CHANGES OF RAMAN SCATTERING IN THE CH-STRETCHING REGION DURING THERMALLY INDUCED UNFOLDING OF RIBONUCLEASE

by

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SUMMARY

Thermally induced unfolding of ribonuclease at low pH produces a large increase of Raman scattering intensity at $\sim 2930 cm^{-1}$ (CH_3-stretching region). Comparisons of the CH_3-stretching spectra of various model compounds in the presence or absence of H_2O (^2H_2O), indicate that the changes observed with ribonuclease arise from the insertion of previously buried, aliphatic amino acid residues into water.

INTRODUCTION

The fluorescence of protein fluorophores in isolated erythrocyte membrane ghosts or vesicles becomes abruptly more sensitive to paramagnetic quenching by nitroxide analogs of stearic acid as the temperature is raised above 37°C, in a process that is irreversible above $\sim$ 42°C (1,2). Furthermore, the discontinuities in paramagnetic quenching correlate with a thermotropic discontinuity in the intensity of protein residue CH_3-stretching, detected by laser Raman spectroscopy (3); this is also irreversible above $\sim$ 42°C. Finally, intact erythrocytes, starved of glucose exhibit an abrupt decrease in membrane potential, which centers at 38°, is reversible up to 41° and irreversible at higher temperatures (4). The membrane responses to temperature revealed by the above-mentioned spectroscopic approaches show a sharp dependence on pH in the near physiological range (1-3). Moreover, the temperatures at which the responses detected by fluorescence-quenching and Raman spectroscopy become irreversible roughly overlap with those where differential thermal calorimetry (5) and proton magnetic resonance spectroscopy (6) indicate membrane protein unfolding at neutral pH.

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It is established that water-soluble proteins can also undergo thermotropic changes in tertiary structure, but at pHs well below their isoelectric points (7, 8). We have therefore suggested (3) that it is the unique environment of integral membrane proteins that may make their structures responsive to temperature- and pH-changes in the physiological, or near physiological range. If this postulate is correct, laser-Raman spectroscopy should reveal changes of CH_3-stretching during the thermotropic unfolding of a soluble protein below its pI that resemble those exhibited by membrane proteins at neutral pH. Unfortunately no information of this type has been reported although extensive studies have been published (9, 10) dealing with other Raman manifestations of the low-pH thermotropism of soluble proteins. We have accordingly evaluated the changes in the CH-stretching region of ribonuclease at low pH (pH < pI), where this protein is known to undergo reversible, temperature-determined changes of tertiary structure.

We find that the thermotropic transition of ribonuclease at low pH produces large changes of Raman scattering in the CH-stretching region. Moreover, evaluation of the results in terms of the behavior of simple model compounds, suggests that, at low pH, an increase of temperature forces methyl residues of ribonuclease into contact with water.

EXPERIMENTAL

We used crystalline bovine pancreatic ribonuclease A purchased from Sigma Chemical Co. (St. Louis, Mo. U.S.A.) and egg lecithin obtained from Lipid Products (South Nutfield, G.B.). Other reagents were of reagent grade and solvents of spectrochemical purity. We used $H_2O/^2H_2O$ mixtures (1/1) to minimize solvent background when following the CH-stretching features of ribonuclease as a function of temperature.

Spectra of ribonuclease in solution were obtained at a protein concentration of 40 mg/ml. To prepare ribonuclease/lecithin mixtures we first sonicated egg lecithin (40 mg/ml) in $H_2O/^2H_2O$ (1/1) at stated apparent pH (1.72, 2.72 and 6.9) and then dissolved the ribonuclease in the lecithin dispersion to give a final protein concentration of also 40 mg/ml. The pH was then again determined.

Samples were placed into thermoregulated capillaries and their CH-stretching spectra recorded as before (e.g. 3) with a Ramalog 4 spectrometer (Spex Industries, Metuchen, N. J.) interfaced to an Interdata computer (model 70), using the 488 nm Ar^+ laser line for excitation at 400 μ slit width for ribonuclease and 60 μ for model compounds. Temperature equilibration was, as in (3) for 20 min before scanning from 2750cm^{-1} to 3050cm^{-1} (2.4 scans; not more than 300 s/scan). The averaged, smoothed (least-squares) spectra were plotted for each temperature. The data points or ranges presented derive from at least four separate experiments.

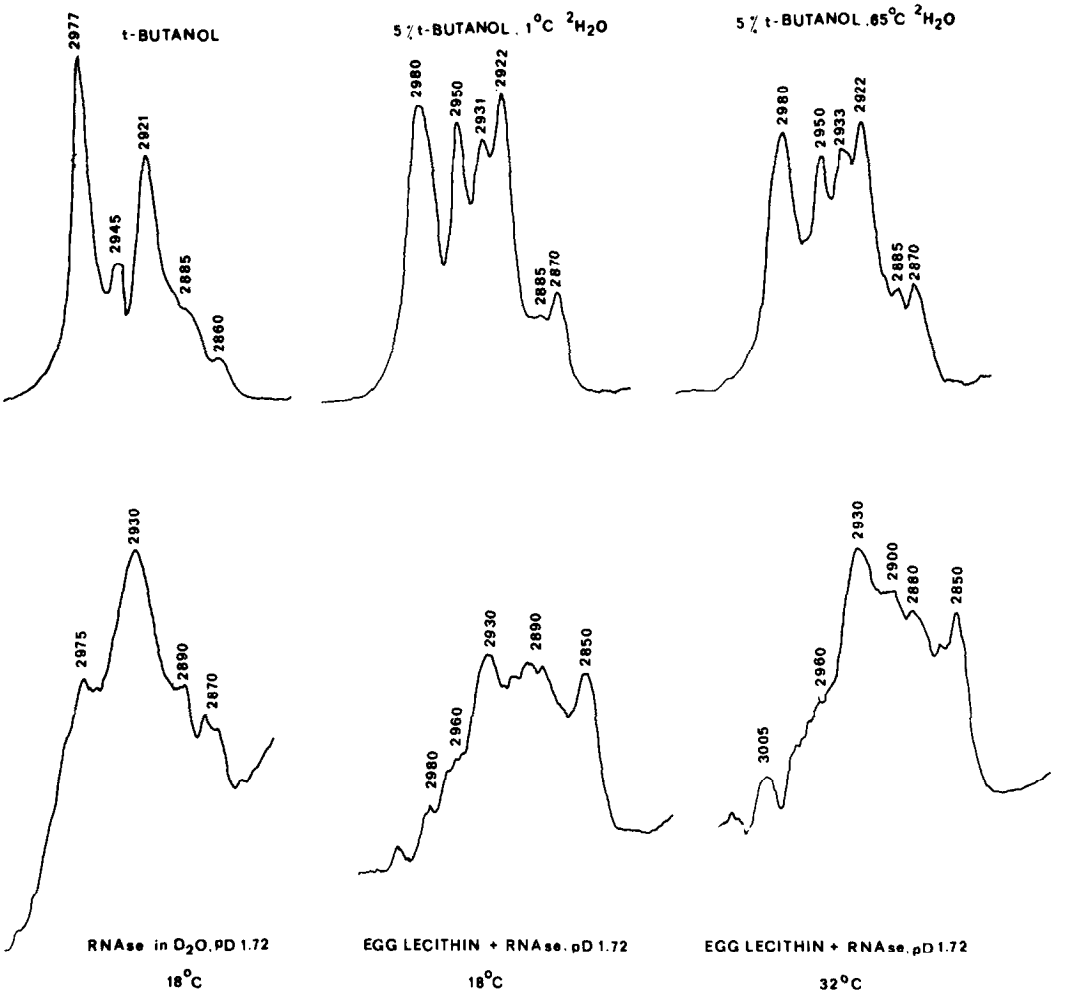

Fig. 1. CH-stretching spectra.

RESULTS AND DISCUSSION

Ribonuclease A, whether crystalline or in aqueous solution, at pH (or pI) 1.72 and 18°C, gives the CH-stretching spectrum shown in Figure 1. The principal feature is the strong band at 2930 ± 2 cm^{-1}. Weaker peaks occur at 2870 cm^{-1}, 2890 cm^{-1} and 2975 cm^{-1}. All of the bands are broad, probably because of slightly dissimilar contributions of various aminoacid methyl and methylene residues with different environments and chemical linkages.

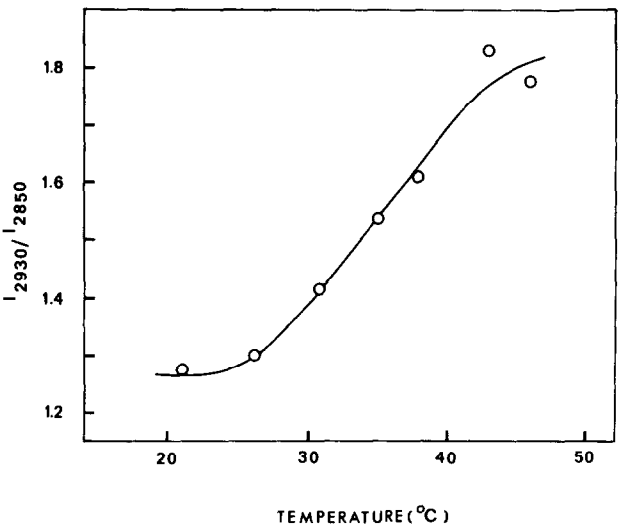

Fig. 2. Variation in intensity of the 2930 cm^{-1} band of ribonuclease relative to that of the 2850 cm^{-1} band of lecithin as a function of temperature. See text for details.

To determine the variation of the ribonuclease CH-stretching spectrum with temperature in the 20°C - 50°C range, an internal standard is required. For this we chose the 2850 cm^{-1} CH_2-stretching band of egg lecithin, and, in fact, measured the thermotropism of lecithin-ribonuclease mixtures (1/1; wt/wt). This choice is justified because (a) the $\sim$ 2930 cm^{-1} band of egg lecithin is weak and stable above the transition temperature, - 5.5°, of this phospholipid (11); (b) the 2850 cm^{-1} band is thermally stable, (c) the lecithin headgroup is a zwitterion at the pH levels employed preventing ionic interactions with lecithin (12). The CH-stretching spectrum of the ribonuclease-lecithin mixture is shown in Fig. 1 for set temperatures of 18°C and 32°C.

The variation of the intensity of the 2930 cm^{-1} band of ribonuclease relative to that of the 2850 cm^{-1} lecithin reference band is given in Fig. 2. The solvent was $H_2O/^2H_2O$ (1/1; v/v), apparent pH 2.72. A broad transition between 26°C and 44°C is evident, which corresponds in position and width to the transition detected at this pH by differential thermal calorimetry (7). The midpoint of the transition

is 38°C (corrected for 2°C heating of the sample by the laser light). The width of the transition is explained by the Raman data of Chen and Lord (9) in the 1800 cm^{-1} - 250 cm^{-1} region, showing that ribonuclease unfolds in sequential steps.

What mechanisms might underlie the change of intensity at 2930 cm^{-1} upon ribonuclease unfolding? Thermodynamic analyses (7) indicate that the enthalpy changes occurring during unfolding are due to the exposure of apolar residues to water. Also Larson and Rand have proposed (13) that Raman-active CH-stretching vibrations vary with solvent polarity. To further test the latter suggestion we have recorded the CH-stretching spectra of methanol, dimethyl sulfoxide and t-butanol for the pure solvents and for various mixtures with H_2O or ^2H_2O.

Pure methanol exhibits two strong peaks at 2834 cm^{-1} and 2942 cm^{-1}. The latter band is flanked by two broad, weak shoulders, centering near 2975 cm^{-1} and 2915 cm^{-1}, respectively. However, in methanol/H_2O or methanol/^2H_2O (1/10) the 2942 cm^{-1} band occurs at 2955 cm^{-1} and the 2834 cm^{-1} feature shifts to 2847 cm^{-1}.

Pure dimethyl sulfoxide exhibits a strong band at 2912 cm^{-1} and a weak one at 2996 cm^{-1}. In mixtures of dimethyl sulfoxide and ^2H_2O (e.g. 5% v/v) these bands appear at 2924 cm^{-1} and 3010 cm^{-1}, respectively. Moreover, the intensity of the low frequency feature relative to that of the high frequency band changes from 3.63 for pure solvent to 4.0 in ^2H_2O at 21°C and 4.28 in ^2H_2O at 58°C.

Pure t-butanol (Fig. 1) exhibits two strong bands, at 2921 cm^{-1} and 2977 cm^{-1}, a weaker band at 2945 cm^{-1} and several shoulders in the low frequency range of the 2921 cm^{-1} feature. In t-butanol/H_2O (15/100) all the bands appear at higher frequencies. In particular, the 2945 cm^{-1} band now occurs at 2950 cm^{-1} with increased intensity (I) relative to that of the lower-frequency band, which has shifted to 2922 cm^{-1}, while the 2977 cm^{-1} peak moves to 2980 cm^{-1}. The ratio of the intensities of these two bands, i. e. $[I_{2945-2950 cm^{-1}}/I_{2921-2922\ cm^{-1}}]$ increases with the proportion of water in the system (Fig. 3). In contrast the intensity of the 2921-2922 cm^{-1} feature, relative to that of the 2977 cm^{-1} - 2980 cm^{-1} peak remains constan (0.86) at all water proportions. When ^2H_2O is substituted for H_2O, a new band appears

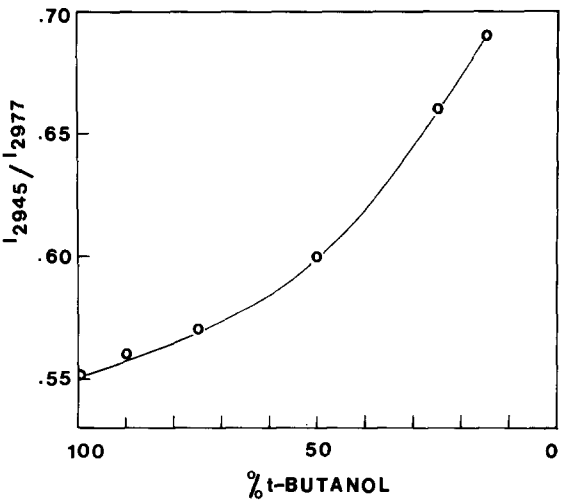

Fig. 3. Variation in intensity of the 2945 cm^{-1} band of t-butanol relative to the 2977 cm^{-1} band as a function of the proportion of water. See text for details.

at 2931 cm^{-1} which is not resolved in pure t-butanol or t-butanol/H_2O (Fig. 1). This band diminishes in intensity with increasing proportions of ^2H_2O. Also t-butanol/^2H_2O mixtures (5/100) give a decrease in [$I_{2945-2950\ cm^{-1}}/I_{2921-2922\ cm^{-1}}$] from 0.91 at 1° - 20°C to 0.87 at 55°C.

In ribonuclease the strong, broad temperature-sensitive band at $\sim$ 2930 cm^{-1} does not change position detectably. This is expected in view of the complex origin of the band. Nevertheless its intensity change, viewed in the light of data obtained by calorimetric methods (7) and the above-described variations of the CH_3-stretching upon exposure of methyl residues to water, indicate that the CH_3-stretching thermotropism of ribonuclease at acid pH, is due in part to the insertion, into water, of residues such as valine, leucine and isoleucine. Reasoning by analogy, the marked intensification near 2930 cm^{-1} that occurs when erythrocyte membranes are warmed to above 40°C (3) may reflect exposure of apolar protein side chains to an aqueous milieux.

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