Raman study and DFT calculations of amino acids

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ABSTRACT

Intense Raman signals were observed of nano gram quantities of three amino acids (histidine, valine, glycine) using visible laser excitation (488nm) at relatively low laser power (5mW) and short acquisition time (2min). Considerable variations in the Raman signal were observed when changing the pH of the amino acid solution. Scanning electron microscopy reveals considerable differences in the crystallite structure of histidine, valine and glycine and for different pH. Narrow vibrational bands are correlated with formation of crystallites. H/D substitution and pH dependence experiments were used to identify vibrational bands associated with the functional groups able to exchange protons. The observed spectral bands are directly compared with density functional calculations to assign the vibrational bands in histidine, valine and glycine. The effect of hydration is studied at neutral pH in the theoretical calculations.

Keywords: Raman spectroscopy, amino acids, density functional calculations, scanning electron microscopy, micro-crystals

1 INTRODUCTION

Raman spectroscopy is a powerful non-invasive tool to obtain information on structure, function and reactivity of biological molecules such as proteins. [1] The vibrational spectra of amino acids in peptides and proteins depend sensitively on organization and interaction with its environment and give hence important information on conformation and function. Amino acids are the basic building blocs of proteins and have been studied by Raman spectroscopy and theoretical simulations have been used to assign the observed spectra. [2-4] The native 20 amino acids have no chromophores in the visible spectral range, no electronic resonances can be used to enhance the Raman signal in the visible spectral range and relatively large samples volumes (powders, crystals) or concentrations are necessary to study amino acids. To take advantage of electronic resonances of chromophores of amino acids such aromatic residue side chains and peptide bonds one can use UV Raman spectroscopy. UV Raman has been increasingly applied to study proteins and their secondary structure. [5,6]

We have recently observed an enhancement of the Raman signal of the amino acid histidine using visible excitation at low laser power (<5mW). This was achieved by drying droplets of mM solutions of histidine on SiO_2/Si surfaces. [7] The drying process formed spontaneously needle shaped micro crystals (diameter 80nm, several micrometers long). We have applied the same method to other amino acids and investigated the influence of pH and H/D substitution on the vibrational spectra. Scanning electron microscopy of the micro crystals is used to clarify if the shape of the crystals influences the observed enhancement. A strong influence of the pH of the solution on the vibrational spectra is found. Amino group were studied by proton/deuterium substitution experiments. We find that intense Raman signals can also be observed for amino acids such as valine and glycine. We compare experimental Raman spectra to *ab-initio* calculations and propose an assignment of the observed vibrational Raman bands.

2 EXPERIMENTAL

Histidine, glycine and valine (Sigma Aldrich) in their zwitterionic form (($^{+}H_3NCHRCOO^{-}$) were first dissolved in 1ml of de-ionized water (or D_2O) at a concentration of 30mM and then single droplets (15µl) were deposited on SiO_2/Si plates. The droplet was dried on a heating plate at 80°C. For the liquid or powder sample spectra, a 15µl droplet or 0.3-0.4 mg respectively was deposited on a glass plate (estimated thickness 50 micrometers).

Raman spectra were recorded (Dilor XY) using 488nm excitation. The incident laser beam was focused on the sample through a microscope with an x100 objective. The resulting spot size is less than 1µm^2. The power on the sample has been 4mW.

3 THEORETICAL CALULATIONS

All calculations were performed using state of the art density functional theory (Gaussian-03 Package). [8] The considered geometries and vibrational frequency were calculated within the Becke's three parameters exchange hybrid functional B3LYP associated with the Generalized Gradient Approximation (GGA) of Lee Yang and Parr. [9,10] The electronic wave functions were described by the 6-311++G** basis set. The polarization and diffuse functions are crucial to the treatment of both electrostatic interactions and hydrogen bonds. [3] This approach has proven to be efficient in describing structural, electronic

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and vibrational properties of many molecular systems such involving amino acids.

4 RESULTS AND DISCUSSION

4.1 Raman intensity and scanning electron microscopy

Glycine is the simplest amino acid and valine has a hydrophobic side chain in contrast to the more hydrophilic side chain in histidine. No Raman signal was observed from a 30mM solution of glycine using the 488nm excitation line at 4mW. But after drying a single droplet of solution we recorded intense Raman signals (Figure 1) which are similar to what is observed for spectra recorded from powder but corresponding to a much larger quantity.

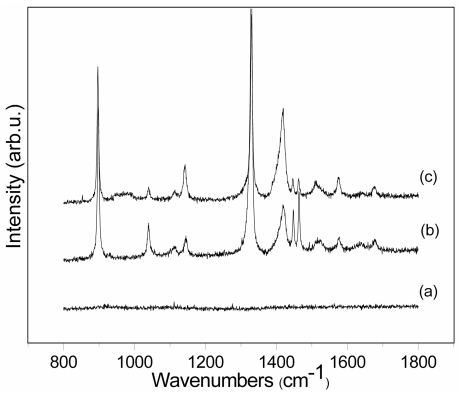

Figure 1: Raman spectra of glycine (a) 30mM in H_2O at pH 7.8 (b) powder (c) solution 30mM in H_2O at pH 7.8 dried on silica

Figure 1 compares the Raman signal of glycine recorded on the SiO_2/Si surface with glycine in powder form. No signal is detected at comparable experimental conditions for the solution and the spectrum is comparable to the spectrum recorded from powder of glycine after crystallization. We find the same behavior for valine. Amino acids can have different protonation states depending on pH which might influence the molecular packing in the micro crystals.

pH values between different pKa values were selected to have the most uniform protonation state. The Raman spectra of dried glycine from a solution at pH 1.2, 7.8 and 12 are shown in Figure 2. The intensities of the Raman signal are comparable at pH 1.2 and pH 7.8. At pH 12, the intensity is significantly lower (Figure 2, 10x longer acquisition time at pH 12). The observed vibrational bands depend clearly on the pH of the solution. Figure 3 shows the pH dependent Raman spectra of valine. We have found similarly intense signals at pH 1, but significant lower signals at pH 12 (acquisition time x10 longer). We recorded spectra for histidine at four different pH values (pH 1.6, 3.8, 7.8, and 12).

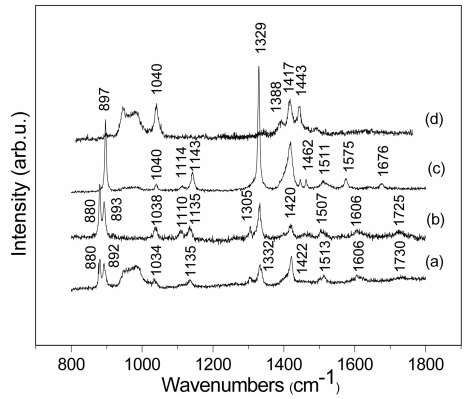

Figure 2: Raman spectra after evaporation of glycine dissolved in H_2O de-ionized at different pH (a) pH 1.2 (b) pH 2.4 (c) pH 7.8 and (d) pH12 on silica.

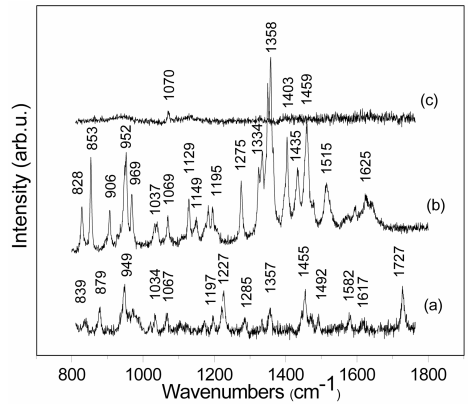

Figure 3: Raman spectra after evaporation of valine dissolved in H_2O de-ionized at different pH (a) pH 1 (b) pH 7.8 and (c) pH 12 on silica.

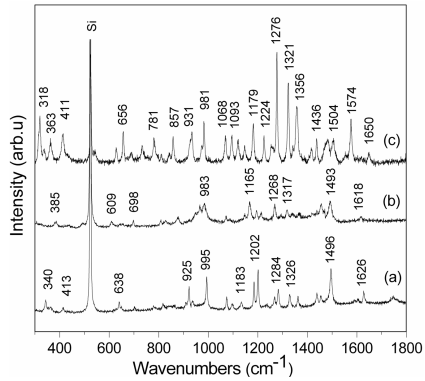

Figure 4: Raman spectra of aqueous histidine at different pH evaporated on silica (a) pH 1.6 (b) pH 3.8 (c) pH 7.8. The intense vibrational band at 521cm^{-1} corresponds to the SiO_2/Si substrate.

Figure 4 shows an intense spectrum for pH 1.6, 3.8 and 7.8. At pH 12 the spectrum disappeared nearly completely.

For all three amino acids micro crystals, no vibrational bands due to the presence of water (OH: 3400cm^{-1}) were observed, in contrast of previous study on larger crystals. [11] This does not exclude the presence of water in the crystalline structure but shows that the amount of water in the crystals must be small.

4.2 Density Functional Calculations

We used density functional calculations to get a better insight on the organization of amino acids and their ionic nature in condensed form. We find through structuctural optimization that a totally dehydrated zwitterionic form of a single histidine molecule is unstable, giving rise to a proton transfer from the NH_3^+ to the COO^- group upon minimization. To explore the zwitterionic form of the amino acids, we focus on the polar regions and investigate how they couple through intermolecular interactions or with residual water molecules. For this purpose, we investigate the vibrational response of the amino acids as a function of the degree of saturation of their polar regions by water. We build different hydration models for the glycine with up to six surrounding water molecules (table 1). For valine and histidine, we only consider the simplest cases using two water molecules. We use the information derived from glycine for the assignment of the main vibrational bands.

Glycine ($^+H_3NCH_2COO^-$) is the simplest amino acid and is abundant in various proteins and enzymes (up to 7.5%). It is the only amino acid which has no stereo isomer (not optically active). Several studies of Raman bands of glycine are reported in the literature. [3,12,13] The observed Raman bands of glycine at pH 7.8 are listed in table 1.

In order to test the assignment, in particular of the vibrational modes that correspond to the NH_3^+ group, we made H/D exchange experiments (Figure 5). The only exchangeable protons of glycine at neutral pH are the three protons of the NH_3^+ group (CH_2 does not exchange and COO^- is de-protonated).

When H was replaced by D in NH_3^+ several vibrational bands appeared at 832cm^{-1}, 971cm^{-1}, 992cm^{-1}, 1006cm^{-1}, 1278cm^{-1}, and some bands disappeared or are attenuated at 1575cm^{-1}, 1511cm^{-1}, 1143cm^{-1} and 897cm^{-1}. The band at 1511cm^{-1} disappears in the D_2O spectrum and corresponds accordingly to the assignment to the NH_3^+ deformation vibration. The bands at 1114cm^{-1} and 1143cm^{-1} disappear in the D spectrum and they correspond to the vibrations related to N (N-H, N-C) according to the assignment. The band at 897cm^{-1} is attenuated in the D spectrum and is attributed to C-C vibration indicating that the D influences the C-C vibration. Some of the vibrations are shifted in energy by incorporating D. The vibrational band at 1329cm^{-1} is shifted to 1320cm^{-1} in 100% D_2O. This is in line with the theoretical calculations which show the contribution of NH_3 to this band. The same is found for the 1420cm^{-1} band which shifts to 1410cm^{-1}.

Wavenumbers (cm^{-1})	Calculation (cm^{-1})	Assignment
360	318	δ(CCN)
597	574	δ(CCN)
696	681	δ(CCN)
897	853 - **891**	ν(C-C), $\delta(COO)_{sc}$
1040	954 - **1017**	$\nu(C\text{-}N)_{st}$
1114	1088 - **1153**	$\delta(CH_2)_{tw}$, $\delta(NH_3)_{tw}$
1143	1122 - **1194**	$\delta(NH_3)_r$, $\delta(CH_2)_w$
1329	1273 - **1371**	$\nu(COO)_s$, $\delta(CH_2)$, $\delta(NH_3)$
1420	1306 - **1357**	$\delta(CH_2)_{tw}$, $\delta(NH_3)_{tw}$
1445	**1416**	ν^8, $\delta(CH_2)_w$, $\delta(NH_3)_r$
1462	1475 - **1482**	$\delta(CH_2)_{sc}$
1511	1618 - **1575**	$\delta(NH_3)_{umb}$
1575		
1676	1776 - **1694**	$\nu(COO)_{as}$
2984	2957	$\nu(CH_2)_s$
3019	3015	$\nu(CH_2)_{as}$

Table 1: List of experimental and calculated vibrational frequencies for glycine (pH 7.8). Bold numbers in the calculation column account for glycine surrounded by six water molecules (normal text accounts for two water molecules around the amino acid).

The vibrational band at 1278cm^{-1} which is not present in the protonated spectrum, can be attributed to a N-D deformation vibration. In agreement with the assignment, we find the bands of CH_2 at 2984cm^{-1}and 3019cm^{-1} which are not influenced by D substitution (i.e. 2986cm^{-1} and 3020cm^{-1}). It is not clear why the band at 1676cm^{-1}, assigned to ν(COO) disappears in the D spectrum. A possible explanation is that the COO is engaged in a hydrogen bond which is modified as a result of H/D substitution. As the signal to noise ratio is the same in 100% H_2O and 100% D_2O we can conclude that the effect of signal increase is active in both cases. The band at 897cm^{-1} is shifted to lower and the band at 1676cm^{-1} is shifted to higher energies when the pH is reduced to pH 1.2. This is consistent with the assignment of the two bands to COO vibrations (see Table 1).

Valine is an amino acid and has a branched-chain amino acid like leucine and isoleucine. Valine contains a single hydrocarbon side chain making it hydrophobic and is usually found in the interior of proteins. Its hydrophobicity contributes with other hydrophobic amino acids to the

tertiary and quaternary structures of proteins. The spectrum of valine contains a larger number of vibrational bands than glycine due to the larger side chain (iosopropyl group).

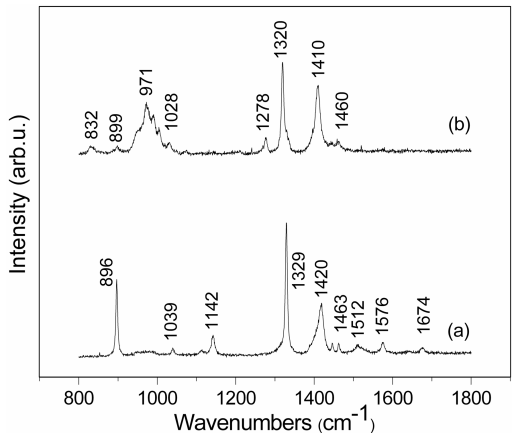

Figure 9: Raman spectra after evaporation on silica of glycine in solution (a) 100% H_2O, (b) 100% D_2O.

Only the Raman spectra at low and neutral pH have shown intense bands. At low pH the COO^- is protonated and forms a COOH. As a result the bands assigned to COO^- are expected to shift most upon reducing the pH. The band of COO^- at 1323cm^{-1}, 906cm^{-1} and 853cm^{-1} are not found at low pH. The bands which shift less (<5cm^{-1}) are in the high frequency range at 2979cm^{-1}, 2921cm^{-1}, 2890cm^{-1} and in the intermediate frequency range at 1358cm^{-1}, 1334cm^{-1}, 1195cm^{-1}, 1069cm^{-1} and 969cm^{-1}. All these bands are assigned to vibrations including C-H of the isopropyl side or C alpha chain. At high pH we observe only one narrow band at 1069cm^{-1}. Its origin is not clear at this point.

We observe that vibrational bands are influenced by crystallization. The bands are narrower, shifted and new bands appear in microcrystalline form. [7] By varying the pH in histidine we find that almost all bands are shifted. This shows that the pH variation affects not only bonds directly related to groups of atoms that are de/protonated (NH_3^+, COO^- and imidazole group), but the entire molecule. We propose that the presence of the imidazole group with its possible delocalized electrons and the formation of hydrogen bonds with the carboxylate group influence the electronic structure of the microcrystal.

In the case of the H/D exchange using D_2O**,** most bands shift in the 800-1800cm^{-1} region. The bands above 2900cm^{-1}, assigned to localized CH_2 vibrations are not affected by the D/H exchange as expected.

5 CONCLUSION

We show that the method to crystallize histidine to observe intense Raman signals using visible laser excitation can be extended to other amino acids. We find that the pH influences the spectrum considerably which shows that the crystalline order depends on the protonation state of the molecule. This simple method allows detecting intense Raman signals of glycine, valine and histidine at neutral and acidic pH at the nano gram level. We expect that other amino acids dried on surfaces the same way exhibit the same enhancment on the Raman signal. We note, however, that high pH resulted in reduction of the Raman signal which could be related to the overall negative charge or the presence of Na^+ ions in the solution.

The observed narrow Raman bands provide an interesting basis for the study of the crystalline structure using state of the art DFT calculations. The results of the theoretical modeling show the importance of the degree of saturation by water of each polar region which affects strongly the calculated vibrational modes. We find that the zwitterionic form of the amino acids give better agreement with observed modes compared to the neutral form where the polar regions appear to be saturated. It is not clear at this point how the saturation is taking place.

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