# Raman spectroscopy of acetylcholine receptor-rich membranes from *Torpedo marmorata* and of their isolated components

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## 1. INTRODUCTION

Raman spectroscopy is a particularly powerful technique to explore the three-dimensional organisation of biological molecules. With proteins, this method lead to the detection of differences in conformation affecting both the backbone and the amino acid side chains [1]. In particular, the analysis of the conformationally sensitive Amide I and Amide III regions of the spectra offered new insights and predictions on the secondary folding of the polypeptide chain. Also, the vibrations of the amino acid side chains, in particular those of the aromatic amino acids tyrosine and tryptophan, brought original information on the state of these residues in proteins [2,3].

With simple lipids and model membrane systems the Raman active (C–H) and (C–C) stretching modes have been used to monitor lateral mobility and *trans-gauche* isomerism [4,5]. Various lipid–protein complexes have also been the object of intense Raman studies [6–8] and the analysis extended to biological membranes from human and rabbit erythrocyte ghosts [9,10], thymocytes [11], sarcoplasmic reticulum [12], hamster lymphocytes [13] and human blood platelets [14].

*Abbreviations:* AcCh, acetylcholine; AcChR, acetylcholine receptor; DDAO, dimethyldodecylamine oxide

Within the framework of the vibrational spectroscopic studies on the conformation of acetylcholine (AcCh) and related compounds (review [15]), and of the analysis of the physiological action of these ligands at the molecular level (review [16]), we have undertaken a laser Raman spectroscopic investigation of the subsynaptic membrane and of its main components. Here we present Raman spectra of membrane fragments rich in acetylcholine receptor (AcChR) purified from *Torpedo marmorata* electric organ, and of some of their macromolecular and lipidic components. Our data provide original information on the conformation of the proteins and of the lipids which compose these subsynaptic membrane fragments.

## 2. MATERIALS AND METHODS

### 2.1. *Biological materials*

#### 2.1.1. Preparation of AcChR-rich membranes

AcChR-rich membranes were purified from freshly dissected electric organs from *T. marmorata* as in [17], in the presence of protease inhibitors and chelating agents to limit proteolysis (buffer A; 50 mM Tris-HCl (pH 7.5), 3 mM EDTA, 1 mM EGTA, 0.1 mM PMSF, 5 units/ml aprotinin, 5 $\mu$g/ml pepstatin; see [18]). The mem-

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branes were stored in liquid nitrogen at a concentration of 20–25 $\mu$M $\alpha$-toxin sites, and thawed at room temperature immediately before use. Specific activities ranged from 1500–2500 nmol $\alpha$-toxin sites per g protein.

2.1.2. Alkaline extraction of the 43 kDa proteins

Alkaline extraction of the 43 kDa and other non-receptor proteins were performed basically as in [19] but at 4°C. The alkaline treated membranes were pelleted, neutralized by resuspension of the pellet in a large volume of buffer B (10 mM Tris-HCl, pH 7.5) and finally resuspended to a final concentration of about 20–30 $\mu$M $\alpha$-toxin sites, after an additional centrifugation. The soluble alkaline extracted material was neutralized upon controlled addition of HCl resulting in the aggregation of essentially the 43 kDa proteins, which were further resuspended after centrifugation in buffer B.

2.1.3. Detergent extraction of the AcChR protein

The AcChR protein was extracted from the membrane bilayer using either the detergents Na cholate or dimethyldodecylamine oxide (DDAO). Alkaline-treated membranes (about 10 mg protein/ml) were first supplemented with 0.6% 2-mercaptoethanol and 3% (w/v) Na cholate or DDAO, shaken for about 1 min and centrifuged at 100000 $\times$ $g$ for 20 min to remove the insoluble material. Further purification was achieved upon centrifugation of the soluble material on 5–20% sucrose gradients supplemented with either 1% Na cholate or 0.5% DDAO, at 40000 rpm for 16 h in a Beckman SW 41 rotor. The fractions corresponding to the 9 S 'light' form of AcChR were dialysed for 5 h against buffer B supplemented with either 1% Na cholate or 0.5% DDAO to remove sucrose, and finally concentrated about 10-times in a 5-ml Diaflo cell using an Amicon XM 30 membrane. Final protein concentration was about 20–25 mg protein/ml, and SDS gel analysis discloses almost exclusively the 4 polypeptide chains of the AcChR.

2.1.4. Assays

$\alpha$-Toxin binding site concentration was determined with $^3$H-labelled *Naja nigricollis* $\alpha$-toxin as in [20]; proteins were assayed as in [21] using bovine serum albumin as a standard; phospholipids were assayed as in [22].

2.1.5. Chemicals

$^3$H-labelled *N. nigricollis* $\alpha$-toxin was a gift from Dr A. Menez; DDAO was from the CEA (France); Na cholate was from Merck and other chemicals from Sigma.

2.2. *Raman spectra*

All the samples (except the purified soluble AcChR) were washed twice before use by centrifugation at full speed in a Beckman airfuge and resuspension in buffer B. Ten $\mu$l of concentrated materials (20–35 mg protein/ml) were introduced into capillary tubes (1 mm diameter) placed within a holder whose temperature was controlled by a thermoelectric cooling system (8°C).

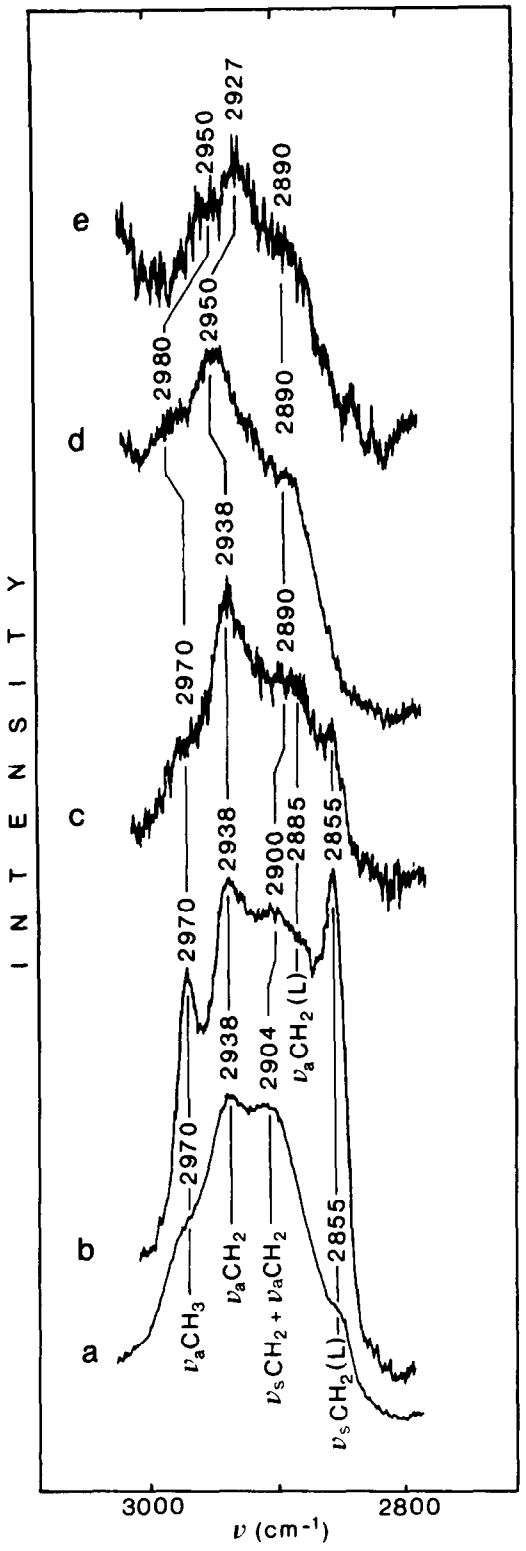
Raman spectra were recorded with a Coderg PHO spectrometer, using a Coherent Model CR-2 argon laser tuned at 488 nm with a power at the sample level of about 400 mW. The spectral slit-width was of the order of 8 $cm^{-1}$.

## 3. RESULTS AND DISCUSSION

Raman spectra of the AcChR-rich membranes fragments and of some of their isolated components, including the detergent extracted AcChR and the alkaline extracted 43 kDa proteins, were recorded in the 400–3000 $cm^{-1}$ range and are presented in fig. 1, 2. Although a significant fluorescence background was sometimes observed in the low-frequency range (see for instance the 400–800 $cm^{-1}$ domain in fig.2a) very intense and well resolved peaks were recorded at the frequencies indicated in the figures. The positions and relative intensities of these peaks were found to be reproducible among the various membrane preparations tested and were therefore analyzed in detail in relation with the protein and lipid composition of the different samples and of the possible interactions between these elements.

### 3.1. *(C–H) stretching vibrations (2800–3000 $cm^{-1}$) and lipid–protein interactions*

It is well established that the characteristic protein vibrations corresponding to the aliphatic (C–H) stretching vibrations of the amino acid side chains take place near 2900, 2940 and 2980 $cm^{-1}$,




and correspond respectively to the methylene symmetric ($\nu_s CH_2$), methylene asymmetric ($\nu_a CH_2$) and methyl asymmetric ($\nu_a CH_3$) vibrations [11]. On the other hand the characteristic lipid vibrations have been established without ambiguity near 2850 cm$^{-1}$ ($\nu_s CH_2$), 2885 cm$^{-1}$ ($\nu_a CH_2$) and 2930 cm$^{-1}$ ($\nu_a CH_2$) [23].

The spectrum of the AcChR-rich membranes (fig.1a) discloses a very strong band near 2940 cm$^{-1}$ and two shoulders near 2904 and 2970 cm$^{-1}$. These frequencies correspond to the protein vibrations in this region. On the other hand, a pronounced shoulder is observed at about 2855 cm$^{-1}$. It most likely corresponds to the $\nu_s CH_2$ vibration of the membrane lipids. The relatively high value of the protein intensities compared to the lipid ones can be related to the exceptionally high protein: lipid ratio of the AcChR-rich membranes, which reaches values as high as 2.2 (w/w) (see [24]). The low intensity of the lipid vibrations might also result from the significant lateral packing of the lipid side chains resulting itself from the dense packing of the AcChR proteins in the subsynaptic membrane (see [25]).

As shown in fig.1a–e, the characteristic protein vibrations are observed to be essentially unmodified with the alkaline treated membranes (fig.1c), but are shifted in the case of the extracted 43 kDa proteins (fig.1e) and solubilized AcChR (fig.1d; see also the increased intensity of the 2970 cm$^{-1}$ vibration in fig.1b). These modifications in frequency and intensity of the $\nu$ (C–H) vibrations are most likely relevant to changes in the protein–protein and/or protein–lipid interactions consecutive either to detergent extraction in the case of the purified AcChR or to partial denaturation and/or aggregation in the case of the isolated 43 kDa proteins.

Particularly evident are the differences in both characteristic lipid vibrations (near 2855 and 2885 cm$^{-1}$) between the various samples tested.

Rather paradoxically, a very intense 2855 cm$^{-1}$

Fig. 1. Raman spectra in the (C–H) region of (a) AcChR-rich membrane fragments, (b) DDAO extracted purified AcChR, (c) alkaline treated membranes, (d) Na cholate extracted purified AcChR, (e) alkaline extracted 43 kDa proteins. Excitation wavelength 488 nm, power at sample level 400 mW, spectral slits 8 cm$^{-1}$, temperature 8°C.

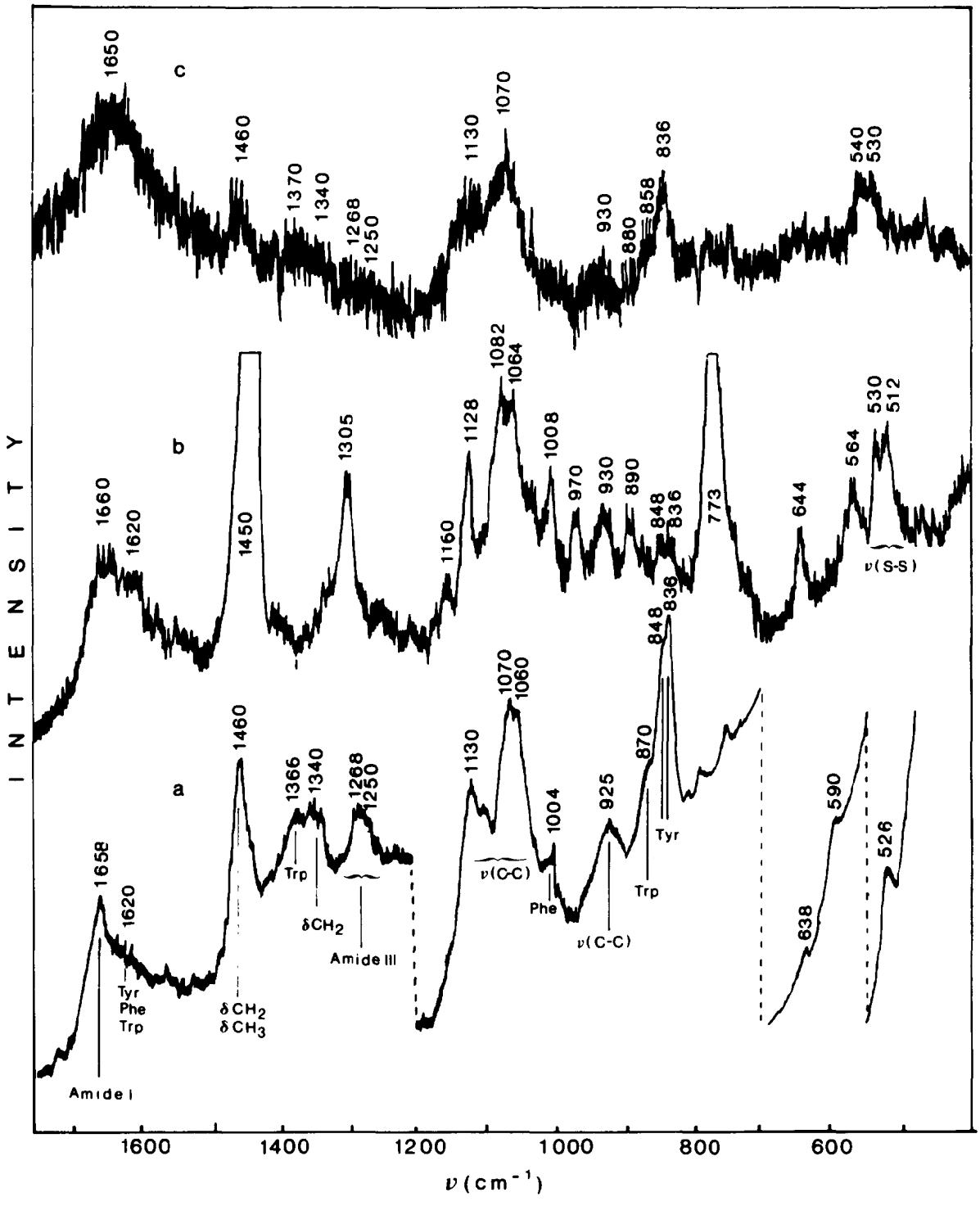



Fig. 2. Raman spectra in the 400–1700 $cm^{-1}$ range of (a) AcChR-rich membrane fragments, (b) DDAO extracted purified AcChR, (c) alkaline extracted 43 kDa proteins. Recording conditions as in fig. 1.

vibration is clearly detected with the soluble AcChR extracted in DDAO (see fig.1b). This strongly suggests that lipids remain associated with the 9 S form of the AcChR purified in the presence of this particular detergent, an interpretation confirmed by direct assay of the phospholipid content of the purified preparation of AcChR (about 50 mol phospholipid/mol protein). The value of the ratio ($I_{2885}/I_{2855}$) of the vibration intensities near 2885 and 2855 $cm^{-1}$ provides information on the physical state of the lipids and of their chain mobility [5,27]. Interestingly, the low value of the $I_{2885}/I_{2855}$ ratio noticed with the purified receptor is a strong indication of a disordered conformation of the lipid chains and of a reduction of their lateral packing. Chemical (presence of detergent) and/or physical (disruption of the membrane bilayer, change in lateral pressure, etc.) factors would then prevent lipids from crystallizing into the gel phase, even at the temperature of the experiment which was as low as 8°C (as expected, no change could be detected when the temperature was raised up to 40°C).

Alkaline extraction of the 43 kDa proteins from the AcChR-rich membranes also results in an increase of the 2855 $cm^{-1}$ vibration in the membrane, and further reveals a stronger intensity of the vibration near 2885 $cm^{-1}$ (see fig.1c). This suggests that important changes in the lipid–lipid and/or lipid–protein interactions take place at the level of the membrane as a consequence of the release of the 43 kDa proteins. These changes might be due to (1) the removal of the 43 kDa proteins themselves or (2) chemical alterations of the membrane lipids during alkaline treatment. The second alternative appears unlikely since alkaline treatment was performed at low temperature as described in [26], and since no change in the Raman spectra could be detected upon further addition during treatment of bovine serum albumin (0.1 mg/ml lipid-free) to trap possible lyso derivatives of the membrane lipids (see [26]). The relatively high intensity of the 2885 $cm^{-1}$ vibration in the alkaline treated membranes strongly suggests that under the conditions of the experiment (i.e. 8°C) the lipids are in an ordered crystal-like state. Preliminary studies on the effect of temperature further show that a transition takes place near 23°C which may correspond to a gel–liquid crystal phase transition.

Finally, the 2855 $cm^{-1}$ vibration is no longer detected with the alkaline extracted 43 kDa proteins (fig.1e) nor with the Na cholate solubilized and purified AcChR (fig.1d). This result is consistent with the extrinsic nature of the 43 kDa proteins which are not embedded in the lipid bilayer and can be extracted without detergent (see [19]). This is also expected from the low lipid: protein ratio of the AcChR in Na cholate as derived by direct phospholipid assay (about 3–10 mol/mol). Disordered lipids thus remain associated with the AcChR in the presence of DDAO but not in the presence of Na cholate. This observation can be related to the finding that in reconstitution experiments lipids are essential to maintain the AcChR in a functional state [28]. Also low-affinity binding of non-competitive blockers of the permeability response have been postulated to occur at the interface between the AcChR protein and membrane lipids [28]. Thus the lipids which remain associated with the AcChR may play a critical role in the regulation of its functional properties.

### 3.2. *Characteristic protein vibrations in the 400–1700 $cm^{-1}$ range*

#### 3.2.1. Amide I and Amide III bands

The Amide I and Amide III frequencies have been widely used as indicators of the secondary structure of proteins [29–32]. It has further been pointed out that a sharp and intense Amide I band indicates uniform H-bonding while a broad band suggests a wide range of peptide conformations [2,29,33].

The Raman spectrum of the AcChR-rich membranes (fig.2a) discloses a sharp Amide I vibration centered at 1658 $cm^{-1}$, strongly suggesting predominantly a $\alpha$-helical conformation [29]. This is further strengthened by the Amide III modes which appears as a strong band at 1268 $cm^{-1}$, characteristic of the $\alpha$-helical conformation. A shoulder is however observed near 1250 $cm^{-1}$, which indicates the presence of a small fraction of the random-coil conformation [29].

Analysis of the spectrum of the DDAO extracted AcChR (fig.2b) discloses the Amide I band at about the same frequency (1160 $cm^{-1}$) as in the native membranes (1658 $cm^{-1}$), but the peak is broader and less intense. Authors in [34] have also observed similar effects (broadening of the Amide

I band without shift in frequency) on lysozyme in the presence of interacting phospholipids. This result might thus be accounted for by changes in the interactions between the AcChR and the still associated phospholipids (see above). The presence of an intense peak at 1305 $cm^{-1}$ in the spectrum obscured the Amide III vibrations especially the region specific for the $\alpha$-helical conformation. The only peak visible is a small one at 1255 $cm^{-1}$ indicating the presence of random coil conformation.

Analysis of the spectrum of the 43 kDa proteins (fig.2c) reveals modified Amide I (broad and shifted) and Amide III (broad and structureless) bands. Since similar effects in proteins were interpreted in [1] as a result of denaturation, it appears likely that the 43 kDa proteins after extraction and resuspension are in a largely disordered conformation.

3.2.2. Aromatic amino-acid ring vibrations

It has been suggested that the intensity of the well-known tyrosine doublet near 850 and 830 $cm^{-1}$ is sensitive to conformational changes [35]. The intensity ratio $I_{850}/I_{830}$ is related to the environment of the tyrosine side chains and should be close to 1.25 for 'exposed' tyrosine residues with moderate hydrogen bonding, and close to 0.5 for strongly hydrogen-bonded 'buried' residues [3]. In the spectrum of the native membranes (fig.2a), the vibration near 848 $cm^{-1}$ appears as a shoulder of the very intense vibration near 838 $cm^{-1}$, suggesting buried tyrosine residues. This remains unchanged in the spectrum of the 43 kDa proteins (fig.2c) while it changes significantly in that of the DDAO extracted AcChR (fig.2b). The overall intensity of the tyrosine doublet appears weaker and the 848 $cm^{-1}$ vibration is at least as intense as the 836 $cm^{-1}$ vibration. It suggests that in the soluble AcChR some of the tyrosine residues are exposed.

Tryptophan vibrations are also sensitive to local environment. Thus, the increase or decrease in intensity of the vibration near 1360 $cm^{-1}$ suggests buried or exposed tryptophans respectively [36]. On the other hand, it has been proposed [37] that the intensity of the vibration near 870 $cm^{-1}$ can also be used as an additional probe for tryptophan environment. Both characteristic vibrations are visible in the spectra of the native membranes and of the alkaline extracted 43 kDa proteins (fig.2a,c), suggesting mostly buried residues. In contrast, they are undetectable in the spectrum of the DDAO extracted AcChR (fig.2b) suggesting, as for tyrosine, that most of the residues are exposed.

3.2.3. Disulfide bonds

The (S-S) stretching vibrations associated with the disulfide bridge can be observed close to 530 and 540 $cm^{-1}$ for the 43 kDa proteins, and close to 510 and 530 $cm^{-1}$ for the DDAO extracted AcChR (fig. 2b,c). According to [38,41], these slight shifts may be relevant to differences in the conformation of the cysteine bridges.

3.3. *Other vibrations*

The Raman frequencies associated with the coupled skeletal stretching vibrations, $\nu$(C–C) and $\nu$(C–N), are sensitive to the amino-acid incorporation into the polypeptide chain [38] as well as to the conformational state of the lipid hydrocarbon chains [39]. A vibration at 1130 $cm^{-1}$ is indicative of all-*trans* chain segments (ordered state), and a band near 1090 $cm^{-1}$ (for lipids) and 1070 $cm^{-1}$ (for proteins) of chains containing *gauche* conformations [23,39]). According to the spectra in fig. 2, the *gauche* conformation of the side chains is the predominant one. The 1082 $cm^{-1}$ peak in the spectrum of the DDAO extracted AcChR most likely corresponds to the symmetric $PO_2$ stretching band of phospholipids ([40] and see above).

The vibration near 1460 $cm^{-1}$ which belongs to the (C–H) deformation modes, $\delta CH_2$,$\delta CH_3$, is a mixture of protein and lipid vibrations. It is sensitive to the chain conformation [42], and becomes very intense in the spectrum of the AcChR (fig.2b) whereas it is a broad and weak peak in the spectrum of the 43 kDa proteins (fig.2c).

Other vibrations can be detected, essentially with the DDAO extracted AcChR (fig.2b). The strong vibration at 1305 $cm^{-1}$ has also been observed with pure lipids [23] and has been assigned to an in-plane methylene twisting motion. The vibration at 1160 $cm^{-1}$ would be assigned to a $\nu$(C–N) motion [43], and the vibration near 1160 $cm^{-1}$ (also visible in fig.2a) to a breathing vibration of phenylalanine [44]. The very intense band at 773 $cm^{-1}$ remains poorly understood at present as well as the one of 564 $cm^{-1}$. Both vibrations at 970 and 890 $cm^{-1}$ are probably the $\nu$(C–C) vibra-

tions of the lipid molecules and the band at 644 $cm^{-1}$ might be a mixture of tyrosine and $\nu$(C-S) vibrations.

## 4. CONCLUSION

The first results on the Raman spectroscopy of the AcChR-rich membranes from *T. marmorata* electric organ clearly show that the method is particularly well suited to explore a wide variety of structural features characteristic of these membranes and of their isolated components. In particular, the method reveals a rich content of $\alpha$-helix in the case of the AcChR, and of random-coil conformation in the case of the isolated 43 kDa proteins. It also indicates that some tyrosine and tryptophan residues of the AcChR protein become exposed upon solubilization by the non-denaturing detergent DDAO. The analysis of vibrations characteristic of the lipids further confirms that, in the native membrane, the lipid:protein ratio is particularly low and that the state of the membrane lipids may change upon extraction of the 43 kDa proteins. It also shows that, after solubilization and purification by the detergent DDAO, lipids remain associated with the AcChR protein. Raman spectroscopy may thus be applied to the analysis of the ligands interactions with the AcChR protein, of the conformational changes of the AcChR which take place under these conditions, and of the functional relationships between the AcChR and neighbouring lipids.

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