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Qualitative Analysis of Collective Mode Frequency Shifts in L-alanine using Terahertz Spectroscopy

Anita R. Taulbee^{*},

Department of Chemistry and Biochemistry, Miami University, Hughes Hall, Oxford, OH 45056

Justin A. Heuser,

Syngenta Biotechnology, Inc 3054 East Cornwallis Road, Research Triangle Park, NC 27709

Wolfgang U. Spindel, and

Department of Chemistry and Biochemistry, Miami University, Hughes Hall, Oxford, OH 45056

Gilbert E. Pacey

Department of Chemistry and Biochemistry, Miami University, Hughes Hall, Oxford, OH 45056

Abstract

We have observed collective mode frequency shifts in the phonon absorbance spectra of deuterium-substituted L-alanine isotopologues. Terahertz (THz) absorbance spectra were acquired at room temperature in the spectral range of 66–90 cm^{-1} , or 2.0–2.7 THz, for L-alanine (L-Ala) and four L-Ala compounds in which hydrogen atoms (atomic mass = 1 amu) were substituted with deuterium atoms (atomic mass = 2 amu): L-Ala-2-*d*, L-Ala-3,3,3-*d*₃, L-Ala-2,3,3,3-*d*₄ and L-Ala-*d*₇. The absorbance maxima of two L-Ala collective modes in this spectral range were recorded for multiple spectral measurements of each compound, and the magnitude of each collective mode frequency shift due to increased mass of these specific atoms was evaluated for statistical significance. Calculations were performed which predict the THz absorbance frequencies based on the estimated reduced mass of the modes. The shifts in absorbance maxima were correlated with the location(s) of the substituted deuterium atom(s) in the L-alanine molecule, and the atoms contributing to the absorbing delocalized mode in the crystal structure were deduced using statistics described herein. The statistical analyses presented also indicate that the precision of the method allows reproducible frequency shifts as small as 1 cm^{-1} , or 0.03 THz to be observed, and that these shifts are not random error in the measurement.

Keywords

Terahertz spectroscopy; L-alanine; Amino acid; Collective mode; Statistical Significance

1. Introduction

The region of the electromagnetic spectrum known as terahertz (1 THz = 10^{12} Hz) has gained increasing interest due to availability of new sources and detection systems. Progress in ultrafast detection schemes, combined with femtosecond (1 fs = 10^{-15} s) laser pumped nonlinear crystals, has brought the technique from specialized laboratories to realizing potential in mainstream applications. THz spectroscopy is a low-power technique, facilitating nondestructive analysis of fragile materials; the energy in a THz pulse is less than typical background radiation present in the environment. The spectral range covers both rotational

^{*}Author email: taulbear@muohio.edu, FAX: (513) 529-5715.

transitions from the microwave regime and vibrational modes from the infrared. This unique overlap of spectral features provides opportunities to analyze samples with completely new perspectives.¹

Terahertz spectroscopy has been shown in recent studies to contain frequencies resonant with delocalized collective modes of crystalline solids, specifically those composed of biomolecules of interest such as amino acids.^{2–8} These modes are attributed to low-frequency vibrations of the hydrogen-bonded crystal lattice, and naturally are also affected by the motion of the amino acid side chains, which contribute to torsion and deformities of the crystal lattice. To properly make spectral assignments in the THz regime, it is crucial to not simply stamp absorbance frequencies with the general term “collective modes,” but to also make every effort to determine which specific atoms in the unit cell are primarily responsible for which mode, when applicable. For example, the spectroscopic examination of tryptophan revealed two distinct collective mode frequencies in the THz region. Mode assignments determined that the carboxylic acid group was the primary contributor to the lower-frequency mode, whereas the rings of the tryptophan side chain produced the higher-frequency mode.⁴ THz absorbance frequencies of alanine (Ala) have also been calculated theoretically and observed experimentally^{5, 6}, but the experimental observations in the THz regime were limited to L-alanine and L-alanine-*d*₇, and the accompanying calculations which were used to confirm the theoretical assignments do not agree with the observed spectra of L-ala-2-*d*, L-ala-3,3,3-*d*₃, and L-ala-2,3,3,3-*d*₄ presented in this work. However, this author agrees that calculations similar to those previously reported may indeed be used to draw conclusions about spectral data without the use of more complicated modeling programs. Statistical methods are presented in this work to incorporate the data from the three aforementioned species in the context of those previously observed.

There is a challenge present in making spectral assignments in the THz regime for crystalline solids. Each absorbance band is produced by intermolecular collective modes, which in turn are affected by the motion of the individual molecules that comprise the crystal. Therefore, to properly conduct a controlled experiment to make spectral assignments in the THz regime, individual segments of the analyte molecule must be isolated, or marked, so that the effect(s) on the THz absorbance frequencies can be traced back to an identifiable source. The research of this author works toward this goal by examining THz absorbance spectra for the five isotopic species of L-alanine, in which varying combinations of hydrogen atoms were substituted with deuterium atoms. Because the crystal structure of L-alanine remains constant with or without the deuterium atoms, there is truly an isolated variable X, the atom(s) of greater mass, which may be concluded to have caused the shift in Y, the absorbance frequency of the mode. The effects on the THz frequencies absorbed are related to the number and placement of the high-mass deuterium atoms; i.e. each THz spectral feature is associated with the corresponding molecular and crystal structural feature(s) contributing to the mode. The association of intra- and intermolecular activity in small molecules like L-alanine with THz absorbance spectra will assist in building the THz spectral library. From this knowledge, trends in THz spectral assignment of simple molecules can be observed, and thus information on complex species containing similar building blocks can be deduced, bringing THz spectroscopy up to speed with its longer-standing predecessors.

2. Experimental Methods

Amino acids L-alanine (99%), L-alanine-2-*d* (98 atom % D), L-alanine-3,3,3-*d*₃ (99 atom % D), and L-alanine-2,3,3,3-*d*₄ (98 atom % D) were purchased from Sigma-Aldrich, L-alanine-*d*₇ from CDN Isotopes, and all compounds were examined without further purification. Differential scanning calorimetry (DSC) was performed on all compounds to confirm their crystallinity; all species were crystalline as purchased. Samples were prepared in triplicate, and for all species except rare compounds L-alanine -2,3,3,3-*d*₄ and L-alanine-*d*₇, additional three

sample sets were able to be prepared for a total number of six. The sample preparation and analysis methods have been previously described by Teraview Ltd. and Heuser.⁹ Briefly, the solid amino acids were weighed into 40 mg aliquots and mixed with 360 mg polyethylene (PE) powder. Using a mortar and pestle, the mixture was ground to reduce heterogeneity and decrease particle size to eliminate scattering of THz radiation and eliminate complications during compression. The 400 mg mixture was poured into a steel die and subjected to 2 metric tons of pressure for 60 seconds, and allowed to rest for 60 seconds before pressure was reapplied. The resulting 13 mm in diameter, 3 mm thick sample discs were extracted and sealed in plastic for at least 24 hours before analysis.

Spectroscopic measurements were performed in transmittance mode at room temperature using the TeraView TPS Spectra 2000, for which the experimental setup has been described previously in detail.¹⁰ Water vapor was purged from the enclosed sample chamber with N₂ gas at 10 L/min. After recording a reference using a 360 mg PE pellet, measurements of each sample were performed 5 times each in rapid scan mode. 3600 averages per scan were performed in the instrument at a rate of 30 scans/s with resolution of 32 gigahertz (1.2 cm⁻¹). A fast Fourier Transform (FFT) was applied to reference and sample waveforms, and absorbance spectra were obtained by dividing sample frequency response by that of the reference. The data were exported into graphing software (Microsoft Excel), and an average of all spectra for each sample aliquot was calculated. The reported species absorbance maxima are a collective average of all sample spectra of that species.

3. Results and Discussion

The precision of the method was tested by collecting multiple THz spectra of a single compound, and evaluating the differences in absorbance maxima between different sample pellets, and day of analysis of the same pellets. (Hydrogenated L-alanine was chosen for precision evaluation due to cost of deuterium-containing species.) No statistically significant differences were found between means of a given absorbance maximum for the same species due to sample preparation or day to day fluctuation of the instrument. A description of the statistical methods, which were applied to both same-species and inter-species evaluations for each absorbance maximum, is summarized here. Student's t-test was performed using the appropriate calculation for standard deviation for each data set. Differences between means were evaluated for statistical significance at the 5% level of significance ($\alpha=0.05$, sometimes referred to as the 95% level of confidence).^{11, 12}

Figure 1 shows the absorbance spectra of all five compounds from 66–90 cm⁻¹ (2.0–2.7 THz). The two absorbance frequencies observed for L-alanine containing only hydrogen atoms agree favorably with data previously reported.^{5, 6} The statistical summary is reported in Table 1; this includes the means and standard deviations for each of the two absorbance maxima of each species, noted as Peak 1 and Peak 2 respectively for the lower-frequency and higher-frequency maxima, along with the sample sizes for the data sets. The three means highlighted in bold in Table 1 were the only absorbance maxima found *not* to be significantly different from one another at the 5% level of significance. The bold values in Table 1 indicate that Peak 1 does *not* shift in proportion to the square root of reduced mass of the L-alanine molecule, as previously thought. Additionally, the fact that Peak 2 *does* shift significantly between the three species in question indicates that the side-chain atoms are contributing more to the higher-frequency mode than to the lower-frequency mode.

Terahertz absorbance frequencies were predicted using the method employed by Yamaguchi et Al. and are displayed in Table 2. The frequency of a vibrational mode is inversely proportional to the square root of the reduced mass of the mode, thus absorbance frequencies were calculated using the molecular weight of the compound as an estimate of the reduced

mass of the mode.⁶ The reduced mass and observed frequency of L-alanine, having been established previously in literature, were used for the calculated ratios of all other species. Agreement between experimental peak values and calculated predictions would indicate that the observed modes truly are vibrational modes that shift in proportion to the molecular weight of the analyte; however, upon examination of the values in Table 2, it is clear that the observed spectra do not conform to the model. A similar discrepancy was encountered when normal mode analysis was attempted using a prominent software program to predict the inelastic neutron scattering spectrum of crystalline L-alanine in previous studies.¹³ As in this study, certain calculated normal mode frequencies were “somewhat too high” compared to the observed frequencies. This discrepancy is especially apparent in the higher-frequency mode shown in Table 2, in which the observed absorbance peak is at a significantly lower frequency than the predicted value. Combined with the results in Table 1, this indicates that while both modes indeed incorporate many molecules in the crystal and cannot be attributed to any one atom or group thereof, certain atoms have a greater influence on one mode than another. The location of these atoms in the crystal structure of L-alanine may be correlated to the observed frequency shift to determine the primary origin of the mode.

As discussed in the introduction, the L-alanine side-chain is not part of the hydrogen bonded framework of the crystal. Upon examination of crystalline L-alanine shown in Figure 2, it is clear that a correlation exists between the atoms' locations in the crystal structure and the shifting THz absorbance maxima. Substitution of hydrogen atoms with deuterium bonded to C2 and C3 had minimal influence on the frequency of Peak 1, but caused significant changes in the frequency of Peak 2. Substitution of hydrogen atoms with deuterium on the C3 side chain had no significant influence on the absorbance frequency of Peak 1, but caused a significant shift in the frequency of Peak 2. Peaks 1 and 2 were also both influenced by the addition of a single deuterium atom on C2, which participates in a weak hydrogen bond with a neighboring oxygen atom. These spectral results indicate that while any change in atomic mass will certainly produce a slight effect across the entire crystal structure, Peak 2 is influenced more than Peak 1 by the amino acid side-chain. It has also been demonstrated that absorbance maximum differences of a single wave number, or 0.03 THz, can be found to be significantly different, and thus represent true spectral shifts that are not attributable to random error in the measurement. Therefore, calculated spectra which report frequencies with differences of 2–3 wave numbers from experimental values do not necessarily indicate “agreement” between experimental data and theoretical models. Finally, the applied statistics show that the experimental methods employed in this experiment are reproducible and capable of significantly differentiating absorbance frequency shifts as small as 0.03 THz.

4. Conclusions

By examining five analyte species which share a common molecular and crystal structure, and changing the mass of specific atoms within said structure, the two THz absorbance maxima of L-alanine in the spectral range 66–90 cm^{-1} have been correlated with the contributions of specific atoms to the delocalized collective modes of the crystal as a whole. A difference in THz absorbance frequency as small as one wave number, or 0.03 THz, has been found to be statistically significant at the 5% level of significance, sometimes referred to as the 95% confidence level, using the methods employed in this experiment. This information is important to making spectral assignments in the terahertz regime, as it demonstrates that frequency shifts of minimal magnitude can be differentiated using this analytical method, and greater standards of precision in collective mode spectral assignment can be met using terahertz spectroscopy.

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Figure 1.

THz absorbance spectra of each analyte from 66 – 90 cm^{-1} ; the y-axis values have been offset for clarity. Spectra displayed are averages of six sample spectra for **a)** L-alanine, **b)** L-alanine-2- d and **c)** L-alanine-3,3,3- d_3 ; averages of three sample spectra are displayed for rare compounds **d)** L-alanine-2,3,3,3- d_4 and L-alanine- d_7 .

Figure 2.
Crystal structure of L-alanine with hydrogen atoms shown in white; black lines indicate strong H-bonding, yellow lines indicate weak H-bonding (structure made using ChemBio3d Ultra and Microsoft Paint).

Table 1

Statistical Summary

Analyte	L-ala	L-ala-2-d	L-ala-3,3,3-d ₃	L-ala-2,3,3,3-d ₄	L-ala-d ₇
Peak 1 (Abs max, cm ⁻¹)	$x = 74.1_7$ $s = 00.0_6$ $n = 6$	$x = 72.9_7$ $s = 00.6_6$ $n = 6$	$x = 73.2_9$ $s = 00.1_5$ $n = 6$	$x = 72.8_6$ $s = 00.0_7$ $n = 3$	$x = 71.7_3$ $s = 00.1_5$ $n = 3$
Peak 2 (Abs max, cm ⁻¹)	$x = 85.6_{11}$ $s = 00.1_4$ $n = 6$	$x = 83.7_{11}$ $s = 00.5_6$ $n = 6$	$x = 81.5_3$ $s = 00.1_9$ $n = 6$	$x = 80.3_2$ $s = 00.0_7$ $n = 3$	$x = 79.4_6$ $s = 00.2_5$ $n = 3$

Table 2

Calculated vs. observed terahertz absorbance frequencies

Analyte	Red. mass est.	Peak 1 Calc.	Peak 1 Obs.	Peak 2 Calc.	Peak 2 Obs.
L-ala-2-d	90.0 ₈	73.7 ₆	72.9 ₇	85.1 ₃	83.7 ₁
L-ala-3,3,3-d ₃	92.0 ₆	72.9 ₆	73.2 ₉	84.2 ₂	81.5 ₃
L-ala-2,3,3,3-d ₄	93.0 ₆	72.5 ₇	72.8 ₆	83.7 ₆	80.3 ₂
L-ala-d ₇	96.0 ₃	71.4 ₃	71.7 ₃	82.4 ₆	79.4 ₆