

Structural Isotopic Effects in the Smallest Chiral Amino Acid: Observation of a Structural Phase Transition in Fully Deuterated Alanine

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A first study of possible changes instigated by deuteration in amino acids was carried out using neutron diffraction, inelastic neutron scattering, and Raman scattering in L-alanine, $C_2H_4(NH_2)COOH$. Careful analysis of the structural parameters shows that deuteration of L-alanine engenders significant geometric changes as a function of temperature, which can be directly related to the observation of new lattice vibration modes in the Raman spectra. The combination of the experimental data suggests that $C_2D_4(ND_2)COOD$ undergoes a structural phase transition (or a structural rearrangement) at about 170 K. Considering that this particular amino acid is a hydrogen-bonded system with short hydrogen bonds ($O\cdots H \sim 1.8 \text{ \AA}$), we evoke the Ubbelohde effect to conclude that substitution of hydrogen for deuterium gives rise to changes in the hydrogen-bonding interactions. The structural differences suggest distinct relative stabilities for the hydrogenous and deuterated L-alanine.

Introduction

Recently, much attention has been devoted to the study of amino acid crystals mainly due to the importance of understanding basic biological molecular systems as well as due to the possibility of applications in technological devices such as harmonic generators for lasers.^{1,2} L-Alanine ($C_2H_4(NH_2)COOH$), in particular, has been the most studied among the amino acids. Being the smallest chiral one, it is expected to play a key role in understanding several physical aspects of proteins and their functions.

The crystal structure of L-alanine has been determined by both X-ray^{3,4} and neutron diffraction.^{5,6} These studies agree that the system crystallizes in an orthorhombic symmetry in the $P2_12_12_1$ (D_2^4) space group, with four zwitterionic molecules ($NH_3^+-C_2H_4-CO_2^-$) in the unit cell joined together by a complex network of hydrogen bonds (HBs). Until now, many studies on physical properties of L-alanine crystal have been undertaken, pointing to very interesting properties. For instance, anharmonic interactions between molecules significantly affect the Raman spectral lines,⁷ dichroic behavior of the infrared bands,⁸ nonlinear behavior of modes at 42 and 49 cm^{-1} due to a dynamic localization of vibrational energy,⁹ and thermally activated dynamic orientational disorder below 20 K¹⁰. Birefringence and light depolarization measurements showed some subtle symmetry breaking around 220 K,¹¹ which is not consistent with previous X-ray^{3,4} and neutron^{5,6} single-crystal

diffraction measurements that revealed the low-temperature structure of L-alanine as being the same as at room temperature. A possible explanation for this discrepancy is the strong dynamic Jahn–Teller effect due to the NH_3^+ charge-lattice coupling, which can account for the lattice instability at 220 K and the discontinuity of the transmitted light depolarization at that temperature.¹² The instability of the hydrogen bond in L-alanine was further evidenced by high hydrostatic pressure ($P \sim 2.1 \text{ GPa}$) induced structural phase transition.¹³

Isotopic replacement of hydrogen (H) by deuterium (2H or D) or tritium (3H) is a common technique for the study of biomolecules using neutron scattering and high-resolution NMR. Usually, it is presumed that the molecular structure remains very similar regardless of whether the H-nuclei are substituted or the molecules are dissolved in water of varying H/D content. However, H/D isotope effects have been observed for the solid-state NMR chemical shifts of a series of HB complexes,^{14,15} in X-ray crystallographic studies of small molecules containing short HB,^{16,17} and most recently, in the small globular protein ubiquitin.¹⁸ This effect occurs in isotopic substitution in HB systems, simply because the strengths of the HBs are strongly influenced. The isotopic substitution can affect the tunneling rates, zero point energies, and motional properties of the molecule, particularly when the HBs are short ($O-H\cdots O$ distances, $r \leq 2.65 \text{ \AA}$). This effect is known as the Ubbelohde effect¹⁹ and has been observed in solids containing spiral HB, networks of cooperative HB extending throughout the crystal lattice, which is a very general structure, as well as an isolated pair of HB.

As amino acids play a central role both as building blocks of proteins and as intermediates in the metabolism, the comprehension of amino acid properties is certainly critical for the understanding of protein structure and biological activity. In

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this paper, we examine the Ubbelohde effect in L-alanine by describing our detailed neutron and Raman scattering studies as a function of temperature for fully deuterated L-alanine powder ($\text{ND}_3^+-\text{C}_2\text{D}_4-\text{CO}_2^-$, hereafter L-alanine- d_7). The temperature evolution of the Raman spectra shows evidence that a structural phase transition takes place at ~ 170 K. Such a phase transition is not observed in the nondeuterated sister crystal, L-alanine ($\text{NH}_3^+-\text{C}_2\text{H}_4-\text{CO}_2^-$, hereafter L-alanine- h_7). Furthermore, results obtained from neutron powder diffraction (NPD) and incoherent elastic neutron scattering measurements support the idea that the difference between the two forms of L-alanine can be interpreted as a consequence of different dimensions and geometries of the HBs that stabilize the crystalline structure.

Experimental Details

Samples of fully deuterated L-alanine ($\text{ND}_3^+-\text{C}_2\text{D}_4-\text{CO}_2^-$) 98% were obtained from Cambridge Isotope Laboratories and used without further treatment. $\text{ND}_3^+-\text{C}_2\text{H}_4-\text{CO}_2^-$ and $\text{NH}_3^+-\text{C}_2\text{D}_4-\text{CO}_2^-$ have been synthesized by Dr. Ehrenstorfer GmbH (Augsburg, Germany) with purity greater than 99%.

NPD experiments on L-alanine- d_7 powder samples were carried out between 300 and 2 K using the powder diffractometers E9 and D2b located at the Hahn-Meitner-Institut (HMI, Berlin, Germany) and at the Institut Laue-Langevin (ILL, Grenoble, France), respectively. These instruments are dedicated to the determination and refinement of crystal structures with very high resolution, allowing for the accurate determination of lattice constants and internal atomic coordinates. The powder diffraction data were analyzed using the GSAS suite of programs.²⁰ For all temperatures, the data were analyzed using the crystallographic model of Lehmann et al.⁵ All atomic position parameters were refined together with lattice constants and instrumental peak shape parameters. Special attention was paid to the modeling of the background, as thermal diffuse scattering contribution is significant. Isotropic atomic displacement parameters were used in the analysis of the data. Here, using the labeling scheme shown in Figure 2, $U(\text{D1})=U(\text{D2})=U(\text{D3})$, $U(\text{D5})=U(\text{D6})=U(\text{D7})$, $U(\text{O1})=U(\text{O2})$, $U(\text{C1})=U(\text{C2})=U(\text{C3})$. This simplification of the crystallographic model may lead to some systematic discrepancies compared to bond lengths and angles obtained from single-crystal neutron diffraction data, where anisotropic displacement parameters are used in the model.

Incoherent elastic neutron scattering measurements were performed on the time-of-flight spectrometer NEAT located at the HMI. The inelastic fixed-window (IFW) technique in the temperature range 100–300 K was used so that the neutron-scattered intensity is monitored within a narrow energy range ($\Delta E = 100 \mu\text{eV}$, $\Delta t \sim$ hundreds of ps) centered at $E = 0$ as a function of temperature and scattering vector Q ; only those neutrons that are within the elastic resolution (ΔE) are counted.²¹

In the Raman experiments, performed using the same L-alanine- d_7 powder samples, the backscattered light was analyzed using a Jobin Yvon Triplemate 64000 micro-Raman system equipped with an N_2 -cooled CCD detector coupled to a microscope Olympus BX40. The slits were set for a typical 2 cm^{-1} spectral resolution. Excitation ($\lambda = 514.5 \text{ nm}$) was supplied by an argon ion laser. Temperature control was provided by a helium flux cryostat that allows the stabilization of the temperature within 0.1 K. At each temperature, an appropriate thermal stabilization time was verified.

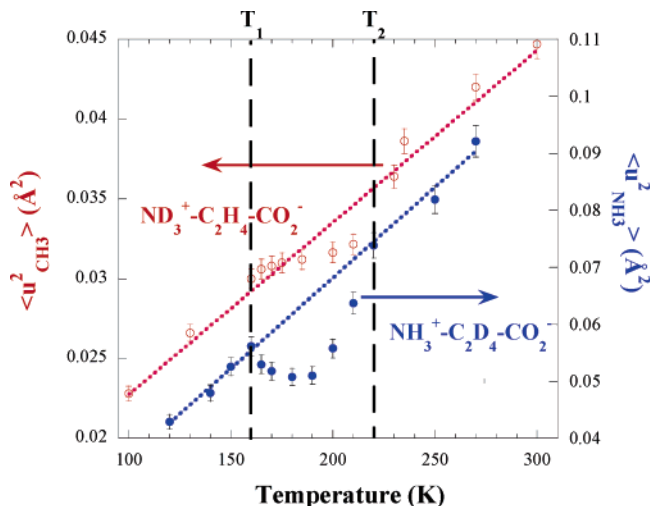


Figure 1. Evolution of the deduced mean-square dependence from the normalized elastic intensity measured on NEAT as a function of the temperature for $\text{ND}_3^+-\text{C}_2\text{H}_4-\text{CO}_2^-$ (left scale, open circles) and $\text{NH}_3^+-\text{C}_2\text{D}_4-\text{CO}_2^-$ (right scale, full circles).

Results

To shed new light on the modifications of the HBs in L-alanine, inelastic fixed-window (IFW) measurements were performed using selective deuteration to allow a differentiation between the dynamics of the different hydrogen groups. As the dependence of the elastic intensity as a function of temperature can be described as $I(T) = I(0) \exp(-Q^2 u(T)^2)$, where $u(T)$ is the mean-square displacement of the scattering nuclei, once the elastic intensity has been experimentally obtained, one can infer the variation of $u(T)^2$. Here, due to the large incoherent cross section of the H, $u(T)$ is assumed to arise solely from the motion of the H-atoms. Furthermore, if a dynamical transition takes place in the system within the time window probed ($\Delta t \sim$ hundreds of ps), one expects to observe a change in the slope of the observed $u(T)^2$.

Figure 1 shows $\langle u_H(T)^2 \rangle$ of $\text{ND}_3^+-\text{C}_2\text{H}_4-\text{CO}_2^-$ (left scale, open circles) and $\text{NH}_3^+-\text{C}_2\text{D}_4-\text{CO}_2^-$ (right scale, full circles). For both molecules, the steady increase of $\langle u_H^2 \rangle$ with increasing temperature originates from small-amplitude, solid-like contributions, and vibrational dynamics, consistent with harmonic behavior (no quasi-elastic broadening was observed outside the resolution of the spectrometer). However, near $T_1 = 160$ K and again near $T_2 = 220$ K, the data shows clear changes in the slope, suggestive of a phase transition (or structural rearrangement), most likely related to thermally activated large-amplitude reorientations of the NH_3 and CH_3 moieties. At higher temperatures, such reorientations will contribute more and more, as indicated by a 25% increase of the Raman line widths of the NH_3 and CH_3 torsion modes between 220 and 300 K.²² In spite of this, and simply because the motions occur on a much faster time scale than that accessible to the instrument (i.e., $\tau_{\text{reorientation}} \sim$ hundreds of ps), no further increase is observed in the mean-square displacement with temperature.²³ A similar behavior was already observed for $\text{NH}_3^+-\text{C}_2\text{D}_4-\text{CO}_2^-$ using NMR spectroscopy,²⁴ where the activation energies found above and below 168 K differ. Further evidence for a phase transition around 170 K was obtained by Lemanov and Popov using phonon spin echo.²⁵ An earlier Raman study⁷ of the intensity and line widths of the NH_3^+ torsional mode also showed a discontinuity at about 220 K. Further anomalies around 220 K are known from previous birefringence, light depolarization, infrared, and inelastic neutron scattering data.¹¹

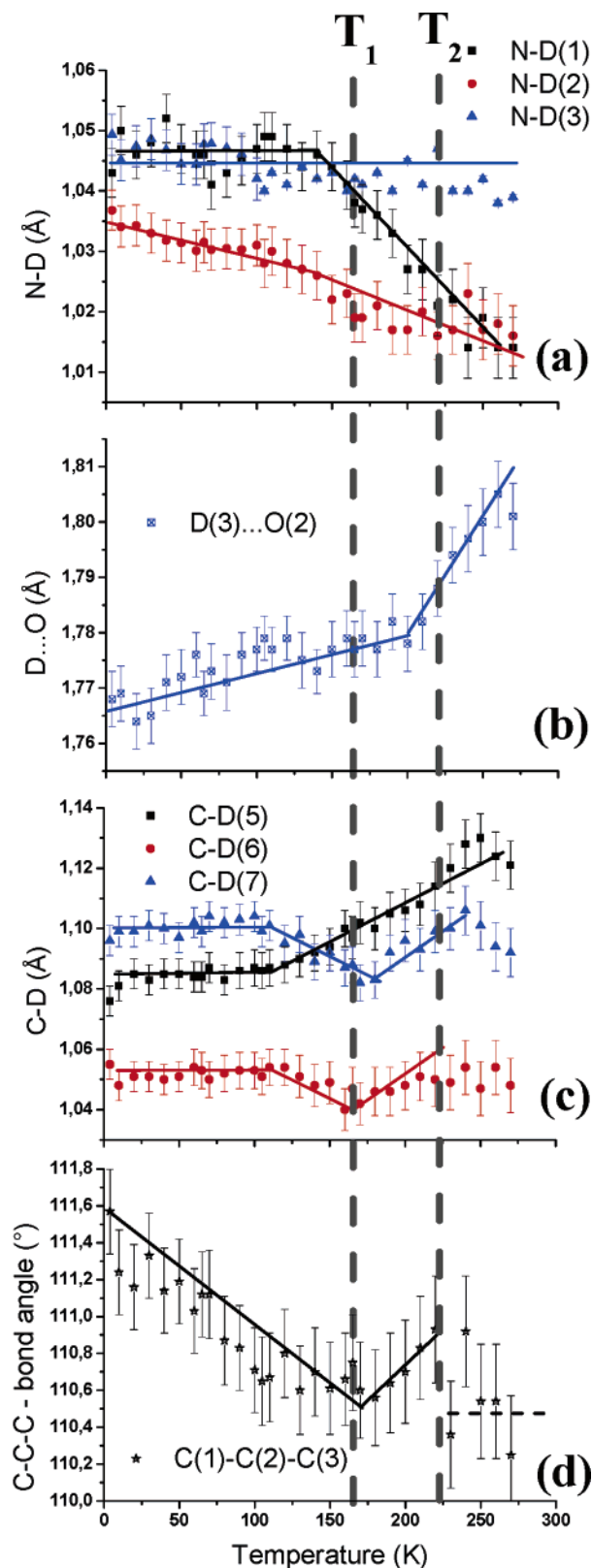


Figure 2. Temperature dependence of the N-D, D...O, and average C-D lengths and C-C-C bond angles in *L*-alanine-*d*₇ obtained from NPD data are shown in panels (a), (b), (c), and (d), respectively. The lines are meant as guides to the eye. The molecule is represented on the top left corner of the figure.

Selective deuteration, which allows us to focus on the dynamics of the different parts of the molecule, is indicative of possible changes in the static averaged structure of crystalline *L*-alanine. Therefore, a detailed structural study of the fully deuterated alanine (*L*-alanine-*d*₇) using NPD was carried out. The refined structure (lattice parameters, atom positions)

determined from the Rietveld analysis²⁰ of the NPD data of *L*-alanine-*d*₇ is in reasonable agreement with the previous determinations.³⁻⁶ However, as shown in Figure 2, a close inspection of the structural data reveals anomalies in the temperature dependence of the bond lengths N-D, D...O, and C-D as well as the C-C-C angle. For instance, as shown in

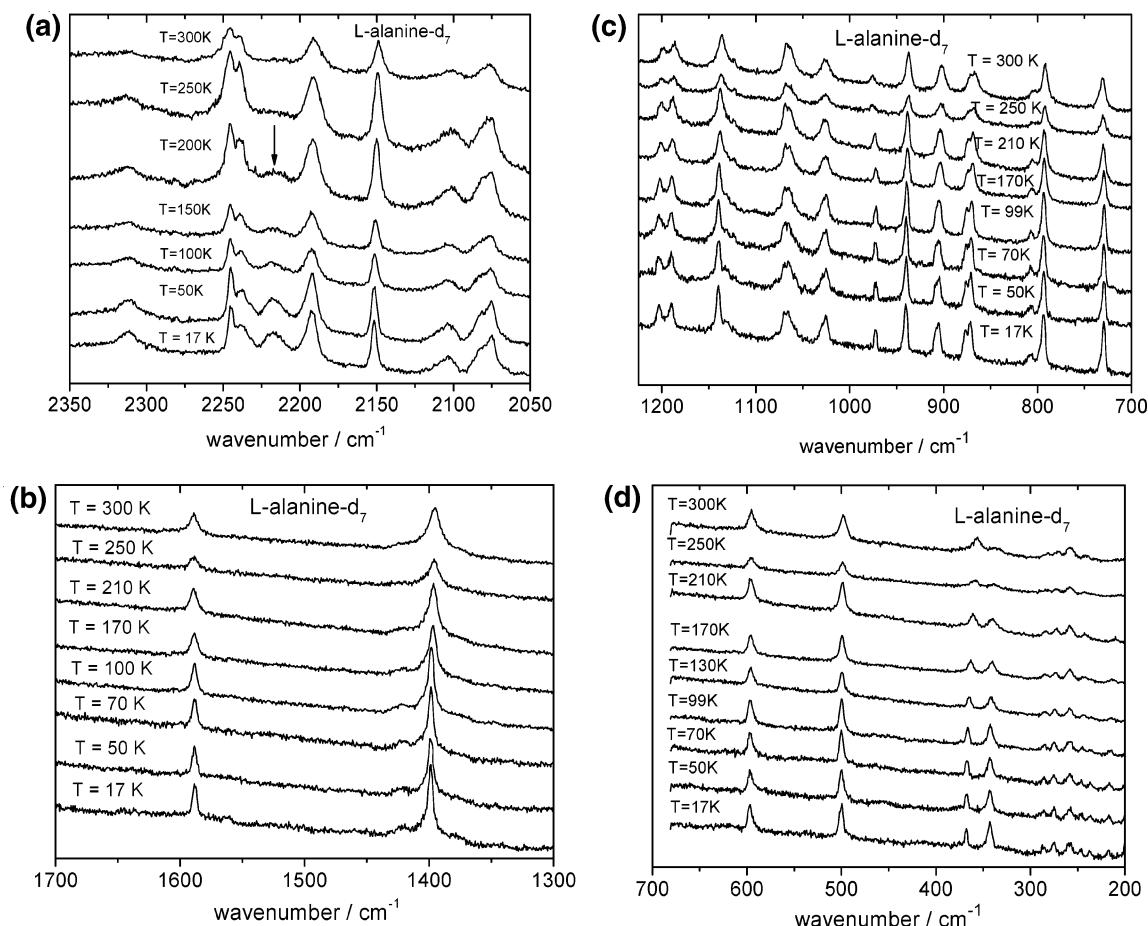


Figure 3. Raman spectra of L-alanine- d_7 for several temperatures between 17 and 300 K. For clarity, the spectra are divided in different regions as follows: (a) from 2050 to 2350 cm^{-1} , (b) from 1300 to 1700 cm^{-1} , (c) from 700 to 1300 cm^{-1} , and (d) from 200 to 700 cm^{-1} . A new band (marked by an arrow) appears in (a) around 2225 cm^{-1} below 200 K.

Figure 2a, below T_1 , the N–D covalent bonds are quite elongated when compared to a normal N–H covalent bond of about 1.01 Å.²⁶ In addition, between T_1 and T_2 , the three N–D bonds are quite different, explaining the splitting of the NH_3 torsional mode observed at 220 K.¹¹ Moreover, as depicted in Figure 2b, the pronounced increase of the HB ($\text{D}(3)\cdots\text{O}(2)$) that links the molecules into columns seems to confirm the breaking of the N–H \cdots O–H bond above 220 K.²² As these structural rearrangements significantly modify the torsion motion, these changes can be directly related to the anharmonicity of the NH_3 torsion mode ($\tau(\text{NH}_3)$). The changes in the C–D bond (Figure 2c) can be explained as resulting from the proximity of methyl groups of neighboring molecules.²⁷ In addition, as it takes less energy to bend a covalent bond than to stretch or shorten it, additional crowding strain in alanine is relieved by opening the C–C–C bond angle (see Figure 2d). Therefore, on the basis of our structural results, the stability of the structure of L-alanine- d_7 is changed between T_1 and T_2 , although results on L-alanine- h_7 ruled out the possibility of a conventional structural phase transition.^{6,12}

To obtain an independent verification of the observed phase transition, we investigated the Raman internal modes of L-alanine- d_7 in the different regions between 17 and 300 K. The results are shown in Figure 3. The assignment of the modes observed in L-alanine- d_7 , given in Table 1, is based in spectroscopic studies performed earlier on L-alanine- h_7 , partially deuterated L-alanine,^{11,27–30} and isoleucine ($\text{C}_6\text{H}_{13}\text{O}_2\text{N}$).³¹ Figure 3a shows seven different bands at room temperature. On cooling, these bands show an increase in the intensity and decrease of the line width. Note that the symmetric stretching vibration of

CD_3 , $\nu(\text{CD}_3)$, has an asymmetric shape consisting of two peaks. This doublet originates from a Fermi resonance.²⁸ The band observed at 300 K around 2102 cm^{-1} also presents an asymmetric profile, which is clearly observable for $T \leq 250$ K. It is also worth mentioning that, starting at about 200 K, a new band (marked by an arrow) begins to appear in the Raman spectrum. In Figure 3b, only two bands are observed between 1300 and 1700 cm^{-1} , while as shown in Figure 3c in the region 700–1250 cm^{-1} , several peaks are observed. The band at 732 cm^{-1} , which is not observed in the spectrum of L-alanine- h_7 is assigned to a rocking vibration of CD_3 , $\rho(\text{CD}_3)$. On cooling, this band becomes very narrow and shows a light asymmetry, possibly due to the increase of the baseline. All bands in the latter spectral region show a decrease of the line width on cooling, which allows a better determination of the number of bands in the low-temperature spectra. An interesting effect is observed in the region between 200 and 700 cm^{-1} (Figure 3d) with decreasing temperature: in contrast to $\delta(\text{NCC})$, which maintains approximately the same intensity, the $\tau(\text{ND}_3)$ band increases in intensity with decreasing temperature. At 170 K, the intensity of the two bands are similar, while at $T = 17$ K, the intensity of the $\tau(\text{ND}_3)$ band is about twice the intensity of $\delta(\text{NCC})$.

Figure 4 presents the Raman spectra of L-alanine- d_7 crystals for several temperatures in the spectral region between 25 and 200 cm^{-1} . This region is known to be very sensitive to a change of the crystal symmetry when an external parameter is modified.^{32,33} At room temperature, nine bands are observed and marked with letters A to I. The low-intensity bands A and B are associated to molecular librations of the L-alanine molecule.³⁴ For both bands, the intensity drastically diminishes on cooling.

TABLE 1: Vibrational Bands (cm^{-1}) and Approximate Assignments for L-alanine- d_7 ($\text{C}_2\text{D}_4(\text{ND}_2)\text{COOD}$)^a

assignment	$\text{C}_2\text{D}_4(\text{NH}_2)\text{COOH}$ and $\text{C}_2\text{H}_4(\text{ND}_2)\text{COOD}$ (cm^{-1}) ^{11,27–30}	$\text{C}_2\text{D}_4(\text{ND}_2)\text{COOD}$ (cm^{-1})
$\nu_a(\text{ND}_3)$	2293	2311
$\nu(\text{CD}_3)$	2248	2246
$\nu(\text{CD}_3)$	2240	2236
$\nu(\text{CD})$	2197	2190
$\nu_s(\text{ND}_3)$	2085	2149
$\nu(\text{CD}_3)$	2050	2078
$\nu_a(\text{COO})$	1591	1585
$\delta_s(\text{CD}_3)$	1355	1395
$\delta_a(\text{ND}_3)$	1189	1197
$\delta_a(\text{ND}_3)$	1182	1187
$\delta_s(\text{ND}_3)$	1148	1137
$\delta_s(\text{CD}_3)$	1053	1065
$\rho(\text{CD}_3)$	1025	1026
$\nu(\text{CCOO})$	917	937
$\delta'(\text{CD})$	900	902
$\nu_s(\text{CCN})$	871	868
$\nu_a(\text{CCN})$	871	868
$\rho(\text{ND}_3)$	775	792
$\rho(\text{CD}_3)$	747	732
		597
$\rho(\text{CO}_2)$	514	489
$\delta(\text{NCC})$	374	360
$\tau(\text{ND}_3)$	335	337
$\delta(\text{CCC})$	261	279
$\delta(\text{NCC})$	258	255
$\tau(\text{CO}_2)$ —band I	190	182
$\tau(\text{CD}_3)$ —band H	155	155

^a ν denotes stretching vibration, a and s denote symmetric and asymmetric modes, respectively, ρ denotes rocking vibration, δ denotes deformation modes, τ denotes torsion modes.

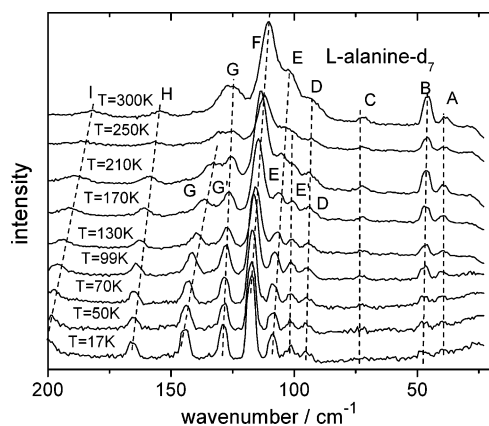


Figure 4. (a) Raman spectra of the external modes of L-alanine- d_7 crystals as a function of temperature. This region is known to be very sensitive to a change of crystal symmetry when an external parameter is modified.

Band C has a very low intensity and can be observed only down to 99 K. Band H, which exhibits a decreasing line width and increasing intensity on cooling, is associated to CD_3 torsion, $\tau(\text{CD}_3)$, according to ref 28. Band I, with a lower intensity, is assigned to torsion of the carboxylic unit, $\tau(\text{CO}_2)$. This band shows a large wavenumber change: at 300 K, $\nu = 182 \text{ cm}^{-1}$; at 17 K, $\nu = 200 \text{ cm}^{-1}$.

Now we turn to the discussion of the bands D, E, F, and G, located at 300 K between 80 and 130 cm^{-1} . The band F, which is the most intense in the Raman spectrum of L-alanine- d_7 in the region shown in Figure 4, is mixed with bands E and G at 300 K. On cooling, the bands become narrower, and it is possible to distinguish the bands in this region more clearly. Below $T \sim 210 \text{ K}$, band E starts to split. At 170 K, the bands

E and E' are clearly distinct, and at even lower temperatures, bands E, E', and D are completely separated. Band G presents a behavior similar to that of band E, splitting in G and G' at 170 K, with G' shifting to lower energies with decreasing temperature. The changes in band localization are indicated in Figure 4.

The gradual splitting of bands E and G in the low wavenumber region provides additional evidence that the symmetry of the unit cell changes with temperature, as already inferred from the structural data. The Raman results confirm that L-alanine- d_7 undergoes a structural phase transition at low temperature.

Conclusion

Anomalies observed in the Debye–Waller factor and in the structural parameters in addition to the evolution of the Raman spectra as a function of temperature show clear evidence of a structural phase transition around 170 K in L-alanine- d_7 . Furthermore, distortions of the NH_3^+ group below 220 K are confirmed.¹² While the evidence of a structural phase transition for the D-enantiomer,³⁵ D-alanine- h_7 , at $\sim 270 \text{ K}$ was refuted,³⁶ it is generally accepted that the L-enantiomer, L-alanine- h_7 , does not undergo a structural change at low temperatures.^{6,12,36} Considering that alanine is the simplest amino acid with respect to molecular structure as well as the most widely used in protein construction, the structural rearrangement observed in L-alanine- d_7 is interesting by both physical and biological points of view.

Although our observations differ from what was found for the nondeuterated sister crystal,^{6,36} L-alanine- h_7 , these differences can be interpreted as consequence of different dimensions and geometries of the HBs that stabilize the crystalline structure. Because of the Ubbelohde effect, the HBs are changed, that is, upon H/D double substitution, the hydrogen bonds are weakened and one expects the respective N–N distances to increase.³⁷ Other striking examples of the Ubbelohde effect are significant modifications in the physical properties of different materials such as changes in the transition temperature,^{38,39} structural isotopic effects in water bilayers absorbed on $\text{Ru}(001)$,⁴⁰ isotope effects on proton-transfer reaction,⁴¹ and deuteration-induced structural phase transitions in HB crystals.⁴² Therefore, our work draws attention to another very important issue: the small size of Ubbelohde effects should not mislead to underestimate its energetic importance. On the basis of an ultrahigh-resolution structure of crambin,⁴³ the electrostatic energy of the HBs can be estimated to be about -938 cal/mol . From our data, we observe that the HB deuteration in L-alanine leads to an average decrease by 0.03 \AA in the NH distance and, due to structural constraints, a similar increase in NO_2 distance between 170 and 300 K. Thus, the electrostatic attraction is reduced by 2.3%, which corresponds to a destabilization of the HB by 22 cal/mol . As the free-energy difference between folded and unfolded states of proteins is small, e.g., $3\text{--}4 \text{ kcal/mol}$ as observed for CspA and RNaseH,⁴⁴ deuteration of all HB in a protein could cause considerable differences in the relative stability of folded and unfolded states. Deuteration of hydrocarbons, for example, results in a more polar, or less lipophilic, molecule, and this effect becomes more pronounced as the content of deuterium in the molecule increases. It was in fact observed^{45–47} that protonated compounds bind more strongly to nonpolar moieties than deuterated ones, thus pointing out that the use of deuterated solutes can have influence on the dynamics of the system.

As a final point, it is worth keeping in mind that the sensitiveness of HB in amino acids to isotope replacement, changes of lattice spacing due to thermal expansion,^{12,48} and to

external compression^{49,50} is perhaps related to the origin of homochirality.^{51–53} Along these lines, a systematic study of the 20 α -amino acids in both L- and D- forms is urgently needed.

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