Peptide conformational heterogeneity revealed from nonlinear vibrational spectroscopy and molecular-dynamics simulations

Sander Woutersen  
Max-Born-Institut für nichtlineare Optik und Kurzzeitspektroskopie, Max-Born-Strasse 2A, D-12489 Berlin, Germany and FOM-Institute for Atomic and Molecular Physics, Kruislaan 407, 1098 SJ Amsterdam, The Netherlands

Rolf Pfister  
Universität Zürich, Wintherthurerstr. 190, CH-8057 Zürich, Switzerland

Peter Hamm  
Max-Born-Institut für nichtlineare Optik und Kurzzeitspektroskopie, Max-Born-Strasse 2A, D-12489 Berlin, Germany and Universität Zürich, Wintherthurerstr. 190, CH-8057 Zürich, Switzerland

Yuguang Mu, Daniel S. Kosov, and Gerhard Stock  
Institut für Physikalische und Theoretische Chemie, J. W. Goethe Universität, Marie-Curie-Str. 11, D-60439 Frankfurt, Germany

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Nonlinear time-resolved vibrational spectroscopy is used to compare spectral broadening of the amide I band of the small peptide trialanine with that of N-methylacetamide, a commonly used model system for the peptide bond. In contrast to N-methylacetamide, the amide I band of trialanine is significantly inhomogeneously broadened. Employing classical molecular-dynamics simulations combined with density-functional-theory calculations, the origin of the spectral inhomogeneity is investigated. While both systems exhibit similar hydrogen-bonding dynamics, it is found that the conformational dynamics of trialanine causes a significant additional spectral broadening. In particular, transitions between the poly(Gly)II and the αR conformations are identified as the main source of the additional spectral inhomogeneity of trialanine. The experimental and computational results suggest that trialanine adopts essentially two conformations: poly(Gly)II (80%) and αR (20%). The potential of the joint experimental and computational approach to explore conformational dynamics of peptides is discussed. © 2002 American Institute of Physics.

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I. INTRODUCTION

The three-dimensional structure of proteins, which is encoded in the one-dimensional sequence of amino acids, is the key point that determines their functionality. Understanding the mechanism of the spontaneous folding of the protein from a one-dimensional chain (random coil) into a three-dimensional structure is one of the largest challenges of biophysical chemistry, known as the protein-folding problem. The major conformational degrees of freedom of the polypeptide chain are the highly flexible dihedral angles (φ, ψ) of the two σ bonds of each amino acid, while the dihedral angle ω of the peptide unit (-CO-NH-) is generally close to 180° because of the partial double-bond character of the C-N bond. A complex balance of forces results in two dominant free-energy minima within the two-dimensional (φ, ψ) configuration space, corresponding to the two most important secondary structure motifs: α-helices and β-sheets. The almost equal depth of these free-energy minima gives rise to the tremendous structural diversity we observe in proteins.

In order to understand protein folding properties, the free-energy potential surfaces of small peptides, which mimic building blocks of the actual polypeptide backbone, have been extensively studied using various levels of theoretical description, such as molecular-dynamics (MD) simulations1−9 and ab initio quantum-chemical calculations10−14. Owing to the shallow free-energy potential surface, one expects to obtain a distribution of conformations, since intramolecular hydrogen bonds that stabilize secondary structures are generally missing in these small systems. However, since a precise description of the conformational distribution would require an accuracy of better than k_B T = 2.5 kJ/mol, various theoretical methods may yield significantly different distributions.9

A new source of reliable experimental data on the conformations of peptide building blocks has become available only very recently with the advent of a novel spectroscopic tool, two-dimensional infrared (2D-IR) spectroscopy.15−23 While the physical background of this methodology is to some extent comparable with 2D-NMR techniques,24 the time scale of the measurement is completely different. NMR techniques intrinsically measure an average over the different conformational states, which for systems as small as those considered here exchange very rapidly on the NMR time scale (1−100 ms). The time scale of IR experiments (1...
ps), on the other hand, is sufficiently fast to resolve the dynamics in individual conformational states.\(^2\)\(^2\)

We have recently adopted 2D-IR spectroscopy to study the conformation of trialanine, a small peptide with two peptide bonds and one set of dihedral angles \((\phi, \psi)\).\(^1\)\(^7\)\(^9\) These experiments suggested that trialanine predominantly adopts only one conformation with \((\phi, \psi) = (-60^\circ, 140^\circ)\), known as poly(Gly)II \((P_{\text{II}})\) structure. This result has been confirmed somewhat later using a combined conventional IR and polarized Raman experiment.\(^5\)\(^5\) However, small contributions of other conformations, as they are predicted by MD simulations,\(^9\) could not be excluded.

In this paper, we compare the spectral inhomogeneity of trialanine with that of N-methylacetamide \((\ce{CH_3CONH-CH_3}, \text{NMA})\), a commonly used model compound consisting of a single peptide unit. Employing classical molecular-dynamics simulations combined with density-functional-theory (DFT) calculations, the origin of the spectral inhomogeneity is investigated. While both systems exhibit similar hydrogen-bonding dynamics, it is found that the conformational dynamics of trialanine gives rise to a significant additional spectral broadening. Moreover, strong evidence for an equilibrium between the \(P_{\text{II}}\) conformation and about 20\% of \(\alpha\)-conformation is found.

II. METHODS

A. Experiment

Our experimental setup has been described in detail elsewhere.\(^1\)\(^7\)\(^2\)\(^6\) We use the output of a commercial Ti:sapphire amplifier to pump a white-light seeded optical parametric amplifier based on BBO and difference-frequency generation of signal and idler in \(\text{AgGaS}_2\) to obtain midinfrared pulses, which at 1650 cm\(^{-1}\) have a duration of 100–150 fs and an energy and bandwidth of 1 \(\mu\)J and 200 cm\(^{-1}\), respectively. A small fraction of the midinfrared pulses is split off to obtain broadband probe and reference pulses. The remainder is passed through an infrared Fabry-Perot filter, resulting in pump pulses with a bandwidth of 10 cm\(^{-1}\), the center frequency of which is varied by adjusting the Fabry-Perot filter. The pump pulses have an intensity envelope that is approximately single-sided exponential with a decay constant of 700 fs. Transient absorption changes are measured by frequency-dispersed detection of the probe and reference pulses.

\(N\)-methyl acetamide (NMA) and trialanine, which was \(^{13}\)C labeled at the center position (Ala-Ala\(^5\)--Ala), were obtained from Sigma-Aldrich and Biosynthan GmbH, respectively, and were lyophilized from \(\text{D}_2\text{O}\) to deurate the NH groups. The deuteration ensures that the amide I mode is cleanly separated from other vibrational modes and that coupling between the amide I mode and the bending mode of water is negligible.\(^2\)\(^7\) All experiments are carried at room temperature on 0.15 mol solutions in \(\text{D}_2\text{O}\) \((p\text{D}=1)\), kept between two \(\text{CaF}_2\) windows separated by a 50-\(\mu\)m Teflon spacer.

The fits were performed using a nonlinear Levenberg-Marquardt algorithm.\(^2\)\(^9\) After normalization of the 2D spectra at different pump-probe delay times in order to eliminate the overall decrease of the signal due to population relaxation, all data points were weighted equally in the fit. This is done even though the experimental noise is getting larger at later delay times in order to be sensitive to spectral diffusion processes, which manifests themselves in particular at late delay times.

The \(^{1}\)H-NMR spectrum has been measured for Ala-Ala-Ala at \(\text{pH}=1\) in \(\text{H}_2\text{O}/\text{D}_2\text{O}=9:1\) solution at 600 MHz (Bruker DRX-6000). Assignment of the NH peaks was facilitated using a \(^{1}\)H-COSY and a \(^{13}\)C-HMBC spectrum. In agreement with previous results of Dorai and Griesinger,\(^2\)\(^9\) we find \(J = 5.27\) Hz for the coupling between the central \(C_\alpha\) and amide-proton and \(3J = 6.22\) Hz for that of the C-terminal group.

B. Theory

Employing classical MD simulations\(^8\)\(^9\) as well as DFT calculations,\(^1\)\(^0\) we have recently performed comprehensive theoretical investigations on trialanine. The MD simulations were performed with the GROMOS96 simulation program package employing the GROMOS force field 43A1.\(^3\)\(^0\) Trialanine was placed in a periodic truncated octahedral box of simple point charge (SPC) water.\(^3\)\(^1\) A minimum solute-towell distance of 1.5 nm was used, giving a total 1263 water molecules. The equation of motion was integrated by using a leapfrog algorithm with a time step of 2 fs. Covalent bond lengths were constrained by the procedure SHAKE\(^3\)\(^2\) with a relative geometric tolerance of 0.0001. A twin-range cutoff of 0.8/1.4 nm was used for the nonbonded interactions, and a reaction-field correction with permittivity \(\epsilon_{\text{RF}}=54\) was employed.\(^3\) The system was weakly coupled to a temperature bath at 300 K and a pressure bath at 1 atm.\(^3\)\(^3\) Following equilibration, a 20 ns simulation was performed and the coordinates were recorded every 0.1 ps for analysis. The MD simulations on NMA were performed in a completely analogous way, using 452 SPC water molecules and 5 ns simulation time.

All \(ab\) \(initio\) calculations were performed on neutral trialanine in the gas phase, using the DFT implementation of GAUSSIAN 98.\(^3\)\(^4\)\(^5\) Being mainly interested in normal-mode vibrational frequencies, we found the combination of the B3LYP functional with 6-31+G(d) basis set to provide good overall agreement with experimental data.\(^1\)\(^0\)\(^3\)\(^5\) To account for higher electronic correlations and vibrational anharmonicities, the amide I frequencies were scaled down by 0.96, which is the recommended scaling factor for B3LYP/6-31+G(d) level of theory.\(^3\)\(^6\) In separate calculations the effects of protonation, solution, and \(^{13}\)C isotope labeling were investigated.\(^1\)\(^0\) While all these modifications result in an overall shift of the vibrational frequencies, the frequency variation \(\delta\omega = \omega - \langle \omega \rangle\) approximately remains the same.

III. EXPERIMENTAL RESULTS

A. Line-shape function of NMA

The absorption spectrum of the amide I mode of N-deuterated NMA dissolved in \(\text{D}_2\text{O}\) is shown in Fig. 1(a). Figures 1(b)–1(d) show 2D plots of the transient absorption change as a function of the pump and probe frequencies, for
pump-probe delays of 1, 2, and 4 ps. These 2D plots are stacks of transient spectra, each horizontal cross section representing a transient absorption spectrum obtained by pumping at the frequency on the vertical axis. In the 2D spectra one observes the bleach and stimulated emission at a frequency which varies with the pump frequency. This implies that there exists a distribution of amide I frequencies and that the narrow-band pump pulse spectrally selects a specific subensemble (hole burning). The spectral hole is broader than the pump spectrum, which means that there is a large homogeneous contribution to the line broadening, as has been reported previously. With increasing delay, the contours become more vertically directed [Figs. 1(c) and 1(d)], i.e., the response of the system becomes less and less sensitive to the frequency at which it has been excited at \( t = 0 \). At a delay time of 4 ps, the contours have become completely vertically directed [Fig. 1(d)], which means that the observed absorption change has become independent of the pump frequency, and that the random fluctuations have completely washed out any memory about the initially selected subensemble. \textit{On the 4 ps time scale, the amide I band of NMA is homogeneous.}

To quantify this result, we determine the line-shape function of the amide I band of NMA. To this end, we use the Kubo picture of stochastic fluctuations of the transition frequency as a result of perturbations by a fluctuating surrounding (which can be inter- or intramolecular degrees of freedom). 49 In this approach, both linear and nonlinear spectroscopies are described in terms of a frequency fluctuation autocorrelation function \( \langle \delta \omega_0(t) \delta \omega_0(0) \rangle \), where \( \delta \omega_0(t) = \omega_0(t) - \langle \omega_0 \rangle \) is the instantaneous deviation of the \( v = 0 \to 1 \) amide I frequency from its average value. Employing time-dependent perturbation theory to first order and a cumulant expansion to second order, the linear absorption spectrum can then be written as

\[
I(\omega) = \text{Re} \int_0^\infty dt \ e^{-i(\omega - \omega_0)t} e^{-g(t)} e^{-t/2T_1},
\]

(1)

where the linewidth of the \( v = 0 \to 1 \) transition is determined by the line-shape function

\[
g(t) = \int_0^\infty d\tau^* \int_0^\infty d\tau \langle \delta \omega_0(t \tau^*) \delta \omega_0(0) \rangle,
\]

(2)

as well as by the vibrational energy relaxation time \( T_1 \).

Within the limits of the second-order cumulant expansion, the formulation is readily extended to describe nonlinear spectroscopy. 40–42 When computing the third-order response functions, the \( v = 2 \) state of the amide I mode is taken into account by assuming that fluctuations modulate the force constant of the amide I mode, which in a harmonic approximation implies \( \delta \omega_0(t) = \delta \omega_{12}(t) \). 41,42 \( T_1 \) vibrational energy relaxation is added to the response functions in a phenomenological way, taking into account that \( v = 2 \to 1 \) relaxation is twice as fast as \( v = 1 \to 0 \) relaxation. 41,42 Since vibrational energy relaxation contributes significantly to the total linewidth of the \( v = 0 \to 1 \) transition (\( T_1 = 1 \) ps, i.e., \( \delta \omega_{T_1} = 5 \text{ cm}^{-1} \)), the broader line shape of the \( v = 1 \to 2 \)

**FIG. 1.** (Color) Linear and nonlinear amide I response of NMA and the center amino acid of isotope labeled trialanine Ala-Ala\(^7\)-Ala. In the contour plots, blue colors indicate negative absorption change, red colors positive absorption change. The 2D spectra have been normalized to eliminate the overall decrease of the signal due to population relaxation. (a) Absorption spectrum of deuterated NMA in D\(_2\)O. (b)–(d) 2D Pump-probe spectra of deuterated NMA, showing the absorption change as a function of pump and probe frequency at delays of 1 ps (b), 2 ps (c), and 4 ps (d). (e) Fitted absorption spectrum and (f)–(h) 2D pump-probe spectra of NMA using the parameters given in Table I. (i) Absorption spectrum of deuterated Ala-Ala\(^7\)-Ala in D\(_2\)O and (j)–(l) 2D pump-probe spectra at delays of 1 ps (j), 2 ps (k), and 4 ps (l). (m)–(p) Fit spectra of trialanine using a Gaussian frequency distribution [Eq. (4)]. The fit parameters are given in Table I.
TABLE I. Spectral parameters for NMA and trialanine obtained from least-squares fitting.

<table>
<thead>
<tr>
<th></th>
<th>( T_2^* ) (ps)</th>
<th>( \tau_c ) (ps)</th>
<th>( \Delta_1 ) (cm(^{-1}))</th>
<th>( \Delta_2 ) (cm(^{-1}))</th>
<th>( (\omega_u - \omega_p) ) (cm(^{-1}))</th>
<th>( p_u )</th>
</tr>
</thead>
<tbody>
<tr>
<td>NMA</td>
<td>0.8</td>
<td>1.6</td>
<td>9</td>
<td>0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Trialanine(^a)</td>
<td>1.5</td>
<td>1.6</td>
<td>11</td>
<td>5</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Trialanine(^b)</td>
<td>1.5</td>
<td>1.6</td>
<td>11</td>
<td>-</td>
<td>14</td>
<td>20%</td>
</tr>
</tbody>
</table>

\(^a\)See fit spectra in Figs. 1(f)–1(h).
\(^b\)Fit according to Eqs. (1), (2), and (4), keeping \( \tau_c \) fixed to the value of NMA. See fit spectra in Figs. 1(m)–1(p).
\(^c\)Assuming a sum of two conformational contributions with frequency splitting \((\omega_u - \omega_p)\) and probability \( p_u \), keeping \( \tau_c \) fixed to the value of NMA. See fit spectra in Figs. 1(q)–1(t).

When simulating solution phase systems, one commonly finds that the frequency fluctuation correlation function decays on (at least) two times scales: \(^a\) (i) an ultrafast, inertial component on a 50–100 fs time scale and (ii) a slower, diffusion controlled component. In the case of vibrational transitions, the ultrafast component is typically in the normal moving limit; i.e., its correlation time \( \tau_c \) is much faster than the effective dephasing time \( T_2^* \). \(^b\) We, therefore, model the frequency fluctuation correlation function by

\[ (\delta \omega_{01}(t) \delta \omega_{01}(0)) = \frac{\delta(t)}{T_2^*} + \Delta_1^2 \exp(-t/\tau_c) + \Delta_2^2. \]  

(4)

The results of the fit obtained by using this approach are shown in Figs. 1(m)–1(p) and the parameters are listed in Table I. To discriminate conformational and hydrogen-bonding contributions to the line-shape function of trialanine, the same correlation time \( (\tau_c = 1.6 \text{ ps}) \) as obtained for NMA was used in the fit. While this is justified because of the similar hydrogen-bonding dynamics of both systems (see below), the strategy allows us to identify additional line broadening processes present only in trialanine. The procedure yields a quasistatic inhomogeneity \( \Delta_0 = 5 \text{ cm}^{-1} \) which accounts for the spectral difference between NMA and trialanine.

It is interesting to note that the one-dimensional amide I absorption spectra of NMA and trialanine [Figs. 1(a) and 1(i)] are essentially indistinguishable and have about the same line width. 2D-IR spectroscopy, however, unambiguously shows that the underlying dynamics is quite different. In order to identify the origin for the spectral inhomogeneity and its underlying dynamics, theoretical investigations are needed that provide detailed information on the interaction between solute and the solvent water molecules.

IV. COMPUTATIONAL RESULTS

A. Hydrogen bonding

Intermolecular hydrogen bonding of the peptide C=O group and the solvent water molecules causes a redshift of its amide I frequency of about 20–30 cm\(^{-1}\). Moreover, it is expected that a significant contribution to the total spectral width originates from the fluctuating hydrogen bond network between the peptide and its surrounding. To find out whether hydrogen-bonding represents the origin of the experimentally observed spectral difference of NMA and trialanine, the hydrogen-bond dynamics of the peptide C=O group of both systems have been analyzed from the MD simulations. To this end, we consider the normalized hydrogen-bond correlation function
The conformational dynamics of trialanine has been studied in detail in recent work. In short, the 20 ns trajectory of trialanine populates three regions in the Ramachandran plot, which are assigned to the \( \beta \) conformation \((\phi, \psi)\approx(-122^\circ, 130^\circ)\) with 42\%, the \( P_{II} \) conformation \((\phi, \psi)\approx(-67^\circ, 132^\circ)\) with 41\%, and the \( \alpha_R \) conformation \((\phi, \psi)\approx(-76^\circ, -44^\circ)\) with 16\%. The \( \beta \) and \( P_{II} \) peaks are separated by only 50° along the \( \phi \) axis with significant overlap between both peaks, giving rise to a high interconversion rate \((0.1 \text{ ps}^{-1})\) between both conformers. The \( \alpha_R \) peak is well separated from the \( \beta \) and \( P_{II} \) peaks and the transition rate between \( \alpha_R \) and extended conformers is predicted to be significantly slower (only 7 such transitions occurred during the 20 ns trajectory). It should be noted that a recent comparative study employing several force fields showed that there is some uncertainty in the location and the relative contribution of the different conformations. Qualitatively speaking, however, the results of the force fields GROMOS96 (Ref. 30), AMBER96 (Ref. 47), and OPLS96 (Ref. 48) were quite similar.

To calculate the dependence of the amide I frequency on the central \((\phi, \psi)\) dihedral angles of trialanine, normal-mode calculations were performed, following a restricted B3LYP/6-31+G(d) geometry optimization with fixed central dihedral angles \( \phi \) and \( \psi \). In this way, a map of the lower amide I frequency as a function of the dihedral angles \((\phi, \psi)\) was assembled. In order to cover the most populated areas in the Ramachandran space (i.e., the \( P_{II}, \beta, \alpha_R \) regions as well as their transition states), in total 180 conformers have been calculated. As a representative example, Fig. 3 shows the variation of the amide I frequency along the line which connects the \( P_{II} \) and \( \alpha_R \) conformation maxima (left panel) and along the line which goes through the \( P_{II} \) and \( \beta \) population maxima (right panel). As trialanine fluctuates in a specific conformer with the root-mean-squared deviations (RMSDs) of \( \Delta \phi, \Delta \psi \approx 25^\circ \), conformational fluctuations clearly contribute to the overall line shape of the vibrational transition. Even more, though, Fig. 3 reveals that possible transitions between the \( P_{II} \) and \( \alpha_R \) conformations would be a major source of the frequency modulation.

Combining the results of the MD simulations and the DFT calculations, the frequency fluctuation correlation function of the amide I frequency is readily calculated. In order to discriminate the effects of conformational fluctuations and conformational transitions, the correlation function has been calculated (i) for the complete MD trajectory (i.e., including conformational transitions) and (ii) for time intervals where the trajectory exclusively populates the \( P_{II} \) conformation (i.e., conformational fluctuations only). Both correlation

![Image](https://example.com/image1.png)

**FIG. 2.** Hydrogen-bonding correlation function [Eq. (5)] associated with the CO groups of NMA (solid line) and trialanine (dashed line) as obtained from the MD simulations.

**FIG. 3.** The lowest amide I frequency of trialanine computed at B3LYP/6-31+G(d) level of theory. The dependence of the amide I frequency on the \( \phi \) dihedral angle (with fixed \( \phi = -80^\circ \)) is plotted on the left panel and the dependence on the \( \phi \) dihedral angle (with fixed \( \psi = 140^\circ \)) is shown on the right panel. The arrows indicate the positions of the maxima of the conformational distribution function.

\[
\langle B(t)B(0) \rangle = \int_0^T B(\tau)B(t+\tau)d\tau / \int_0^T B^2(\tau)d\tau, \tag{5}
\]

where \( B(t) = 1 \) if there is an intermolecular hydrogen bond of the C=O group in study and \( B(t) = 0 \) otherwise. As criterion for a hydrogen bond, a maximum proton-acceptor distance of 2.5 Å and a minimum donor-proton-acceptor angle of 125° was required. A time interval of \( T = 5 \text{ ns} \) was used to achieve convergence. The correlation function decays rapidly; i.e., essentially all memory is lost beyond 4 ps. Hence, we conclude that the slow spectral diffusion component observed in trialanine but not in NMA cannot be associated with hydrogen-bond dynamics.

**B. Conformational dynamics**

In contrast to NMA, which contains only a single peptide unit, trialanine may exhibit conformational dynamics due to its highly flexible intramolecular degrees of freedom \((\phi, \psi)\) of the peptide bond. As the amide I frequencies of trialanine significantly vary with the central \((\phi, \psi)\) dihedral angles (see below), conformational fluctuations as well as transitions between various conformations are expected to considerably contribute to the overall frequency fluctuations of the transition. To quantify this contribution, the frequency correlation function \( \langle \delta \omega_{ij}(t)\delta \omega_{ij}(0) \rangle \) corresponding to the conformational dynamics has been calculated using a combined MD and quantum-chemical approach.

The conformational dynamics of trialanine has been studied in detail in recent work. In short, the 20 ns trajectory of trialanine populates three regions in the Ramachandran plot, which are assigned to the \( \beta \) conformation \((\phi, \psi)\approx(-122^\circ, 130^\circ)\) with 42\%, the \( P_{II} \) conformation \((\phi, \psi)\approx(-67^\circ, 132^\circ)\) with 41\%, and the \( \alpha_R \) conformation \((\phi, \psi)\approx(-76^\circ, -44^\circ)\) with 16\%. The \( \beta \) and \( P_{II} \) peaks are separated by only 50° along the \( \phi \) axis with significant overlap.
functions are shown in Fig. 4. They exhibit the same initial decay within the first 4 ps, after which the conformationally restricted correlation function has decayed completely. In striking contrast, the unrestricted correlation function exhibits a significant long-time tail, which accounts for conformational transitions that are slow on the time scale shown in Fig. 4. A closer analysis shows that the \( P_{\text{II}} \rightarrow \alpha \) transformation causes a shift of \( \approx 12 \text{ cm}^{-1} \), while fluctuations within the \( P_{\text{II}} \) conformation give rise to less than \( \approx 3 \text{ cm}^{-1} \) shift. A fit of the unrestricted correlation function with a functional form similar to Eq. (4) yields a quasistatic inhomogeneity of \( \Delta_0 = 3.8 \text{ cm}^{-1} \). This result is quite close to the experimental value of \( \Delta_0 = 5 \text{ cm}^{-1} \), thus indicating that conformational transitions are the main contribution of the observed spectral inhomogeneity.

V. DISCUSSION

The frequency fluctuation correlation function of the amide I mode reflects a superposition of various dynamical processes, which are difficult to separate. As intermolecular hydrogen bonding of solvent water molecules and the peptide C=O group is known to cause a notable redshift of the amide I frequency, we have first studied the hydrogen-bonding dynamics of NMA and trialanine. The calculated hydrogen-bond lifetime of NMA was found to be in the same order as the measured spectral diffusion correlation time \( \tau_c = 1.6 \text{ ps} \) and the corresponding amplitude \( \Delta_1 = 9 \text{ cm}^{-1} \) is in the expected range.\(^ {45,46} \) Hence, we conclude that the line-shape function of NMA is indeed dominated by hydrogen-bond dynamics. Since the MD simulations predict quite similar time scales of hydrogen bonding for both NMA and trialanine, however, hydrogen bonding cannot be responsible for their distinctly different spectral response, namely, the slow spectral diffusion process observed in trialanine.

The combined MD and quantum-chemical study suggests that this additional broadening process is related to a variation of the amide I frequency as a function of the highly flexible conformational \( (\phi, \psi) \) degrees of freedom of the peptide bond. The MD simulations using the GROMOS96 force field predict three main peaks of the conformational distribution, the \( P_{\text{II}}, \beta, \) and \( \alpha_R \) conformations.\(^ {8,9} \) Due to the well-known large uncertainty of the conformational population probabilities, however, these theoretical results still need to be validated by comparison to experimental data. With the \( ^3J \) coupling between the C\( \alpha \) and the N proton (5.27 Hz), we find \( \phi = -69^\circ \).\(^ {49} \) This angle is in favor of both the \( P_{\text{II}} \) and \( \alpha_R \) conformation, which have roughly the same \( \phi \) dihedral angle, but largely excludes the \( \beta \) conformation. On the other hand, our 2D-IR experiments\(^ {17,19} \) as well as IR-polarized-Raman studies\(^ {25} \) strongly favor the \( P_{\text{II}} \) conformation. Putting these results together with the experimental observation of the spectral inhomogeneity and the results of the MD simulations, we conclude that trialanine may adopt two conformations: predominantly the \( P_{\text{II}} \) conformation with probability \( (1 - p_a) \) and to a smaller extent, the \( \alpha \) conformation with probability \( p_a \). However, it should be noted that recent experiments by Schweitzer-Stenner suggest that the \( \beta \) conformation plays a role as well.\(^ {50} \)

In order to check whether our interpretation is consistent with the spectroscopic data, we have refined the fit of the trialanine 2D-IR spectra by summing up two independent contributions corresponding to the \( P_{\text{II}} \) and \( \alpha_R \) conformations, describing each by the line-shape function derived from the NMA data, and with center frequencies \( \omega_{\alpha_R} \) and \( \omega_{\alpha_R'} \) respectively. This approach goes beyond that of Eqs. (1), (2), and (4) since it may account for the non-Gaussian frequency distribution of two discrete conformations of the molecule. Using the frequency splitting and the \( \alpha \) probability as free parameters, the fit converged to \( \omega_{\alpha_R} = 14 \text{ cm}^{-1} \), in good agreement with the quantum chemistry calculation of Fig. 3, and to \( p_a = 20\% \) (see Table I). Figures 1(q)–1(t) show the resulting 2D-IR fit spectra which properly reproduce the asymmetry of the 2D-IR spectra. The quality of the fit (measured by the sum over the squares of the deviation between model function and data points \( x^2 \), which improves by ca. 10%) is notably better than that using the approach of Eqs. (1), (2), and (4).

It is worth noting that even though two discrete ensembles with different center frequencies have been assumed in the fit shown in Figs. 1(q)–1(t), the linear absorption spectrum in Fig. 1(q) does not at all separate into two peaks. This is because (i) the probability of the second component \( p_a \) is relatively small and (ii) because the intrinsic linewidth of each component is larger than the separation \( \omega_{\alpha_R} - \omega_{P_{\text{II}}} \) between both components. The presence of the second conformation manifests itself in a weak asymmetry of both the 2D spectra and the linear absorption spectrum towards higher frequencies (in agreement with the quantum chemistry calculation; see Fig. 3, left panel). However, it should be stressed that it is not only the difference between both fit models, which is indicative for the presence of two conformations in trialanine, but for the most part the different long-time responses of trialanine as compared to NMA. As the fast dynamics of both molecules is expected to be dominated by hydrogen bond breaking and formation, which according to the MD simulations occur on equal time scales in both cases, we can use NMA as a reference molecule to separate
of the contribution of conformational heterogeneity in the case of trialanine.

It is instructive to discuss this result from yet another, much simpler perspective. Consider the standard deviation of a two-point distribution function (corresponding to both conformers)

$$\Delta a = \sqrt{p_a(1-p_a)(\omega_a - \omega p_a)},$$

which is the quantity that has to be compared with the observed inhomogeneity. When using the result form the fit according to Eq. (4) for the inhomogeneous width $\Delta a = 5$ cm$^{-1}$ together with the frequency splitting estimated from the DFT calculation, $(\omega_a - \omega p_a) = 12$ cm$^{-1}$, we obtain $p_a = 25\%$ for the contribution of the $\alpha$ conformation, in good agreement with the number given above (see Table I). In particular, Eq. (6) indicates that the effects of conformational heterogeneity are expected to be much more pronounced in the nonlinear 2D-IR spectrum than in the linear absorption spectrum. Within in this simple model, this may be explained by the fact that for small $p_a$ the factor $\sqrt{p_a(1-p_a)}$ in Eq. (6) is much larger than $p_a$, while the contribution of the $\alpha$ conformer to the linear absorption spectrum scales only linearly with $p_a$.

The present work refines our previous structure determination,17,19 in which we have concluded that trialanine adopts mostly the $P_{II}$ conformation. However, since the contribution of the $\alpha_R$ conformation is small (20%), the experimental observables used for structure determination in Refs. 17 and 19 (i.e., the cross peak strength and anisotropy) are affected only negligibly. As shown in Fig. 9 of Ref. 19, if the fraction of the $\alpha_R$ conformation would have been larger, we certainly would have seen its contribution also in the cross peak region. Here, we analyze spectral inhomogeneity of the diagonal peaks, which according to Eq. (6) is particularly sensitive to small contributions of other conformations. However, it should be noted that we would not be able to assign the second component solely from the 2D-IR spectroscopy without support from other methods such as MD simulations and NMR spectroscopy.

VI. CONCLUSIONS

A combined experimental and computer simulation study has been presented that reports on the conformational distribution of trialanine, the two main degrees of freedom of which are the $(\phi, \psi)$ dihedral angles, which also determine the backbone conformation of proteins. Out of the three main conformational states in Ramachandran space, trialanine has been found to populate the $P_{II}$ conformation with about 80% and the $\alpha_R$ conformation with about 20%, whereas the $\beta$ conformation seems to play only a minor role. To our knowledge, the present study combined with Refs. 17, 19, and 25 provides the by-far most detailed experimental information to date on the conformational distribution of a small protein building block.

It has been demonstrated that a combination of 2D-IR spectroscopy and computer simulation can truly boost the microscopic understanding of condensed phase systems. First, 2D-IR spectroscopy provides dynamical observables that can readily be extracted from MD simulations. These data may therefore be used to validate the approximations underlying the theoretical description. In a second step, MD simulations may provide a microscopic means to unambiguously assign spectroscopic observations to molecular processes. For example, from the experiment alone we would not have been able to distinguish between the spectral broadening caused by hydrogen bonding and the spectral broadening caused by conformational transitions of the peptide. The intrinsic time scale of 2D-IR spectroscopy of a few picoseconds perfectly matches the time scale of solution phase dynamics and is also easily accessible to MD simulations. Hence, detailed insight into solution phase dynamics is expected to be obtained from this combined approach in the near future.

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The Liouville-space pathways with time-ordering “pump-probe-pump” give rise to coherent coupling. Here, the first two field interactions generate a spatial grating, which scatters pump light from the third field interaction into the direction of the probe light. Hence, the coherent coupling signal varies with pump frequency and causes a small tilt of the negative $v = 0 \rightarrow 1$ bleach and stimulated emission contribution, even when no inhomogeneity is present. This signal decays on a time scale given by the pump pulse duration and should not be misinterpreted as inhomogeneous broadening. However, model calculations show that this tilt is significantly smaller than that found in the data (Fig. 1) and, more importantly, that the tilt of the $v = 1 \rightarrow 2$ excited state absorption, which is not resonant with the pump light, is not at all affected by coherent coupling.