Vol. 47, No. 3, 1972 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS

Raman Spectroscopic Studies of Ligand-Protein Interactions: The Binding of Methyl Orange by Bovine Serum Albumin*

by

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Received April 3, 1972

SUMMARY: Laser Raman spectroscopy has been shown to provide a new approach for studying small molecule-protein interactions by vibrational spectroscopy. An important feature is that ordinary water solutions at pH values near 7 and low ligand concentrations ($\sim 10^{-5}$ molar) can be employed. Its use depends on ligands which yield intensity enhanced Raman spectra and is illustrated in a study of the interaction of methyl orange with bovine serum albumin.

INTRODUCTION: The feasibility of using laser Raman spectroscopy to obtain information about protein-ligand interactions is demonstrated in the present study. Vibrational spectra are obtained for the ligand, hence providing information of its physicochemical state, and also of that of its protein environment. The biologically relevant conditions of low (10^{-5} molar) ligand concentrations in aqueous (H_2O) solutions are employed, obviating the difficulties previously preventing the use of infra-red spectroscopy. In addition, the spectra refer only to the ligand, simplifying assignment and interpretation. The present application of the technique depends on the use of ligands exhibiting enhanced (resonance) Raman spectra. These are produced by exciting with light in an absorption band of the ligand. For practical purposes, the exciting light must be provided by a laser of relatively high power. Aromatic azo compounds exhibit relatively

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*N.R.C.C. publication number 12566.

simple resonance Raman spectra. This initial study was focussed on the interaction of methyl orange (MO) with bovine serum albumin (BSA), and was chosen because the extensive data available for this and similar interactions were expected to be useful in evaluating the results.

EXPERIMENTAL: Raman spectra were obtained using Jarell Ash 25-100 and Spex 1400 spectrometers with 300-800 mW of Ar^+ laser power at 4880 Å. Rotating sample cells were used for solid MO (1) and protein-free MO solutions (2). The intensity of the trans azo N=N vibration did not vary with time of irradiation, indicating the absence of cis-trans isomerization or bleaching of the dye. Thermal effects of irradiation were minimal since only a small thermal lens effect (3) was observed for non-rotating MO-BSA solutions. Infra-red spectra were obtained using a Perkin Elmer 257 spectrometer.

The BSA (Miles Laboratories Inc., Kankakee, Ill.) was dialysed at 4°C, first against water and then buffer, to remove impurities causing spectral interference. All solutions were made up in a phosphate buffer pH 7.6 $\mu = 0.15$. The BSA concentration was 3% and the MO concentration 10^{-5} molar. Under these conditions more than 90% of the MO was bound by the protein as computed from binding constants (4). The MO was a commercial reagent grade (Baker and Adamson) and was shown by elemental analysis to contain two water molecules of crystallization.

RESULTS: Essential features of the interaction can be obtained from the following assignments. One of the peaks of the doublet at 1415 and 1423 cm^{-1} (Figure 1a, Table 1) is assigned (5) to the ν_1 azo group mode (the N=N symmetrical stretch). The peak at 1151 cm^{-1} is assigned to a phenyl - N stretching vibration of the azo group (5,6) and the peak at 1373 cm^{-1} to a C-N stretching of the aromatic amine

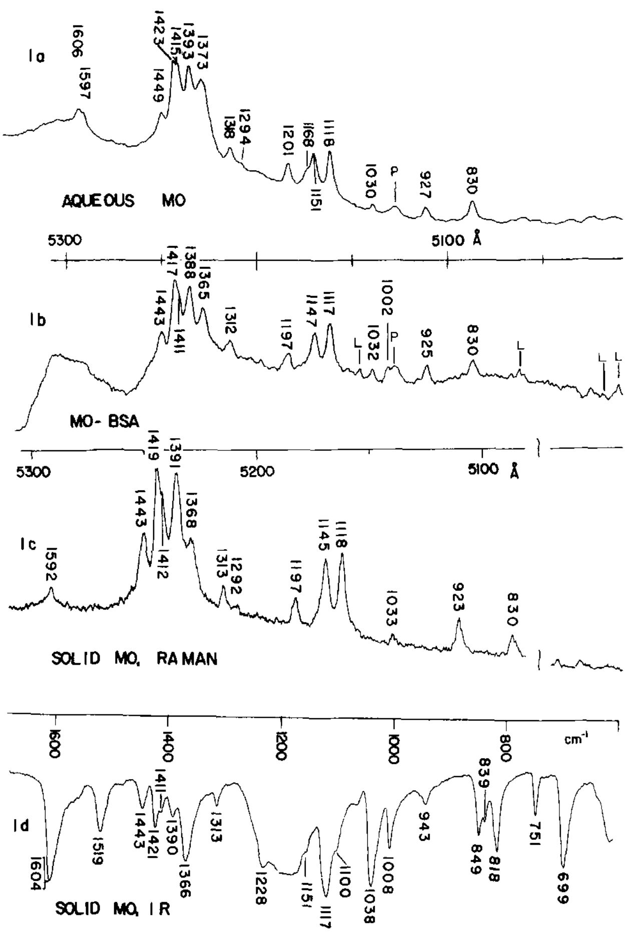

Fig. 1 - la, Raman spectrum of 10^{-5} M methyl orange in phosphate buffer pH 7.6; lb, Raman spectrum of 10^{-5} M methyl orange bound to 3% bovine serum albumin, buffer same as la; lc, Raman spectrum of solid methyl orange; ld, infrared spectrum of solid methyl orange in a KBr pellet. Spectral slide width = 3.5 cm^{-1} (solid), 5 cm^{-1} (aq. solns.), time constant = 4, typical count rate 10,000 counts per second. Note - abscissa for lc not the same scale as for la and lb. P = phosphate peak, L = laser plasma line.

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on the basis of literature data (7,8) as well as Raman and IR studies on the MO analogue N,N-dimethyl-p-phenylazoaniline (DMA) (10).

An initial conclusion is that bound MO has been essentially removed from an aqueous environment and is effectively buried in the protein, rather than being adsorbed at its surface. This view is based on the striking similarity between the spectra of the MO-BSA

TABLE 1

LINE POSITIONS OF METHYL ORANGE IN RAMAN AND INFRARED SPECTRA

RAMAN			IR SOLID MO
AQ MO	MO-BSA	SOLID MO	
-	-	-	699,751,818
829.8	830.4	830.0	
-	-	-	839(s),849
926.8	925.0	923.2	-
-	-	-	943
987.2	991.1	-	-
-	1002.4	-	-
-	-	-	1008
1030.4	1032.1	1033.0	-
-	-	-	1038
-	-	-	1100(s)
1117.8	1116.5	1117.7	1117
1151.2	1147.0	1144.5	1151(s)
1167.8(s)	-	-	1178(B)
1200.6	1197.1	1197.0	-
1293.7	-	1292.3	1228(B)
1318.4	1312.2	1313.1	1313
1372.7	1365.4	1368.0	1366
1393.3	1387.9	1390.8	1390
1415.3	1410.7(s)	1412.0(s)	1411(s)
1423.0	1417.4	1419.3	1421
1448.9	1443.0	1443.2	1443
-	-	-	1519
1596.7	-	1591.7	1592(s)
1605.6	-	-	1604

s = shoulder, B = broad. Each line position is the mean of two sets of data, the estimated error is 1 cm^{-1} (solid MO Raman) and 1.5 cm^{-1} (aq. MO, MO-BSA, solid MO IR). In the aqueous MO each line has a depolarisation ratio of 0.3-0.4.

complex and solid MO, (Figures 1b and 1c) where the average discrepancy in line positions is 1.2 cm^{-1} with a maximum difference of 2.4 cm^{-1}. Since solid MO has only two water molecules of crystallisation it would be expected that water would not have general access to the bound MO. In both solid and bound MO there is a slight general shift to lower frequencies (2-5 cm^{-1}) relative to unbound aqueous MO due to a change in dielectric constant.

The free as well as the bound form of MO are in the trans azo conformation and there is no twisting of the phenyl groups from their aqueous conformation, as indicated by the 1415 and 1423 cm^{-1} feature due to the azo group merely being shifted in the bound form to the slightly lower frequencies expected from a change in dielectric constant. Twisting of the phenyl rings would be expected to produce a large increase in frequency of one of the bands because the position of the N=N vibration will depend strongly on the degree of conjugation of the electrons and this will, in turn, be seriously affected by such twisting. The presence of the cis form would have produced a shift of nearly 100 cm^{-1} (5). The amino and azo nitrogens, which might be thought prone to specific interactions with BSA (e.g. by the formation of a hydrogen bond), do not seem to have been attacked since the positions of the C-N, N=N, and azo N-Ph vibrations in the aqueous, protein and solid phases show only the small dielectric frequency shift.

Conclusions regarding the SO_3^- group involve the disappearance of the shoulder at 1168 cm^{-1} from the Raman spectra of MO-BSA and solid MO. This shoulder is attributed to a skeletal mode made sensitive to interactions at the SO_3^- group by vibrational coupling. Bands directly assignable to the SO_3^- group, however, do not appear in the resonance Raman spectrum. Neither the SO_3^- bands (readily seen in the IR spectrum) nor the C-S stretch are observed.

Also, all spectral features found in MO appear in some analogs which do not contain SO_3^- (9,10). Further, very little conjugation is expected between the S=O bonds and the skeletal π electrons (11). However, the 1168 cm^{-1} band is, in all likelihood, sensitive to change in the SO_3^- group as indicated by a study of the arsonate analog [p-(p-dimethylaminophenylazo)-benzenearsonic acid] (10). Resonance Raman spectra of this compound are very similar to that of MO and include features closely resembling the 1151 and 1168 cm^{-1} bands. Moreover, bands directly attributable to the arsonate residue are absent. However, protonation of the doubly changed anionic form $-AsO_3^=$ to produce $-AsO_3H^-$ (by lowering the pH from 9.5 to 6.5) increases the frequency of the shoulder by 5 cm^{-1} and increases its intensity relative to the main band by 40%. We propose that the 1168 shoulder in MO spectra shows a similar sensitivity to changes at the anion and that a pronounced effect will only be seen upon destroying the local C_{3v} symmetry of the S=O bonds in water (as with $-AsO_3^= \rightarrow -AsO_3H^-$). Thus, we propose that in solid MO and MO-BSA, ions and/or water molecules are asymmetrically distributed about the SO_3^- group.

A potential additional source of information on the interaction is the intensity redistribution in the spectrum upon complex formation. For example, the intensity of some aromatic ring modes might be sensitive to the degree of π electron overlap resulting from the stacking of a MO phenyl group on a protein aromatic residue, such as the phenolic residue of tyrosine. Similar phenomena have been observed with nuclei acids (12,13). This awaits further study.

DISCUSSION AND CONCLUSIONS: The Raman data are consistent with the results of other studies suggesting that bound MO is in a medium of lower dielectric constant than water and the view that the SO_3^- interacts with a charged region of the protein (14). However, the data goes further by indicating that strong interactions do not occur with the dimethylamino group or the azo group and that positive charge(s) or water molecules or both are asymmetrically distributed about the SO_3^-. The data also provide direct evidence that the bound phenyl groups are not twisted out of the aqueous conformation, in disagreement with a suggestion stemming from binding induced shifts in visible spectra (15).

Although the above conclusions are of intrinsic interest, and can be expected to be amplified on more extensive analyses of the spectra, the most immediate and salient aspect of the data is considered to be the fact that vibrational spectra can be obtained for ligands bound to proteins. An additional important point is that the spectra can be obtained under physiologically relevant conditions. The spectra provide information about changes occurring when particular functional groups of the ligand combine with the protein, providing highly specific information about the nature of the interaction, certainly insofar as the ligand is concerned. These aspects indicate that it should now be possible to exploit more fully the potential of vibrational spectroscopy for providing information about the detailed nature of protein-ligand interactions.

ACKNOWLEDGEMENT: We would like to thank Mr. R. Carriere for careful technical assistance.

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