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RESONANCE COHERENT ANTI-STOKES RAMAN SCATTERING (CARS) SPECTRA OF FLAVIN ADENINE DINUCLEOTIDE, RIBOFLAVIN BINDING PROTEIN AND GLUCOSE OXIDASE

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SUMMARY: Coherent anti-Stokes Raman scattering spectra, in resonance with the isoalloxazine visible electronic transition, have been obtained down to 300 cm^{-1} for flavin adenine dinucleotide, riboflavin binding protein and glucose oxidase, in H_2O and D_2O. Several isoalloxazine vibrational modes can be identified by analogy with those of uracil. Of particular interest is a band at ~1255 cm^{-1} in H_2O, which is replaced by another at ~1295 cm^{-1}, in D_2O. The H_2O band appears to be a sensitive monitor of H-bonding of the N3 isoalloxazine proton to a protein acceptor group. It shifts down by 10 cm^{-1} in riboflavin binding protein, and disappears altogether in glucose oxidase. Other band shifts, of 3-5 cm^{-1}, are similar for the two flavoproteins, and may reflect environmental changes between aqueous solution and the protein binding pockets.

We have reported the resonance CARS spectra of FAD and glucose oxidase, which provided the first vibrational frequencies of flavin in aqueous solution and in an enzyme active site (1). The ordinary Raman spectrum of flavin is swamped by the high fluorescence associated with the chromophore. By generating the Raman signal as a coherent beam of light, CARS spectroscopy permits virtually complete fluorescence rejection via spatial filtering. The CARS amplitude is proportional to the square of the Raman amplitude, and is similarly subject to resonance enhancement (2). Excitation in the electronic absorption bands of flavin derivatives produces resonance enhanced vibrational bands associated with the isoalloxazine rings.

CARS = Coherent Anti-Stokes Raman Scattering
FMN = Flavin Mononucleotide
FAD = Flavin Adenine Dinucleotide

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These can be monitored for evidence of structural change upon protein binding and activation.

Our initial CARS spectra were limited to the region above 1200 cm^{-1}. Lower frequencies were obscured by stray light in the vicinity of the pump laser frequency. This interference has now been substantially reduced with the addition of a second monochromator to the signal filtering system. Herein, we report resonance CARS spectra down to 300 cm^{-1} for FAD and also for riboflavin binding protein and glucose oxidase in H_2O and in D_2O. Frequency shifts are observed which suggest differences in hydrogen bonding of the isoalloxazine N3 proton.

EXPERIMENTAL

We have modified our CARS spectrometer, described in detail in reference (3), in order to achieve low frequency capability. The modification consists of the addition of a second filter monochromator in the optical path of the signal beam to enhance the separation of the CARS beam from the pump lasers. The dual monochromator system, set up in a tandem configuration, is stepped synchronously with the scanning dye laser. The optical bandwidth of the filter monochromator as a whole greatly exceeds that of the dye laser beams, and thus has no effect whatsoever on the spectral resolution. The large gain in optical purity of the CARS signal more than compensates for the rather insignificant decrease in signal intensity introduced by the additional monochromator.

FAD and glucose oxidase were bought from Sigma. Riboflavin binding protein was a generous gift from Professor C.O. Clagett.

RESULTS AND DISCUSSION

CARS spectra of FAD and of riboflavin binding protein in H_2O and in D_2O are shown in Figure 1 and 2. Table 1 contains a list

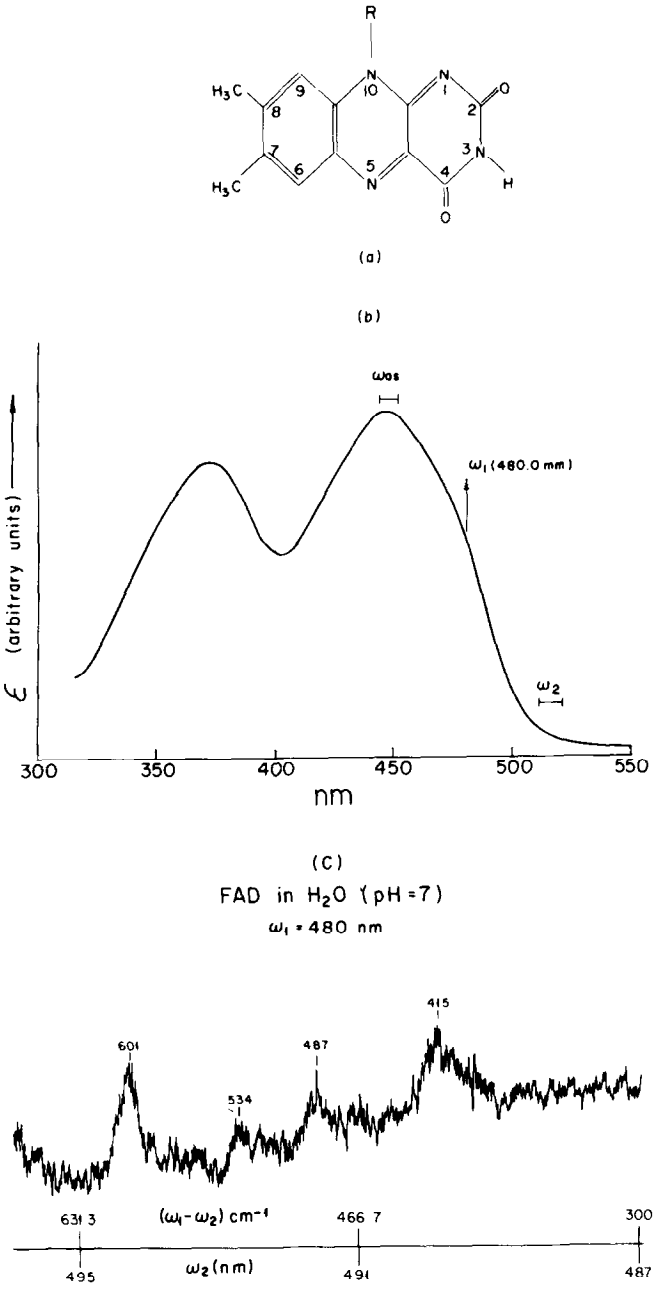

Figure 1 (a) Structure of the isoalloxazine ring in flavins. R is the ribose-phosphate-adenosine unit of FAD.

(b) Absorption spectrum of FAD, showing the positions of ω_1, ω_2 and ω_{as} used to record CARS spectra.

(c) Low-frequency CARS spectra of FAD.

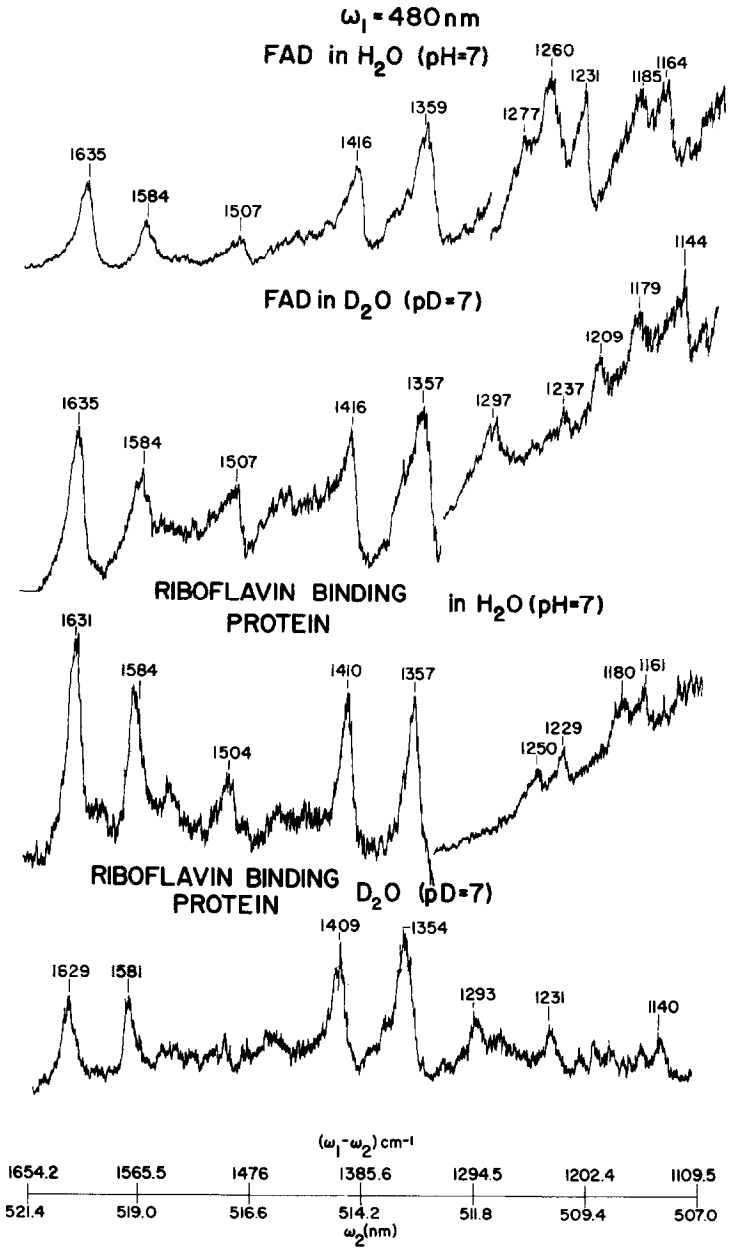

Figure 2. CARS spectra of FAD, deuterated FAD, riboflavin binding protein in H_2O and D_2O; ω_1 = 480 nm, ω_2 scan speed = 0.6 nm/min; laser repetition rate = 10 pulses/sec; 30 pulses averaged. Laser pulse energies of ω_1 and ω_2 were ~10-25 μJ at the sample.

TABLE I

CARS PEAK FREQUENCIES, cm^{-1}

FAD		Riboflavin Binding Protein		Glucose Oxidase	
H_2O	D_2O	H_2O	D_2O	H_2O	D_2O
1164w	1144	1161	1140	1158	1137
1185w	1179w	1180		1182	1169
	1209w	1229	1231	1230	
1231w	1237w	1250	1293		1287
1260m	1297m			1345	1348
1277vw		1357	1354	1364	1364
1359s	1357s	1410	1409	1404	1408
1416s	1416s	1504		1501	1502
1507m	1507m	1584	1581	1578	1578
1584s	1584s	1631	1629	1626	1627
1635s	1634s				

The absolute positions of the frequencies were determined relative to 992 cm^{-1} of benzene. The values in reference (1) for glucose oxidase were determined without a standard.

of the observed peak frequencies. Figure 1 shows the electronic absorption spectrum of FAD (that of the binding protein is very similar) and a structural diagram of the isoalloxazine chromophore. The CARS pump laser was tuned to 480 nm, where the least distortion from Lorentzian CARS peaks is observed. Although this wavelength is on the side of the visible absorption band, both low temperature spectroscopy and an analysis of CARS lineshapes indicate that it coincides with the origin of the electronic transition (4). The resonance condition produces enhancement of vibrational modes localized on the isoalloxazine ring; no vibrational modes of the N10 substituent (R = ribose for riboflavin, adenosine diphosphate ribose for FAD) are expected or observed. FMN (R = phosphoribose) gives the same resonance CARS spectra (not shown) as FAD, even

though there is some evidence that the adenine and isoalloxazine rings of FAD interact (5). Riboflavin is too insoluble to permit acquisition of its CARS spectrum; there is no reason to think that it would differ from those of FMN or FAD.

The isoalloxazine vibrational spectrum can be analyzed by analogy with those of simpler heterocycles, particularly uracil, isoalloxazine contains the uracil ring, which holds the one readily exchangeable proton, N3-H. The low frequency region (Figure 1c) contains a single strong band, near 600 cm^{-1}, which is unshifted in D_2O. This is probably an in-plane ring-breathing mode; the uracil Raman spectrum shows a similar feature at 784 cm^{-1} (6). Several bands are observed above 1300 cm^{-1}, which are also unshifted in D_2O. Their assignment will require other isotope substitution studies. The two highest energy bands, 1635 and 1584 cm^{-1}, may correspond to C=O stretching modes, their frequencies shifted down from those of uracil, 1679 and 1634 cm^{-1}, by electron delocalization onto the rest of the isoalloxazine ring system.

Marked changes between H_2O and D_2O are observed in the 1100 - 1300 cm^{-1} region, where substantial contributions from N-H deformation are expected. The spectra are sufficiently complex that it is difficult to correlate corresponding bands between H_2O and D_2O. It does appear, however, that a weak but distinct band at ~1160 cm^{-1} in FAD is shifted down by 20 cm^{-1} in D_2O. The most striking change is the replacement of a strong band, which we call band H, at ~1255 cm^{-1} in H_2O with another one, band D, at ~1295 cm^{-1} in D_2O. An apparent 40 cm^{-1} increase on deuterium exchange is puzzling, but there is a striking parallel in the uracil Raman spectrum (6,7): the most intense band in this region is at 1233 cm^{-1} in H_2O, but at 1249 cm^{-1} in D_2O.

Tsuboi's normal coordinate analysis indicates that there is a substantial change in normal coordinate composition for this uracil mode (7). After deuteration it has less N-H bending, and more C=O stretching character.

A 10 cm^{-1} decrease in the band H frequency is observed between FAD and riboflavin binding protein, while the band D difference is only 4 cm^{-1}. We are unable to locate band H at all in the CARS spectrum of glucose oxidase, although band D is observed at 1287 cm^{-1}, 10 cm^{-1} below its FAD frequency. These difference are significant and very likely reflect changes in the hydrogen bonding of N-3 proton, to protein acceptor groups in glucose oxidase and riboflavin binding protein, and to water for FAD. Further analysis of these complex normal modes will be needed to establish the nature of these differences.

There are also 3 cm^{-1} to 5 cm^{-1} frequency decreases between FAD and the binding protein for bands at 1635, 1416, 1185 and 1164 cm^{-1}. Similar shifts are observed in glucose oxidase (Table 1), and may reflect the environmental change between aqueous solution and the protein binding pockets.

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References

1. Dutta, P.K., Nestor, J.R., and Spiro, T.G. (1977) Proc. Natl. Acad. Sci. (USA) 74, 4146-4149.
2. Begley, R.F., Harvey, A.B., and Byer, R.L. (1974) Appl Phys. Lett. 25, 387-390.
3. Nestor, J. (1978) J. Raman Spect., in press.
4. Dutta, P.K., and Spiro, T.G. (1978) J. Chem. Phys., in press.
5. Kyogoku, Y. and Yu, B.S. (1968) Bull. Chem. Soc., Japan, 41, 1742.

6. Lord, R.C., and Thomas, G.J., Jr. (1967) Spectrochim. Acta 23A, 2551-2591.

7. Tsuboi, M. (1973) in Physico-Chemical Properties of Nucleic Acids, ed. Duchesne, J. (Academic Press, New York), pp. 91-146