Vibrational Spectroscopy 80 (2015) 59–65

Contents lists available at ScienceDirect

Vibrational Spectroscopy

journal homepage: www.elsevier.com/locate/vibspec

Continous gradient temperature Raman Spectroscopy identifies flexible sites in proline and alanine peptides

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ARTICLE INFO

Article history:
Received 2 April 2015
Received in revised form 10 July 2015
Accepted 11 July 2015
Available online 20 July 2015

Keywords:
Gradient temperature Raman spectroscopy
Proline
Alanine
Peptide structure
Raman spectroscopy
Differential scanning calorimetry

ABSTRACT

Continuous gradient temperature Raman spectroscopy (GTRS) applies the temperature gradients utilized in differential scanning calorimetry (DSC) to Raman spectroscopy, providing a straightforward technique to identify molecular rearrangements that occur near phase transitions. Herein we apply GTRS and DSC to the solid dipeptides Ala-Pro, Pro-Ala, and the mixture Ala-Pro/Pro-Ala 2:1. A simple change in residue order resulted in dramatic changes in thermal stability and properties. Characteristic Pro vibrations were observed at ~75 °C higher temperature in Pro-Ala than Ala-Pro. The appearance/disappearance of characteristic vibrational modes with increasing temperature showed that a double peak in the Ala-Pro major phase transition (174–184 °C) was due to a *gauche* to *anti* 165° rotation of $H_3C—C^*—NH_3$ about C*. CH_2 rocking and wagging frequencies present in Pro-Ala were not observed in Ala-Pro. For Ala-Pro, the Ala $^+NH_3$, and Pro COO^- sites were flexible whereas the Pro ring moiety was not; since the O=C—N(—C)$_2$ amide bond is planar the C—N—C moiety keeps the Pro ring rigid. For Pro-Ala, CH_2 sites in the Pro ring were flexible and the O=C—NH amide bond is perpendicular to the Pro ring. Since the mass of the Pro ring is significantly larger than the mass of the flexible Ala $^+NH_3$ moiety, Pro-Ala absorbs more thermal energy, corresponding to a higher phase transition temperature (240–260 °C). Ala-Pro, Pro-Ala, and Ala-Pro/Pro-Ala 2:1 exhibited α-helix, β-sheet, α-helix secondary structure conformations, respectively.

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1. Introduction

Although flexibility in peptide and protein structures is critically important [1–6], identifying flexibility at multiple sites simultaneously is elusive. In particular, structural changes involved in the thermal adaptation of proteins are considered manifold and complex. Every day, billions of people consume animal or plant proteins subjected to high degrees of thermal stress (*i.e.* cooking, canning, processing); yet quantifying the processes involved in the thermal transformation of proteins remains as much a culinary art as a science [7–10]. Most current analytical techniques are not designed to collect data continuously over wide temperature ranges [11–14].

A correlation between thermostability and rigidity of protein structures is well documented. The corresponding state hypothesis argues that proteins show a similar structural flexibility within temperature range that particular organisms which synthesize the proteins are adapted to. At room temperature, thermophilic proteins exhibit reduced flexibility and appear more rigid compared to mesophilic or psychrophilic proteins. However thermophilic proteins become more flexible at the relatively higher temperatures at which they function [10–13,15].

Proline is an atypical amino acid with the N atom part of a five membered ring, thus it contains a $^+NH_2$ zwitterion site instead of $^+NH_3$, and the phi (φ) angle range in Pro-containing polypeptides is highly restricted. Proline can readily adopt *cis*- and *trans*-configurations. The *cis*- configuration accounts allows prolyls to bend the regional amino acid alignment and therefore fold the protein [6,16]. Although *cis*/*trans*- switching in proline rich tandem-repeat domains can be a factor in overall protein flexibility, polypro linker peptides (all *trans*-) are highly rigid and linear [17,18].

On average thermophilic/hyperthermophilic proteins have relatively higher concentrations of Pro that mesophilic/psychrophilic proteins, with Pro tending to be at the N-terminal of an

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http://dx.doi.org/10.1016/j.vibspec.2015.07.003
0924-2031/Published by Elsevier B.V.

α-helix [19]. Proteins are universally stabilized by Pro residues at the N-terminal of an α-helix, regardless of their overall thermostability. Exposed and flexible sites are more conducive to the Pro effect [20].

Raman spectroscopy has been utilized to characterize primary and secondary structure in small unfolded peptides and proteins [21–25]. Raman has significant advantages over IR spectroscopy in this capacity because it is insensitive to water absorption. Vibrational modes from crystalline, solid (hydrated or not), amorphous aggregate and aqueous phases can be characterized with equal precision. Our research group has developed the technique of gradient temperature Raman spectroscopy (GTRS), which applies the precise temperature gradients utilized in differential scanning calorimetry (DSC) to Raman spectroscopy [26,27]. DSC is powerful technique to quantify the heat absorption of amino acids, peptides and proteins, providing information regarding phase transitions and fundamental thermodynamic properties [28,29], but not on the mechanisms by which heat is absorbed at the molecular level. However, the GTRS technique identifies specific temperature ranges where flexible structures absorb heat and the molecular-level response to that thermal stress. GTRS provides a very rapid and straightforward technique to identify theoretically proposed molecular rearrangements that occur just prior to or at phase transitions.

For example, Raman spectra of the organochlorine pesticide endosulfan were acquired at 1 °C intervals from 50 to 102 °C for α-endosulfan, β-endosulfan and a 60/40 mixture. A phase transition observed at 97–102 °C for β-endosulfan corresponded to the largest shifts in the GTRS spectra. An irreversible pathway for the isomerization from the symmetrical β-isomer to the nonsymmetric α-isomers was confirmed [26].

Similarly, Raman spectra were acquired for urea, biuret, cyanuric acid and melamine (pure and at 1% in dried milk powder) from 50 to 310 °C. Vibrational modes that were mainly associated with ring breathing, stretching and in-plane deformation shifted with respect to temperature in all four molecules [27]. Specific frequencies significantly increased/decreased in intensity within narrow temperature ranges independent of whether the amine was mixed in milk. Correlation of Raman and DSC data identified and assigned the moieties which are most temperature sensitive, especially just prior to phase transitions. Results suggest flexible molecular sites in solid structures absorb thermal energy before more rigid sites do. Herein we apply the GTRS technique to the dipeptide structural analogs, Ala-Pro and Pro-Ala, and correlate the results with DSC.

2. Materials and methods

The GTRS system utilizes a Raman spectrometer (Raman Explorer 785, Headwall Photonics, Fitchburg, MA) fitted with a 16-bit CCD camera (1024 × 256 pixels; Newton DU920N-BR-DD, Andor Technology, South Windsor, CT). The spectrometer detects a Raman shift range of 102.2–2538.1 cm^{-1} with a spectral resolution of 3.7 cm^{-1}. A 785-nm laser module (I0785MM0350MF-NL, Innovative Photonic Solutions, Monmouth Junction, NJ) serves as the excitation source. A fiber optic Raman probe (RPB, InPhotonics, Norwood, MA) is used to focus the laser and acquire the Raman signals. A bifurcated fiber bundle delivers the laser radiation to the probe and transmits the Raman signals to the spectrometer. System software was developed using LabVIEW (National Instruments, Austin, TX) to fulfil functions such as camera control, data acquisition, temperature measurement and synchronization. Data can be collected at temperature intervals as short as 0.2 °C.

SigmaPlot 13 (Systat Software, Inc.) generated 3D contour plots (frequency, temperature, signal intensity). Relative intensity is scaled for the signal intensity range which occurs within each of the six spectral wavelength sets. Maximum to minimum intensity colors are red > orange > yellow > green > blue > black. Each contour data array contains about 20 Mb. First derivative contour plots were calculated intensity using a three-point running average, *i.e.* the first and last points calculate the slope of the middle point. The contour plots for the second derivative intensity were calculated using the three-point running average data from the first derivative spectra.

Commercially prepared peptides (Sigma Chemicals, St. Louis, MO) >98% purity were utilized. The mixture Ala-Pro/Pro-Ala 2:1 was also investigated to minimize ambiguity in spectral

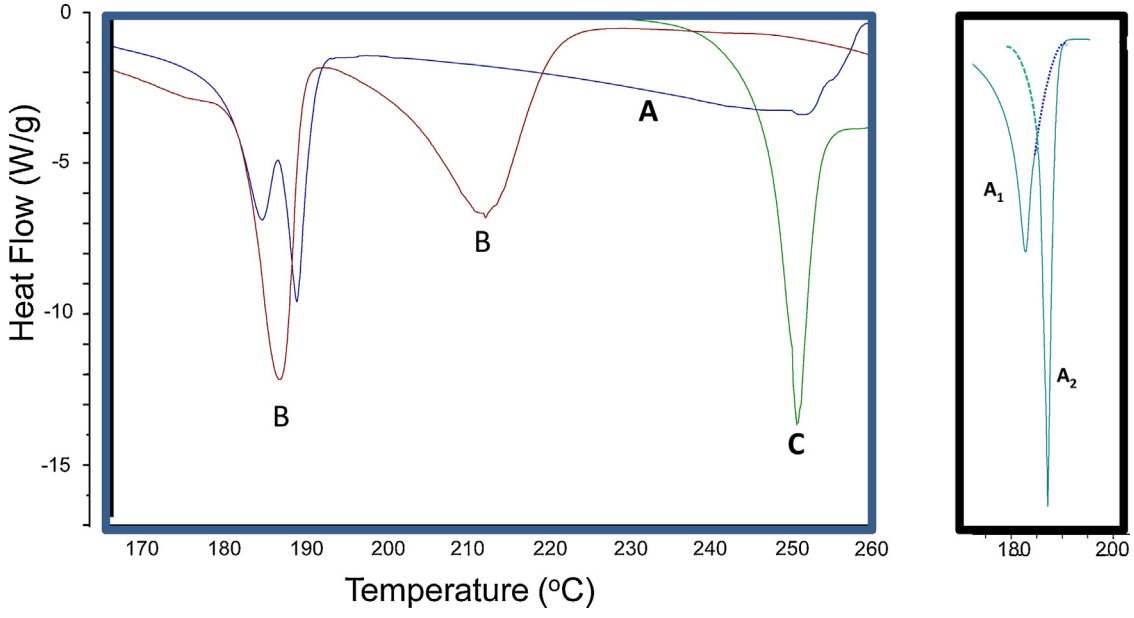

Fig. 1. Differential scanning calorimetry heat absorption data for Ala-Pro, Pro-Ala and a 2/1 mixture. Inset expands the A_1 and A_2 proposed phase transition at 183 °C. Reducing the DSC heating rate broadens the A_1 to A_2 transition but does not change the transition temperature.