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Raman scattering in lysozyme solutions

F. Aliotta, M. P. Fontana,[a] R. Giordano, P. Migliardo, and F. Wanderlingh

Istituto di Fisica dell' Universita' and GNSM del Consiglio Nazionale delle Ricerche, Messina, Italy
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Previous measurements on the OH stretching behavior in lysozyme solutions do not agree with each other. A careful measurement is performed using an isotopic substitutional technique in order to improve the precision. It is shown that, taking into account the contribution of the OH groups of lysozyme, elsewhere neglected, the interaction between lysozyme and water does not give any significant contribution to the Raman spectrum of the OH stretching. The conclusion is also supported by depolarization ratio measurements.

I. INTRODUCTION

Much attention has been devoted over the past years to the interaction of proteins with water, especially to the structural properties of protein solutions. In 1976 Cavatorta, Fontana, and Vecli[1] (hereafter referred to as CFV) reported measurements of Raman spectra in aqueous lysozyme solutions. They compared the intensity ratio of the C–H stretching band to that of the OH stretching with the concentration, and found a systematic deviation from linearity. In the hypothesis of a linear increase of the intensity of the C–H band, it was suggested that the Raman efficiency of the OH stretching mode of water decreases at increasing concentrations.

Such an effect becomes quite evident at concentrations larger than about 17% by weight. Later the same kind of measurement was reported by Samanta and Walrafen[2] (hereafter referred to as SW) the result of which seems to lead to a different conclusion. In fact SW compared their results with those of CFV, and found a noticeable disagreement. However the disagreement is much less than that claimed in Ref. 2 because of an incorrect evaluation of the different unities used to measure concentrations in Refs. 1 and 2.

In order to clarify the problem, we have performed very careful measurements of Raman spectra, of lysozyme solution, in the region of the O–H and C–H stretching bands. Let us now consider the main sources of error that can affect the correct evaluation of the integrated Raman intensities.

First of all the CH and OH stretching bands partially overlap, so that the decomposition of the Raman spectrum into the two separate contributions is not easy. In addition it is to be noted that lysozyme contains some OH groups whose contribution should be taken into account for the evaluation of the OH contribution of water. We succeed in eliminating these sources of error by making measurements in solutions of lysozyme in normal and heavy water. In the latter case, the OH stretching band is located to higher wave numbers from the OD stretching band, and is quite well separated from it. In addition the small (but not negligible) contribution due to the OH group of the lysozyme becomes evident (see Fig. 1). We should like to stress that the lysozyme used in our experiments is perfectly anhydrous, so that we can excluse that the OH contribution in the D_2O solutions arises from hydration water contained in the lysozyme itself. However, as far as the evaluation of data is concerned, it will be seen that our conclusions are left unchanged even if the above-mentioned OH contribution arises from hydration water.

II. EXPERIMENTAL SETUP AND PROCEDURE

We used high purity anhydrous lysozyme six times crystallized by the Miles Biochemicals Research Products, double distilled water and heavy water of a purity of 99.8%. The spectrum of pure heavy water showed no contribution in the OH region. Measurements were performed with a Spex Ramalog System, with an 800 mW mean power of the Ar^+ laser source operating at 5145 Å wavelength. Care was taken, especially at high concentrations, in order to minimize the fluorescence effect. The Raman spectra were taken with polarized exciting light; in the following, we discuss the I_{vv} spectra where both polarization vector at incident light and analyzer axis are normal to the scattering plane, after the baseline is properly subtracted.

Once the spectra have been obtained, we divide the entire range (1800–3850 cm^{-1}) into three intervals:

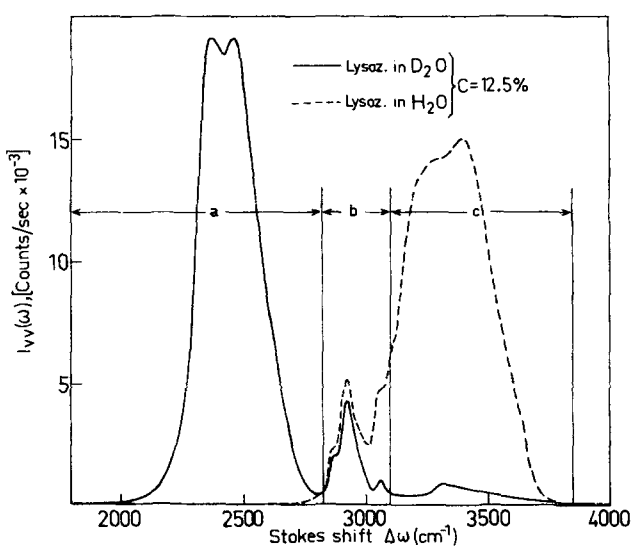

FIG. 1. Raman spectra of a 12.5% by weight lysozyme solution in normal (dashed line) and heavy water (full line).

[a] Permanent address: Istituto di Fisica and GNSM del CNR, Universita' di Parma, Parma, Italy.

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(i) 1800–2830 cm^{-1}. Here the main contribution to the Raman intensity is due to OD stretching. In light water solutions the intensity in this region is practically zero. (ii) 2830–3100 cm^{-1}. Here the main contribution is due to the CH stretching. In the heavy water solutions this is practically the only contribution while in the light water solutions there is a sizable contribution from the OH stretching. (iii) 3100–3850 cm^{-1}. Here the main contribution is due to the OH stretching. In the heavy water solutions such a contribution is due to the OH group of the protein, while in the light water solutions there is also the bigger contribution of water itself.

III. DATA HANDLING AND DISCUSSION

We call a, b, c the integrated Raman intensity of the three abovementioned intervals, and use primed letters to indicate the heavy water solutions.

We have very carefully compared the Raman efficiency, as detected in our experimental apparatus, in the OD and OH stretching spectral range. For this we have monitored both incident and transmitted beam intensities while taking the Raman spectra. These measurements were then repeated several times to check the reproducibility of our results. From these measurements we can construct the ratio between the OH and OD stretching integrated intensities. Such a ratio turns out to be very reproducible. Its value is $\gamma = 0.956 \pm 0.005$. (It is to be noted that such a value cannot be interpreted as an absolute measurement of the ratio of the scattering efficiency of the OH and OD, because of a possible difference in the performance of the experimental apparatus in the spectral regions in which the two spectra take place.)

We now make the hypothesis that the behavior of the solutions at different concentrations is the same in the normal and heavy water solutions. Such a hypothesis is usually made in neutron scattering experiments,[3] for instance.

Thus we can construct a normalization factor K:

$$K = \frac{a+b+c}{\gamma a' + b' + c'} \tag{1}$$

and assume that integrated intensities measured in the heavy water solutions multiplied by K furnish the corresponding contribution in the normal water solution. In such a way we can calculate (from the measured value b') the CH stretching contribution in normal water solutions,

$$I_{(CH)} \equiv Kb' \tag{2}$$

and the OH contribution in normal water solution due to the water only:

$$I_{(OH)} \equiv (b+c) - K(b'+c') \ . \tag{3}$$

Then it is straightforward to construct the ratio $I_{(CH)}/I_{(OH)}$. In such a way our results are independent from any hypothesis on the behavior of the various contributions, as a function of concentration, and also from possible fluctuation in experimental conditions in the different measurements.

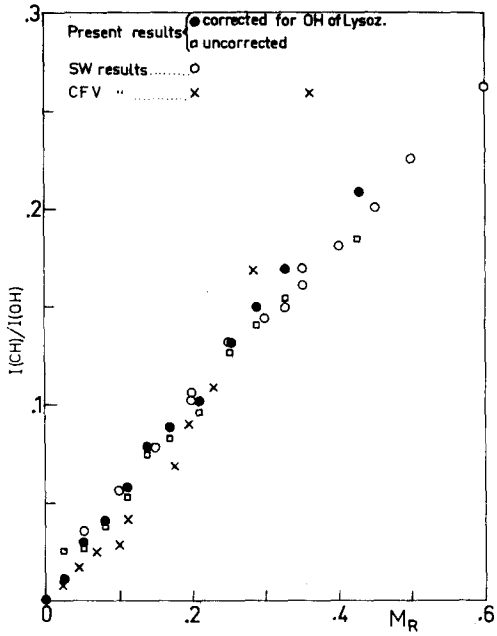

FIG. 2. Integrated intensity ratio $I_{(CH)}/I_{(OH)}$ as a function of relative concentration.

The results are shown in Fig. 2. Here black dots indicate our results, open dots the SW results and crosses the CFV results.

M_R is the ratio between weights of lysozyme and water, as found by SW. Data of CFV are properly reduced to the unities of measurement used by SW and by us. It is worthwhile noting that if we calculate $I_{(OH)} = (b+c) - Kb$, i.e., disregarding the contribution due to the OH groups of the lysozyme, then the present results practically coincide with those of SW (square in Fig. 2). A quadratic fit performed with our uncorrected data gives, in fact, practically the same results as those reported by SW. If, however, one uses the corrected data, i.e., takes into account the contribution of the OH groups of lysozyme, one obtains

$$\frac{I_{(CH)}}{I_{(OH)}} = 0.540\,655\ M_R - 0.102\,123\ M_R^2$$

with an rms of 4.62×10^{-4}.

The smaller value of the quadratic coefficient, compared with the value reported by SW (0.216 37) shows that the deviation from linearity comes mainly from neglecting the contribution of the OH groups of lysozyme.

It is to be noted that one can fit our present data with a linear equation, obtaining

$$\frac{I_{(CH)}}{I_{(OH)}} = 0.499\,157\ M_R$$

with an rms of 6.63×10^{-4}, that is only slightly worse than the preceding one.

Actually the data concerning the contribution of the OH group of lysozyme come from the difference between large numbers, so that some uncertainty is to be expected.

On the other hand, the fit of data is scarcely affected

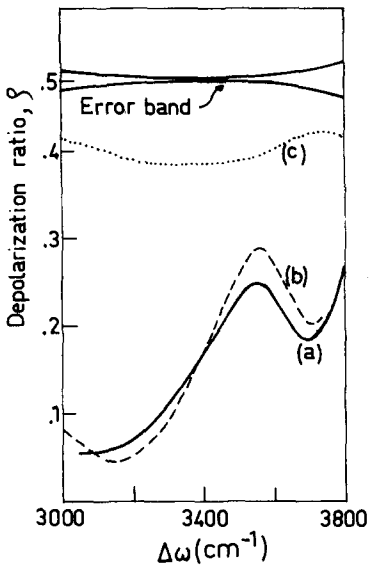

FIG. 3. Depolarization ratio in pure water (curve a) and 30% by weight lysozyme solution (curve b). For comparison 9.1 M $ZnBr_2$ aqueous solution (curve c) (unpublished data).

by the quadratic term, as shown above. Apart from numerical results, however, the general trend of the data clearly shows that, taking into account the contribution of the OH group of lysozyme, a linear relationship can adequately describe the experimental results, showing that the interaction between water and lysozyme, if it exists, is very small.

It is to be stressed that also if the OH band appearing in the spectra of the D_2O solutions is attributed to hydration water initially present in the lysozyme, our conclusion is left unchanged.

In such a case, in fact, while the intensity data are left unchanged, the values of the concentration M_R are to be properly reduced to take into account the additional amount of water contained as hydration water in the lysozyme. Such an amount could be evaluated by the integrated intensity of the OH band in the D_2O solutions. If such a calculation were performed one could show that again the data tend to a linear behavior, the correction being in the same direction and of the same magnitude as that in the hypothesis of a contribution coming from the OH group of lysozyme.

The suggestion that the interaction between water and lysozyme, if any, is very small can be checked, at least qualitatively, by comparing the depolarization ratio in the lysozyme solution and in an electrolytic solution.

In Fig. 3, we show the depolarization ratio for pure water (full line) for the 30% by weight lysozyme solution (dashed line) and for comparison, for a 9.1 M solution of $ZnBr_2$ (dotted line). The latter is known to bind strongly to water and disrupt water structure. It can be seen that while the electrolyte strongly modifies the depolarization ratio, because of its influence on water structure, the effect of lysozyme is definitely much less severe.

The suggestion that lysozyme interacts only weakly with water is not surprising. Actually, it is known that the amount of water tightly bonded to the protein is of the order of $0.2 + 0.5$ mol of water/100 g protein,[5] i.e., in our case of the order of 5% at the higher concentrations. A higher amount of water is also more or less affected by the macromolecules; however, such an interaction (although it could give rise to the phenomena shown in Refs. 2 and 6) seems to be quite ineffective with respect to the OH stretching Raman efficiency.

Therefore, the "weakness" of the interaction suggested by our results can be interpreted in terms of a relatively small amount of water implicated, while the interaction itself could actually be strong.

To sum up, we have remeasured the Raman spectra of lysozyme solutions both in D_2O and H_2O. In such a way a careful evaluation of the integrated Raman intensity for the OH and CH bands can be evaluated. In addition we show an OH contribution also in the D_2O solutions, that allows us to properly correct the data.

Uncorrected data practically agree with those of SW. The corrected data however show that actually the interaction between water and lysozyme, if any, is very small, in the way sketched above.

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