 ps was used to separately couple protein, lipid, and the water molecules. Anisotropic coupling to a Parinello–Rahman⁵⁵ pressure bath with a time constant 3 ps and pressure 1 bar was also employed. A 1 nm cutoff was used for van der Waals interactions. The electrostatic interactions were computed with the Particle Mesh Ewald (PME)⁵⁶ method with a 1 nm cutoff for direct space calculation. The time-step of the simulation was set to 0.5 fs. To equilibrate the protein with its membrane environment, the membrane–protein–water system was first energy minimized using the steepest descent method. It was then equilibrated in two structurally constrained stages and one unconstrained stage. The first stage consisted of a 0.5 ns MD run with positional restraints on the protein and lipid molecules allowing the water to equilibrate the system. In the second stage, another 0.5 ns of MD run was carried out with positional restraints on the protein only, while constraints for the membrane and water molecules were relaxed. After the final unconstrained equilibration for 7.5 ns, the system was subjected to a production run of 500 ps that is used to calculate the amide I frequencies. During the production run, the coordinates of the C, O, N, and H atoms for each amide bond were saved every 5 fs along with the sum of the electrostatic forces onto those atoms.

Two methods are presented below to convert the molecular dynamics simulations to frequencies of the amide I vibrators. In method 1, we use a slightly modified empirical correlation between frequency and hydrogen-bond length previously employed in simulations of 1D and 2D IR spectra.^{5,35,57} In this correlation, the hydrogen-bond length between residues n and $n+4$ is converted to frequency using:

$$\omega = a + b \bullet (2.6 \text{ \AA} - r_{\text{O-H}}) \quad (1)$$

where $r_{\text{O-H}}$ is the distance between the n th oxygen and $n+4$ th amide hydrogen. Previous studies set a and b equal to 1653 and -30 cm^{-1} , respectively, based on the gas- and condensed-phase frequencies of the amide I vibration in *N*-methylacetamide.³⁶ Equation 1 by itself underestimates the amide I line width. For this reason, previous simulations added 24 cm^{-1} of frequency disorder that is uncorrelated from the hydrogen-bond fluctuations of eq 1.⁵ In this paper, we follow a similar model with slightly modified parameters. We choose a and b as 1620 cm^{-1} and $-60 \text{ cm}^{-1}/\text{\AA}$, respectively. 12 cm^{-1} of additional uncorrelated broadening is included by decreasing the homogeneous dephasing time. Parameter a is lower than in previous reports to account for the decreased frequency of the isotope label. Parameter b is set so that the simulations span about the same range of line widths as the experiments and the additional dephasing is correspondingly decreased in magnitude (each scaled by a factor of 2). Furthermore, because we cannot calculate hydrogen-bond lengths between the peptide and

water, we used a cutoff value in eq 1 that sets the maximum frequency shift to be no larger than that created by a 7.5 Å peptide hydrogen bond. We also note that, because the last turn of the helix lacks peptide hydrogen bonds at the C-terminus, this method could only be used to calculate the frequencies for residues Ace-30 through Ala-48.

In model 2, the electrostatic forces on each of the C, O, N, and H groups that were saved during the production run were converted to an amide I frequency using the ab initio derived frequency correlation of Schmidt et al.⁴² Model 2 could be applied to all 22 residues of the simulated peptide. To investigate the individual contributions to the frequencies from lipid, water, and peptide, the frequencies were recalculated from the saved molecular dynamics simulation trajectories with the electrostatic forces of the appropriate components zeroed.

The experimental data presented in the Introduction of this paper come from previously published experiments.^{11,12,30} Sample preparation and experimental methods can be found in these references as well. To extract the amide I frequencies from the FTIR experiments, the spectra were not fit, but the maximum peak frequency was used. The frequencies extracted from the FTIR and 2DIR measurements were consistent. Multiple spectra were used to generate the error bars in Figure 1c and d.

Results and Discussion

In this section, we simulate the frequency averages, standard deviations, frequency correlation functions, and 2D IR spectra of the membrane peptides using two different models. In model 1, we correlate the hydrogen-bond lengths of the helical protein with the frequency of the amide I band using the empirical relationship explained in Methods. Using the same molecular dynamics run as for model 1, model 2 utilizes the ab initio derived correlation model of Schmidt et al.⁴² The ab initio method allows the individual contributions of the lipids, water, and protein to be computed individually. The simulation results are presented, followed by a discussion of the two models' limitations. Finally the simulations are compared to the experiment.

Model 1: Hydrogen-Bond Analysis

Averages and Standard Deviations—Shown in Figure 2a and b are the average frequencies and standard deviations of the 19 amide I bands in the peptide bundle calculated from structures saved every 5 fs during the 0.5 ps molecular dynamics trajectory as described in Methods. The frequencies of the 4 peptides in the bundle are averaged because the experiment does not discriminate between amide I modes on different protomers. As can be seen in Figure 2a, the amide I frequency is highest at the terminals and lowest near the middle. Because the frequency shifts in this model are proportional to the hydrogen-bond lengths, this trend reflects the fact that the CD3ζ peptides form tight α-helices near the center of the membrane and are less structured at the ends.

The standard deviations of the frequency fluctuations are shown in Figure 2b. This plot includes frequency fluctuations caused by hydrogen-bond structural disorder as well as the 12 cm⁻¹ of uncorrelated broadening.^{11,12} Because the uncorrelated broadening contributes equally to all of the amide I groups, the trend in the standard deviations is created solely by the hydrogen-bond dynamics (which contribute between 10.5 and 24.5 cm⁻¹ to Figure 2b) and reflects the structural disorder of the peptide backbone. The largest standard deviations appear at the end of the peptides, which correspond to backbone structural fluctuations of about 0.4 Å. The most ordered part of the peptide is in the center of the membrane (residue 42), exhibiting 0.17 Å structural fluctuations. There is also a significant amount of frequency disorder near the peptide kink around residue Leu-39. As discussed below, the experimental data suggest that the structural disorder at the kink is about one-half that of the N-terminal end.

Hydrogen-Bond Dynamics—The standard deviation of the frequency distribution measures the width of the frequency fluctuations about the mean, but it does not describe the time scale of the dynamics. Dynamics must be included in the analysis because dynamics will motionally narrow the line widths. Consider a frequency distribution with a standard deviation $(\langle \delta\omega^2 \rangle - \langle \delta\omega \rangle^2) = \Delta$ that decays on a time scale of τ as described by the correlation function $\langle \delta\omega(t) \delta\omega(0) \rangle = \Delta \cdot \exp(-t/\tau)$.⁵⁸ According to Kubo,⁵⁹ when $\Delta \cdot \tau \ll 1$ the system has fast frequency fluctuations that give a Lorentzian line width with a full-width-at-half-maximum (fwhm) of $\Delta^2 \cdot \tau$. In this limit, the system is motionally narrowed because the observed line width is narrower than the frequency distribution actually sampled. When $\Delta \cdot \tau \gg 1$, then the frequency fluctuations are slow and the system is inhomogeneously broadened. It is in the latter situation that the standard deviation is a good measure of the line width. In the analysis that follows, we use model 1 and the molecular dynamics trajectory to calculate the frequency correlation function for each amide I mode in the peptide. The correlation functions of the four equivalent peptides are then averaged.

Three representative frequency correlation functions are shown in Figure 2c for residues Leu-31 (dashed), Gly-37 (dot-dashed), and Thr-47 (solid). These three residues lie at both ends of the helix and near the middle of the peptide, sampling different degrees of backbone disorder. Furthermore, these three residues lie in very different regions of the membrane environment. Nonetheless, all three decay on about the same time scales within the first 300 fs, indicating that they have similar homogeneous lifetimes. However, their long-term dynamics differ. At 10 ps, these three residues reach different values because they have different distributions of hydrogen-bond lengths, with larger frequency (and thus structural) distributions at the ends. The offsets roughly correlate to the standard deviations in Figure 2b. The fast decays followed by nearly static offsets are indicative of Bloch dynamics and support the interpretation of the experimental data in terms of homogeneous and inhomogeneous widths.^{11,12}

The frequency correlation functions are related to the 2D IR line shapes through a Fourier transform given by:

$$f(\omega_1, \omega_3) = \frac{2}{\pi^2} \int_0^\infty dt_1 \int_0^\infty dt_3 e^{-i\omega_1(t_1+t_3)} \bullet (1 - e^{i\Delta t_3}) \bullet e^{-2g(t_1)-2g(t_3)+g(t_1+t_3)} e^{-(t_1+t_3)(1/2T_1+1/T_2)} \quad (2)$$

for $t_2 = 0$. In eq 2, T_1 is the experimentally measured population relaxation time (600 fs),^{11,12} and T_2 accounts for 12 cm⁻¹ of uncorrelated broadening described in Methods. Δ is the anharmonicicity of the amide I mode. $g(t)$ is the line shape function given by:

$$g(t) = \int_0^t dt_1 \int_0^t dt_2 \langle \delta\omega(t_2) \bullet \delta\omega(0) \rangle \quad (3)$$

Equation 3 arises when the cumulant expansion⁶⁰ is truncated to second order. The second-order cumulant approximation is valid for these simulations because the frequency distributions for the amide I modes are close to Gaussian. Shown in Figure 2d are the diagonal 2D IR line widths simulated using model 1 and eq 1–eq 3. The diagonal widths are the quantities experimentally found to be most sensitive to environment (Figure 1c) and are closely related to 1D absorptive line widths. As compared to the standard deviations, the hydrogen-bond dynamics narrow the 2D line widths to one-half the frequency range of the standard deviation (neglecting the 11 cm⁻¹ lifetime broadening included in the 2D line widths but not the standard deviations). Thus, the observed frequency distribution is only 50% of the frequency range actually sampled by the amide I oscillators. The N-terminal has slightly less motional narrowing than the other residues. This end is partially unfolded, and the amount of motional narrowing is sensitive to the cutoff used in model 1. We set a cutoff of 7.5 Å (see Methods)

because this distance best reproduces the experimental data, shown below. Notice that model 1 predicts larger line widths for the C-terminus than the N-terminus, that the narrowest line width is at residue Gly-43, and that there is a region of increased width in the center of the peptide near residue Leu-39 that is caused by the kink. These three features resemble the trends in the experimental data (Figure 1c). The comparison is discussed in more detail below. Also shown in Figure 2d (dashed) are line widths simulated from the distance between the n th residue oxygen and the $n+5$ th amide hydrogen (using modified parameters in eq 1). The similarity between these two sets of line widths demonstrates that it is primarily the structural fluctuations of the peptide backbone that causes the line widths, not changes in the hydrogen-bond angles.

Model 2: Electrostatic Field Analysis

Much work has recently gone into developing sophisticated models for correlating the amide I frequency to environmental electrostatic forces. Using coefficients obtained from ab initio calculations, the experimental frequency correlation functions of *N*-methylacetamide in water can be extremely well reproduced from molecular dynamics simulations using a projection of either the environmental electrostatic potential or the field onto the atoms of the amide I group.^{17,38–45} In model 2, we use the correlation coefficients of Schmidt et al.⁴² to convert the electrostatic fields experienced by the CD3 ζ amide I modes to frequencies. We then calculate average frequencies, standard deviations, frequency correlation functions, and 2D IR spectra, as was done above for model 1. According to model 2, the environment has a large influence on amide I modes. Near the surfaces of the membrane, water and lipid headgroups create large amounts of inhomogeneous broadening. In contrast, there is relatively little dephasing caused by interactions between peptides in the bundle, and most of the dephasing in the middle of the membrane is largely caused by fluctuations of the helices themselves.

Averages and Standard Deviations—Shown in Figure 3a are the average frequencies of the 22 amide I bands in the peptide bundle calculated from the same molecular dynamics trajectory that was used for model 1. The data are plotted as the frequency shift away from the gas-phase frequency of *N*-methylacetamide (1717 cm⁻¹). The oscillatory features in the frequency shifts are a result of the different electrostatic environment for residues on the inside of the peptide bundle as compared to the outside, which are especially pronounced near the ends where the residues are partly solvated.

To better understand the nature of the forces that contribute to the frequencies, the average frequency shifts created by the lipids, water, and peptide were each calculated separately according to the procedure outlined in Methods. Shown in Figure 3b are the frequency shifts created by each component individually. The lipids and peptide contribute the most to the frequency shift, while the water contributes relatively little. What is interesting about these plots is the strong anti-correlation between the components. Shown in Table 1 are the cross-correlation coefficients between the three components calculated from their frequency trajectories. The cross-correlation coefficients for the lipid–peptide and lipid–water are mostly anti-correlated, whereas the water and peptide forces are mostly uncorrelated. As a result, residues in the middle of the peptides experience forces from the lipids that would produce blue-shifted frequencies if it were not for counter balancing forces from the peptides and water molecules. The forces switch signs at the terminal ends. One consequence of these strong anti-correlations is that the amide I frequencies calculated from the total electrostatic forces lie within a much narrower frequency range than any one component would predict individually.

Shown in Figure 3c are the standard deviations of the amide I frequency fluctuations calculated from the total electrostatic forces that are the sum of all lipids, water, and peptides. The standard deviations are highest at the termini and minimal near the middle of the peptide. To gain a better understanding of this trend, we also decomposed the total standard deviation calculations

into lipid, water, and peptide components (Figure 3d). Because the peptide is a relatively uniform α -helix, the standard deviations caused by the peptide electrostatics are about the same for all residues, whereas the standard deviations created by the water and lipids are highest at the ends where the water and lipid headgroups are in close vicinity to the peptide. It is surprising that all three components contribute about equally to the standard deviations, especially near the ends of the peptides, considering that the frequency shifts created by water are so much smaller than the shifts caused by the lipids and peptides (Figure 3b). The reason that water has a larger effect on the standard deviations than the mean frequencies is because the water is very dynamic, creating frequency shifts that are about as likely to be positively or negatively signed. We noted in our previous publication that the line widths scale with water and lipid headgroup concentration and that the asymmetry of the peptide bundle creates asymmetric solvation.¹² Besides these observations, there are two other structural features apparent in these simulations. First, the peptide kink creates a large deviation in the peptide frequencies near residues 35. Although the kink itself begins at residue 39, it is the carbonyl group from residue 35 that is hydrogen bonded to 39 that exhibits the effects of the structural fluctuations. Second, the spike in the water standard deviation at residue Tyr-42 is caused by a single water molecule trapped between the α -helices in the peptide bundle. Molecular dynamics simulations without this water molecule lack this feature and predict a uniform standard deviation from residues Leu-39 to Ile-46. In many ways, the standard deviations of the three individual components are intuitive; the frequency distribution scales with the dynamical propensity of the peptide and its environment. However, we note that the standard deviation of the total electric field (Figure 3c) does not reflect the environmental trends as strongly as each of the components do separately (Figure 3d) because of the strong anti-correlations (Table 1).

Dynamics of Frequency Fluctuations—Shown in Figure 4a are plots of the normalized correlation functions for residues Leu-31 (dashed), Gly-37 (dash-dotted), and Thr-47 (solid). All three exhibit a fast decay in under 200 fs followed by a slowly decaying baseline, similar to the frequency correlation functions predicted by model 1. Once again, this bi-exponential behavior is indicative of Bloch dynamics with the fast decay causing motional narrowing, and the slowly decaying baseline gives an inhomogeneous distribution that is nearly static on the time scale of our experiment.

The frequency correlation functions were calculated individually for the lipids, water, and peptides and are shown in Figure 4b for residue Thr-47. Notice in Figure 4b that the three components exhibit different dynamics. The peptide correlation function is well-described by Bloch dynamics. In contrast, the water correlation function decays continuously over 10 ps caused by water rearrangement. The lipid dynamics lie in between. The offsets of the water and lipids at long times scale with the amount of water or number of headgroups near the amide I mode being simulated. One might expect that the water and, to a lesser extent, the lipid dynamics would create spectral diffusion, but because their dynamics are anti-correlated, the total correlation function more closely follows Bloch dynamics, as described above.

Shown in Figure 4c are the 2D IR diagonal line widths for each residue, calculated individually for each of the three components. The line widths were generated from the correlation functions using eq 2 and eq 3, following the same procedure as for model 1. The purpose of including Figure 4c is to show how the vibrational dynamics narrow the observed distribution of frequencies given by the standard deviations in Figure 3d. Motional narrowing is about equal for all residues, so that the line widths and standard deviations have similar trends (remember that population relaxation contributes 11 cm^{-1} to the line widths but is not included in the standard deviations). The peptide electrostatic fields experience more narrowing than do the lipids or water because of the fast decay in the peptide correlation function at short times (Figure 4a). Notice that the peptide kink and trapped water molecule still create substantial broadening even with the dynamics included.

Finally, the diagonal 2D line widths calculated from the total electrostatic fields are shown in Figure 4d. The vibrational dynamics reduce the observed frequency distribution to 25% of the standard deviations, a much larger narrowing than was observed with model 1. Furthermore, the line width distribution now assumes the asymmetric trend observed in the experimental data and model 1, with a larger line width predicted for the C-terminal than the N-terminal. In addition, the single trapped water molecule creates a significant amount of broadening at residue 42. This single water molecule has a disproportionate influence on the line width because it appears in a region of the peptide with a very weak electrostatic environment. It was this water molecule that gave rise to our previous hypothesis that a water pocket exists in the bundle.¹² Without trapped water, model 2 predicts no significant broadening for residues in the middle of the peptide.

Limitations of Models 1 and 2

Before comparing the simulations to experiment, we first address some of the limitations of the above models. Model 1 relies on a linear relationship between the hydrogen-bond length and the amide I frequency. The slope and intercept of this linear correlation were derived from the gas- and condensed-phase frequencies of *N*-methylacetamide.³⁶ While simple and often used to model 1D and 2D IR data, it alone is not sufficiently accurate to precisely reproduce the frequency fluctuations of *N*-methylacetamide. One reason might be that the carbonyl oxygen of peptides can donate two hydrogen bonds while the amide hydrogen can accept one.⁶¹ It would be difficult for a single correlation parameter to account for all three hydrogen bonds. It is also interesting to note that the intuitive correlation of model 1 does not seem to hold for the CD3 ζ membrane peptide. As was briefly mentioned in the Introduction and will be addressed again below, the ends of the CD3 ζ peptide have lower amide I frequencies than do the middle residues (Figure 1d). According to model 1, lower frequencies indicate tighter hydrogen bonds and thus a more structured α -helix. However, we expect from the molecular dynamics simulations that the ends are less structured. Hydrogen bonding to water does not resolve this discrepancy because few waters replace peptide hydrogen bonds according to the molecular dynamics simulations. It is more likely that model 1 fails to predict the frequency shifts because it neglects environmental forces other than hydrogen bonding. The environment surrounding the transmembrane domain of CD3 ζ varies between hydrophilic and extremely hydrophobic. Environmental forces from the second solvation shell around *N*-methylacetamide account for at least 30% of its frequency shift.⁴² This may indicate that model 1 also has limited applicability to large proteins, which often have hydrophobic interiors. Nonetheless, even though model 1 does not agree with the experimental frequencies, it does predict the correct trends in line width. As discussed in more detail below, we believe this indicates that the frequency fluctuations can be approximated with a linear response to the hydrogen-bond length, even if the absolute frequencies themselves cannot.

In contrast to model 1, model 2 can in principle account for both hydrogen bonding and electrostatic forces. The primary limitation of model 2 is that the correlation parameters by Schmidt et al.⁴² are derived for *N*-methylacetamide in D₂O, and we do not know if they accurately extrapolate to a membrane environment. Similar correlation parameters developed by Cho and co-workers for the electrostatic potential rather than the field work reasonably well for *N*-methylacetamide in DMSO-*d*₆ and CDCl₃,⁴³ even though they were developed for *N*-methylacetamide in D₂O. Neither the Cho nor the Schmidt parameters distinguish between peptide/peptide hydrogen bonds and peptide/water hydrogen bonds, which may have different covalent contributions.⁶² Even if small, the differences between peptide/peptide and peptide/water hydrogen bonds may be important in the middle of the membrane where peptide/water hydrogen bonds do not exist. In fact, it appears that model 2 overestimates the environmental contributions because the simulated frequency shifts are around 130 cm⁻¹ (and as large as 200 cm⁻¹) whereas the experimental values are only about 65 cm⁻¹. Developing correlation

coefficients explicitly for peptide/peptide interactions and coefficients for membranes versus polar solvents might improve the agreement. Nonetheless, even though the frequencies themselves are poorly predicted, we find that the line widths are much better simulated by model 2, as is discussed below.

Not included in either model 1 or model 2 is the effect that coupling to other protein modes might have on the line widths. The strongest coupling interaction between two amide I bands in proteins occurs between nearest neighbors and is less than 10 cm^{-1} .^{63,64} It is mostly this coupling that gives rise to the characteristic amide I frequency shifts of α -helices and β -sheets. The next closest band in frequency is the amide II band, which is coupled to the amide I band by about 27 cm^{-1} .⁶⁵ Other modes are much further away and less likely to contribute to the amide I band frequency. The $1\text{-}^{13}\text{C}=^{18}\text{O}$ isotope-labeled amide I bands lie about 60 cm^{-1} from their unlabeled amide I counterparts and about 40 cm^{-1} from the amide II band. Thus, if a structural change occurred that caused a coupling to change by 10 cm^{-1} , the $^{13}\text{C}=^{18}\text{O}$ band will shift by less than 2 cm^{-1} (estimated by diagonalizing a 2×2 Hamiltonian). A structural change large enough to cause a 10 cm^{-1} change in coupling is unlikely to occur in this system or in other systems with stable helices. One should also consider frequency shifts caused by coupling to helical modes that are not IR active. The frequency range of the amide I band covers roughly 15 cm^{-1} , so the separation may be smaller than estimated here, but even when considering IR dark bands, it seems unlikely that couplings cause larger than 1 cm^{-1} fluctuations.

Of course, both models are limited by the accuracy of the peptide structural model. The structural model relies on infrared-derived angular restraints of the amide I modes relative to the membrane normal.³⁰ The bundle geometry itself was developed using these restraints in a constrained molecular dynamics simulation. Because of the nature of the measurements, there are no experimental constraints on the separation between the peptides in the bundle or the depth of the bundle in the membrane.

Comparison to Experiment

The 2D IR experiments measure the amide I frequencies, homogeneous line widths, inhomogeneous widths, vibrational lifetimes, and spectral diffusion time scales. Of these quantities, the experimental inhomogeneous widths and vibrational frequencies (Figure 1c and d) are the most sensitive to the structure of the peptide bundle and the surrounding environment. Models 1 and 2 are attempts to quantify the link between structure, environment, and the vibrational dynamics. Except for the vibrational lifetimes that were included empirically, we have used models 1 and 2 to address each of the experimentally measured quantities. In this section, we call attention to four characteristic features in the experimental data and address their physical origins using models 1 and 2.

There are four distinct trends in the experimental data (Figure 1c and d). These are as follows: (1) The homogeneous line widths of most amide I modes are the same to within 1 cm^{-1} (previously reported but not shown).¹² (2) The experimental data are asymmetric, with the C-terminal end having lower frequencies and broader line widths than the N-terminal. (3) Residues in the middle of the peptide between Leu-39 and Val-44 have broader line widths and lower frequencies than their neighbors. (4) The least inhomogeneous line width appears at residue Ile-45.

The simulations provide physical insight into the origin of these four trends. (1) Both models 1 and 2 predict that the vibrational dynamics can be approximated with Bloch dynamics because the correlation functions decay on two clearly separate time scales (Figure 2c and Figure 4a). The fast time scales (with time constants of 200–300 fs) are similar for most residues, leading to motional narrowing that is about the same for most residues and agrees with the experimental

observation that the homogeneous line widths are nearly all identical. (2) Although neither model 1 nor model 2 predicts the correct frequencies (compare Figure 2a and Figures 3a to Figure 1d), both models correctly predict larger line widths at the C-terminal than the N-terminal, creating the asymmetric trend in the line width data. (3) Model 1 predicts that the peptide kink produces an increased amount of broadening in the middle of the peptide between residues 38 and 42. However, the kink does not create a substantial amount of broadening according to model 2. Instead, model 2 predicts very little inhomogeneous broadening in the middle of the peptide unless the peptides trap water. (4) Model 1 predicts that residue Gly-43 has the least inhomogeneous broadening, whereas model 2 predicts that a range of residues near the middle of the membrane should have little inhomogeneous broadening (residues 38–46).

Overall, the comparison of simulations to experiments reveals that there is a distinct link between peptide structure, environment, and the vibrational line widths (although the link between the vibrational frequencies and the two models is less clear, which is discussed below). On the femto-/picosecond time scale, only the water molecules exhibit significant amounts of structural dynamics, while the lipids and peptides are almost structurally static. Water dynamics create spectral diffusion, but these dynamics have a small effect on the line widths as compared to the concentration gradient of the water across the membrane and the heterogeneous structures of the headgroups and peptides. This small contribution of spectral diffusion is why the vibrational dynamics appear close to the Bloch limit. With this in mind, the spectral variations in the 2D IR line widths are better interpreted in terms of structural disorder rather than structural dynamics. The structural disorder of the peptides and their environment varies across the membrane with changing concentrations of water molecules, lipid headgroups, and hydrophobic lipid tails. The membrane headgroups and solvent partially unfold the ends of the peptides, causing large amounts of structural disorder that lead to broader line widths as do their stronger electrostatic fields from the headgroups and water. In comparison, the membrane interior provides a more homogeneous environment with less peptide disorder and weaker electric fields, thus giving narrower line widths. The structural and environmental variations are strong enough that the line width data are even sensitive to the asymmetry of the peptide bundle created by a 9° kink in the helices.

One point where the two models sharply disagree is on the origin of the broadening between residues 38 and 44 (feature 4). According to model 1, this feature is created by the peptide kink. At the kink, the peptide backbone bends, creating extended hydrogen bonds with larger amounts of structural disorder. Hence, the line widths broaden. According to model 2, the kink has little influence on the line widths. Instead, the amide I bands in the hydrophobic interior of the membrane should appear homogeneous unless there is water trapped in the bundle. In our earlier paper, we suggested that a water pocket might exist to explain this feature.¹² This suggestion was motivated because we found from our earlier simulation that a single water molecule can induce a significant amount of line width broadening according to model 2. Our work here confirms that prediction (Figure 4d). The Arkin structural model^{30,31} is not accurate enough to conclude whether such a water pocket exists. However, even if a water pocket were present, it does not appear that it could fully explain the experimental data. Water trapped inside the bundle will only create larger frequency fluctuations for residues on the inside of the helices, facing the bundle, and little broadening for residues on the outside of the bundle near the lipid tails. That is why only residue 42 exhibits increased line broadening in model 2. Thus, for model 2 to reproduce the experimental data, there would not only need to be water trapped inside the bundle but also water bound on the outside. The trapped water concentration would not need to be large, because a single water molecule in the middle of the peptide can create as much broadening as numerous waters at the headgroup region, but the water would have to permeate the bundle. Considering that nearly all of the CD3ζ residues are hydrophobic, it seems

unlikely that such a water distribution would be present. Instead, it appears more likely that feature 4 is created by structural fluctuations of the peptide kink as predicted by model 1.

It is also interesting that the molecular dynamics simulations predict that the most stable part of the peptide occurs in the exact middle of the membrane at residue Gly-43 (model 1). The peptide has the smallest structural perturbations in the middle of the membrane because the lipid chains cause much less structural fluctuations than does water or the headgroups. That is the reason that Gly-43 exhibits the narrowest line width in model 1 (Figure 2d). According to the 2D IR experiments, the residue with the narrowest measured line width is at Ile-45. If the structural fluctuations are really linked to the amide I line width as model 1 suggests, then the depth of the peptide bundle may need to be corrected in the CD3 ζ structural model. Better frequency models need to be developed and more rigorous simulations run before this hypothesis can be quantitatively tested. Yet it is entirely possible that the bundle depth is incorrect because there are no experimental constraints available to place the bundle in the membrane.^{30,31}

While the agreement between the simulated and experimental line widths is only qualitative, the simulations provide a physical basis for interpreting the experimental 2D IR line widths. We have confidence in the physical basis of the line widths because our simulations predict the same four features as observed experimentally. However, this agreement is in stark contrast to the experimental frequencies that are poorly reproduced by either model. We believe that the line widths are better predicted than the frequencies themselves, because the frequencies require that the absolute electric field be properly calculated or the hydrogen-bond lengths be perfectly correct, whereas the line widths only require the correct changes in electric field or hydrogen-bond length. In other words, the vibrational line widths follow a linear response with respect to the dynamics. Of course, the best model would be able to reproduce both the experimental line widths and the frequencies. Hopefully, this can be accomplished in the future with models explicitly designed to simulate the frequencies of membrane proteins.

Conclusion

We have found through experiment and simulations that the structural heterogeneity of the peptides and their environment is reflected in the vibrational dynamics of the amide I modes of the CD3 ζ transmembrane bundle. Environmental forces from the water and lipid headgroups partially denature the helices, resulting in shifted infrared frequencies and broadened line widths for residues near the membrane surface. Residues near the middle of the membrane have higher frequencies and more homogeneous line widths because the peptide backbones are less structurally disordered and environmental forces are smaller. The CD3 ζ peptides have kinked helices, which creates an asymmetric structure of the peptide bundle in the membrane. The bundle asymmetry is revealed by lower frequencies and larger infrared line widths for the C-terminal than the N-terminal. The kink itself appears as a region of increased line width because the peptide backbone is less structured. The amide I bands with the narrowest line widths are those lying precisely in the middle of the membrane.

This comparison of experiment and simulations helps reveal the microscopic origins of the amide I vibrational dynamics. Development of frequency models explicit for membrane peptides should lead to more quantitative interpretation of the data. Yet already from our results, it is clear that these modes are sensitive indicators of their local structure and environment. As we have done here, the vibrational dynamics of individual amide I modes can be probed with isotope labeling, which does not alter the peptide structure unlike ESR labels. Furthermore, the vibrational dynamics are sensitive to much faster time scales than is ESR or NMR. Interestingly, because only water molecules exhibit significant structural dynamics on the femto-/picosecond time scale, the 2D IR experiments are better interpreted in terms of structural

disorder rather than structural dynamics. As a result, linear-infrared spectroscopies such as FTIR may be sufficient for monitoring transmembrane peptide structural and environmental disorder because there is such a small contribution from spectral diffusion. Considering that the time-resolution of 1D and 2D IR spectroscopies is just a few picoseconds, in the future it may be possible to probe an ensemble of membrane proteins while they fold or a channel while it opens by monitoring the evolving vibrational dynamics.

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Figure 1.

(a) Structure of the CD3ζ transmembrane peptide bundle inside the bilayer membrane. Water and the lipid molecules are shown in red and gray, respectively. The tetrameric helical bundle is shown in green. This tetramer forms a funnel-like structure with the N-terminal residues (the top residues) approaching the membrane surface more gradually than the C-terminal (bottom). (b) Structure of a single helix. There is a kink in the peptides at Leu-39. (c) Experimentally measured 2D IR line widths of the 11 different 1-¹³C=18O isotopically labeled residues reported previously. (d) Infrared absorption frequencies of the 11 different isotopically labeled residues measured from FTIR and 2D IR experiments.

Figure 2.

(a) Average frequencies and (b) standard deviations simulated using model 1 and the 0.5 ns molecular dynamics simulation with an additional uncorrelated 12 cm^{-1} additional broadening. (c) Normalized frequency correlation functions of the residues Leu-31 (dashed), Gly-37 (dot-dashed), and Thr-47 (solid). (d) 2D IR line widths calculated from the $n/n + 4$ hydrogen-bond fluctuations (solid) and $n/n + 5$ (dashed) using eq 1 of model 1. Additional broadening due a frequency disorder of 12 cm^{-1} and population relaxation of 11 cm^{-1} were also added.

Figure 3.

(a) Mean frequency shifts calculated using model 2. (b) Contribution of lipid, water, and peptide to the average frequency shifts. (c) Standard deviation of the frequency fluctuations from the total electric field. (d) Standard deviation of the frequency fluctuations due to individual contributions of water, lipid, and peptide.

Figure 4.

(a) Frequency correlation functions of Leu-31 (dashed), Gly-37 (dot-dashed), and Thr-47 (solid) calculated using model 2. (b) Frequency correlation function calculated separately for water, lipid, and peptide electrostatic forces for Thr-47. (c) Contributions of the lipids, water, and peptide molecules to the total diagonal 2D IR line width of the residues. (d) Diagonal 2D IR line widths calculated from the frequency correlation functions of the residues using model 2. The line widths include 11 cm⁻¹ of population relaxation, as described in the text.

TABLE 1Joint Correlation Coefficients between Lipid Frequencies, Water Frequencies, and Peptide Frequencies^a

residue name	$R_{(\text{lipid-protein})}$	$R_{(\text{protein-water})}$	$R_{(\text{lipid-water})}$
Acc-30	-0.12	-0.02	-0.20
Leu-31	-0.21	-0.03	-0.30
Gly-32	-0.37	-0.01	-0.30
Tyr-33	-0.08	-0.15	-0.40
Leu-34	-0.49	-0.02	-0.20
Leu-35	-0.44	-0.51	0.01
Asp-36	-0.43	-0.21	-0.10
Gly-37	-0.40	-0.12	0.20
Ile-38	-0.14	0.01	-0.20
Leu-39	-0.52	-0.18	0.10
Phe-40	-0.08	-0.02	-0.30
Ile-41	-0.01	0.03	-0.30
Tyr-42	-0.17	-0.09	-0.20
Gly-43	0.02	-0.08	-0.50
Val-44	0.05	-0.12	-0.60
Ile-45	-0.07	-0.01	-0.60
Leu-46	0.00	-0.01	-0.50
Thr-47	0.12	-0.02	-0.50
Ala-48	0.01	-0.05	-0.60
Leu-49	-0.06	-0.02	-0.60
Fla-50	-0.31	0.05	-0.60
Leu-51	-0.54	0.18	-0.50

^a Given by $R_{a-b} = \langle \delta\omega_a \delta\omega_b \rangle / \sigma_a \sigma_b$, where σ_a and σ_b are the standard deviations of the frequency fluctuations for components a and b, respectively, and $\delta\omega_a$ and $\delta\omega_b$ are the frequency shifts contributed by components a and b, respectively.