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Investigation on native vesicles containing the nicotinic acetylcholine receptor using FTIR-spectroscopy

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Abstract

The ligand gated ion channel nicotinic acetylcholine receptor is responsible for the electrochemical signal transduction in nerve cells and at the motor endplates. In the recent years the structure of the channel has emerged to a resolution of 4.6 Å [J. Mol. Biol. 288 (1999) 765]. We have used ATR–FTIR and SEIRA spectroscopy to investigate the extramembraneous structure of the receptor. The adsorption of nicotinic acetylcholine receptor rich vesicles on the surface of Ag-cluster leads to the detection of high content of helical structure in the extra membranous parts of the receptor. Spectra indicate a β-sheet structure perpendicular to the crystal plane.

Keywords: Ion channel; nAChR; FTIR-spectroscopy; SEIRA spectroscopy

1. Introduction

Changes of protein structure induced by ligands are often minor and difficult to detect by FTIR-spectroscopy. A solution to these problems might be the use of a method, which enhance the IR signal [1]: Isolated Ag-cluster, which are very much smaller than the wavelength of light are placed on IR transparent material. The island effect of the Ag-cluster is essential for the SEIRA spectroscopy [2]. The incident IR beam polarises the Ag-cluster and induces a dipole moment $P = \alpha VE$, where α is the polarizability, V the volume of the clusters and E is the amplitude of the incident electromagnetic field. The dipole induces an electromagnetic field around the cluster with the amplitude of the field E: $E = 2P/d^3$, with d as the distance from the centre of the clusters. The electromagnetic field is much stronger than the incident field, which leads to an enhancement of the bands in the spectra of molecules, which are in the immediate vicinity of the cluster.

The enhancement factors of particular vibrations (relation of the intensity of a vibration with Ag-cluster to a vibration without) are usually around 10–500. However, the enhancement effect rapidly declines with increasing distance from the cluster surface. Experiments with lipid membranes showed that the optimised enhancement is achieved for a monolayer of lipid (thickness ca. 2.5 nm thick) [3]. Experiments with *p*-nitrothiophenol support the finding of decreasing SEIRA effect with increasing distance from the cluster surface [4]. Especially vibrations with a transition dipole moment perpendicular to the cluster surface will be enhanced [5].

In an earlier study, it was shown that the spectra of a protein recorded with Ag-clusters might experience a shift of the overall amide I band envelope [6]. In this

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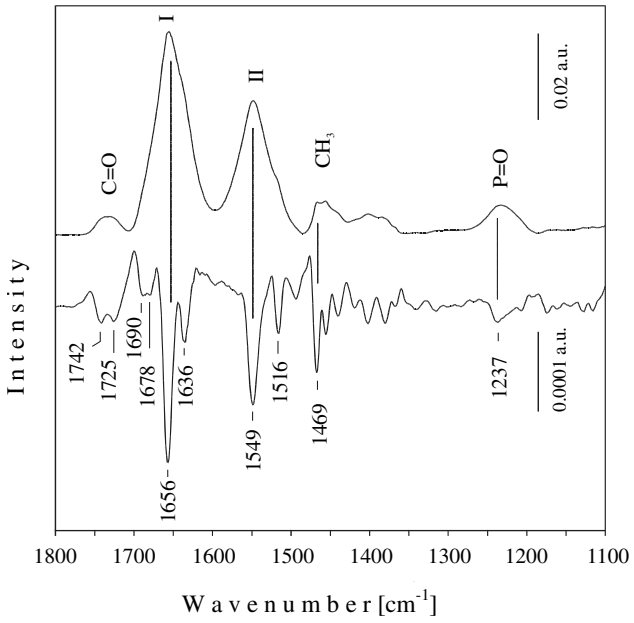

Fig. 1. Transmission spectra of a suspension of nAChR rich vesicles in solution (top trace). Second derivative spectra (17 point, Savitzky-Golay) (bottom trace).

study extremely diluted samples of protein rich vesicles were placed either above or below a layer of Ag-cluster. However, these spectra were recorded in a dried stage. With the present study the investigations are extended to the analysis of the adsorption of protein rich vesicles onto Ag-cluster coated Ge crystals in an aqueous environment.

The protein rich vesicles consist of the nicotinic acetylcholine receptor (nAChR). The nAChR is the structurally best-characterised prototype of the ligand-gated neuroreceptors [7–9], which is responsible for the electrochemical signal transduction in nerve cells and at the motor endplates. FTIR-spectroscopy has also been used to identify the content of individual secondary structural elements [10–12].

2. Experimental

nAChR rich vesicles were prepared from the electric organ of the ray *Torpedo marmorata*. Approximately 200 g of the electric organ were homogenized and enriched with the receptor via several steps of centrifugation [13]. nAChR rich vesicles (ca. 3 mg ml^{-1}) with ca. 5 nM receptor per mg were obtained [14]. The vesicles were treated with a pH 11 extractions to remove the 43 kDa protein and dissolved in NaCl (250 mM), KCl (5 mM), and NaH_2PO_4 (5 mM) at pH 7.4. Aliquots of 0.5 ml were stored at −80°C. Activity of acetylcholine esterase using an activity test [15] was not observed. For the measurements ca. 200 μl of the suspension were washed twice by centrifugation at 8000 U min^{-1}, 2°C and for 3 min (Eppendorf 5417-R) and suspension in buffer of NaCl (150 mM), $CaCl_2$ (2 mM), $MgCl_2$ (2 mM), KCl (4 mM), and HEPES (10 mM) at pH 7.

FTIR-spectra were recorded either on a BRUKER IFS 88 equipped with a MCT-detector (FTIR–ATR and SEIRA) or a Nicolet 5 PC with DTGS-detector (transmission). The spectrometer was purged with dry air (dye point −70°C). The spectral resolution was 2 cm^{-1} using, a Happ–Genzel apodization function. No zero-point filling was applied. 1024 interferograms were added for one spectrum representing ca. 3 min of data collection (FTIR–ATR and SEIRA). Spectra in transmission mode represent an average over 512 interferograms. All spectra were compensated for the contribution of buffer in the spectra by interactive subtraction of buffer spectra until a straight baseline was obtained for the water band at

Table 1
Band positions and percentage contribution of the sub bands of the amide I band envelope. Secondary structures are adopted from Ref. [11]

Band position	% In solution (CaF_2-windows)	Band position	% on Ge	% on Ge/Ag	Description
1690	4	1690	2	2	Turns
1678	11	1678	13	19	β-Sheet
1666	16	1669	4	1	Turns
1656	28	1656	31	51	α-Helix
1647	12	1645	6	6	Unordered
1636	25	1635	45	22	β-Sheet
1624	4	–	–	–	β-Sheet

2300 cm^{-1}. Spectra were also compensated for contributions of water vapour by interactive subtraction of a water vapour spectrum recorded on the same day under the same spectral parameters [16]. The spectra in the figures represent an average of five consecutive spectra. For these averaged spectra a multiple point baseline correction was applied. For analysing the spectra GRAMS 3.0 software (Galactic Industries, US) was used. To analyse the amide I band contour a curve fitting routine (Curve Fit, GRAMS 3.0) was applied to obtain the integrated areas of the sub bands. Band positions were taken as fixed input parameters in the fit routine derived from the second derivative spectra. The sub bands were fitted by a variation of height and half width. Samples were adsorbed on one side of a Ge crystal ($72 \times 4 \times 6\ mm^3$) with an angel of incidence of 45°. For recording the SEIRA spectra the Ge crystal was covered with a layer up to 3 nm of Ag-cluster with a cover rate of 5 Å s^{-1}. Spectra were recorded at 22°C. For recording the transmission spectra CaF_2 windows were separated by a 10 μm thick teflon film (Goodfellow, Bad Nauheim, Germany).

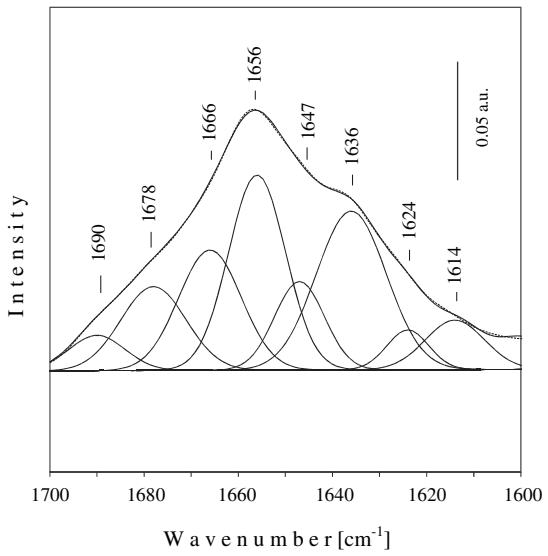

Fig. 2. Deconvolved spectra from Fig. 1 ($\gamma = 2.4$, FWHH = 11.26, Smoothing 75%) including the sub bands from the curve fit routine (Gaussian). Dotted line: summation over all sub bands.

3. Results

As a reference for the analysis of changes in the secondary structure of the nAChR adsorbed to the surface of either germanium (Ge) or germanium coated with silver clusters (Ge/Ag) spectra of nAChR, rich vesicles were recorded in solution. In these spectra structural changes induced by the surface are negligible. Fig. 1 shows nAChR rich vesicles in solution in the amide I and II region. Maxima are found at 1656 cm^{-1} (amide I) and 1549 cm^{-1} (amide II), which are typical for proteins with a considerable amount of α-helix [17]. The second derivative spectra resolves bands at 1690 (turns), 1678 (β-sheet), 1656 (α-helix), and 1636 cm^{-1} (β-sheet) in the amide I region and two bands at 1549 cm^{-1} (α-helix) and 1516 cm^{-1} (see Table 1). Above 1700 cm^{-1} two carbonyl bands (C=O) at 1742 and 1725 cm^{-1} are visible. The contribution of the sub-bands to the overall band envelope of amid I band is given in Fig. 2. The relative content of the sub-bands and their correspondence to secondary structural elements are listed in Table 1. The helical content counts for ca. 28% of the complete band envelope. The second most prominent band corresponds to the 1636 cm^{-1} band

Table 2
Contribution of the secondary structures in %. Values are summarized from Table 1

IR material	α-Helix	β-Sheet	Turns	Unordered
CaF_2-windows	28	40	20	12
On Ge	31	57	6	6
On Ge/Ag	51	41	3	6

assigned to a contribution of β-sheet with 25%. However, other β-sheet bands like the bands at 1624 cm^{-1} (4%) and 1678 cm^{-1} (11%) add up to a total contribution of ca. 40% β-sheet (see Table 2) in the receptor. Structures like turns (1690 cm^{-1} (4%) and 1666 cm^{-1} (16%)) and random coil (1647 cm^{-1}) contribute in total to 19% and 12%, respectively. These findings are in agreement with data from other groups [10,11].

Fig. 3 shows the spectra of nAChR rich vesicles adsorbed on Ge and Ge/Ag within the first 15 min

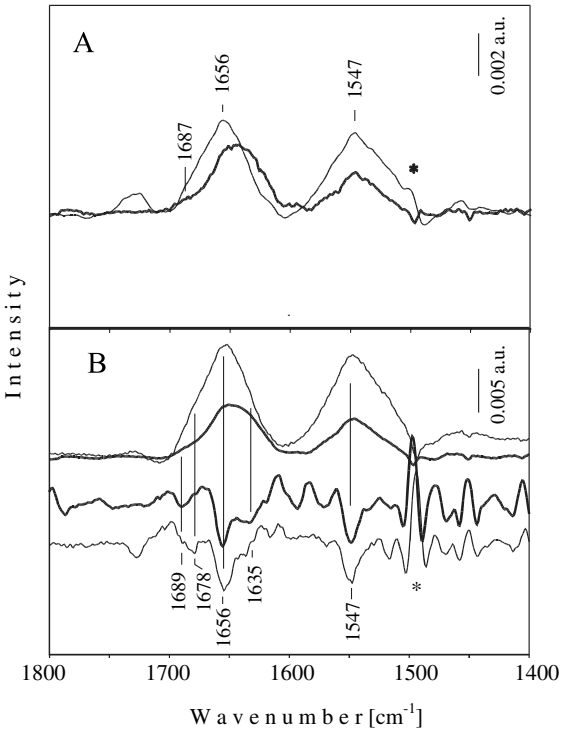

Fig. 3. Spectra of adsorbed nAChR rich vesicles recorded with Ge (thick line) and Ge/Ag (thin line) after 15 min (A) and 1 h (B) of adsorption. In (B) second derivative spectra of the traces (17 points, Savitzky Golay). *Band artefacts due to uncompensated baseline.

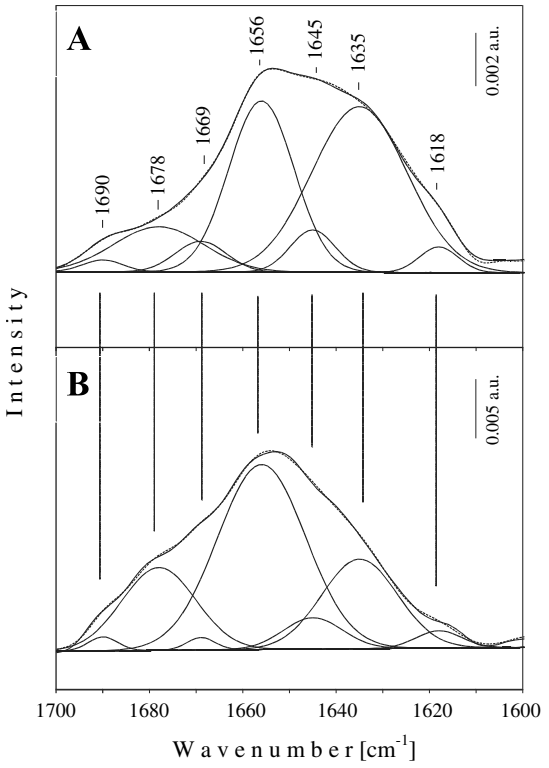

Fig. 4. Deconvolved spectra from Fig. 3B ($\gamma = 2.4$, FWHH = 11.26, smoothing 75%) including the sub bands from the curve fit routine (Gaussian). Dotted line: summation over all sub bands. (A) Recorded with Ge, (B) recorded with Ge/Ag.

(Fig. 3A) and after 1 h (Fig. 3B). The spectra recorded on Ge (Fig. 3A thick line) within the first 5 min exhibit the amide I and II bands at ca. 1650 and 1547 cm^{-1}, respectively. The overall shape of the amide I band is not symmetrical. Spectra recorded with Ge/Ag (Fig. 3A thin line) show a maximum at 1656 cm^{-1}. After ca. 1 h this intensity distribution within the amide I band envelope is still visible. Second derivative spectra reveal bands at 1689, 1656, and 1635 cm^{-1} (only Ge) for recordings with Ge and Ge/Ag. For the latter an additional band at 1678 cm^{-1} is present. For both spectra the position of the amide II band remains constant at 1547 cm^{-1}.

A quantitative analysis of the band envelopes obtained after 1 h of adsorption reveals the percentage contribution of the individual secondary structural elements on the overall band envelope. Fig. 4 shows the deconvolved spectra recorded on Ge (Fig. 4A) and Ge/Ag (Fig. 4B) after ca. 1 h of adsorption inclusive

the sub-bands obtained after applying a curve fit routine on the deconvolved spectra. The relative percentage values are summarised in Table 1. Adsorption on Ge leads to ca. 31% α-helix. This is less than the contribution of β-sheet with 13 and 45% for the bands at 1678 and 1635 cm^{-1}, respectively. Adsorption on Ge/Ag is accompanied with ca. 51% of α-helical substructure. For β-sheet bands at 1678 and 1635 exhibit values of 19 and 22%, respectively Turn and random coil structure is found each with less than 6% (see Table 2).

To summarise the findings, adsorption of the nAChR rich vesicles on a Ge surface drastically increases the size of the bands due to β-sheet up to 57% compared to 40% recorded in transmission (Table 2). The Ge/Ag surface leads to an increase of the contribution of α-helix 51% compared to 28% recorded in transmission and 30% with the Ge surface. The β-sheet content does not exceed 41% recorded with Ge/Ag but shows an increase to 19% for its high frequency band (1678 cm^{-1}) compared to 13% with Ge surface.

4. Discussion

4.1. Intensity enhancement: SEIRA versus adsorption

During the time course of experiment band intensities of the amide I and II bands increase in both spectra recorded with Ge and Ge/Ag. In case of the adsorption on Ge/Ag intensities of the amide I band increases much faster than for Ge. Intensities are also levelling off at higher values (data not shown). The enhancement factor (ratio between intensities recorded with Ag-cluster and without) increases up to about 2 after ca. 1 h of adsorption. A maximum cannot be found, which should occur if the thickness of the adsorbing layer increases [1]. The amide I band shapes of the spectra of the adsorbing proteins in this study do not change even after ca. 1 h. Subtraction of the spectra recorded after ca. 45 min from those recorded after ca. 1 h do not result in any new band shape (data not shown). These results indicate that the intensities are enhanced during the entire duration of adsorption in this study. SEIRA spectra are obtained from parts of the molecules orientated on the Ag-cluster in a vicinity of ca. 2.5 nm [3]. Thus, one can conclude that the event of sedimentation based adsorption and subsequently the covering of the complete Ge/Ag surfaces with a monolayer of receptor molecules has not yet finished. The data represent parts of the protein in the immediate vicinity of the crystals and under the influence of the field enhancement due to the Ag-cluster.

In a recent study [18] we pointed out that also the different penetration depth of the evanescent wave in the Ge/Ag system compared to a Ge crystal will contribute to the shift of the amide I band. However, this might not completely explain the intensity distributions found for the sub-bands. Further studies using polarised spectroscopy should address this question in more detail.

4.2. Secondary structure assessment

The amide I and II maxima found in this study are typical for proteins with high helical content. Whereas, the amide I band consists primarily of the carbonyl stretching vibration the amide II consists preferentially of the NH in plane bending motion [17]. In case of a SEIRA effect a helix with its axis perpendicular to the surface of the Ag-cluster should give rise to an enhancement effect in such a way that the amide I frequency will be increased compared to its amide II frequency. However, we do not find a difference in the overall intensity distribution between the amide I and II band in the spectra recorded with Ge/Ag and Ge. How can we explain the high helical content? 3_{10}-helical structures give rise to bands around 1666–1662 cm^{-1} with week contributions at 1681–1678 cm^{-1} and 1646–1644 cm^{-1} [19]. Such structure could contribute to the overall band envelope of the amide I band. A high contribution of α-helix was also found by others [12].

In the spectra recorded with Ge/Ag the intensity of the bands at 1678 cm^{-1} is enhanced compared to the band at 1636 cm^{-1}. An opposite distribution is observed in the spectra recorded with Ge. Such an enhancement should be observed if the β-sheet structure is oriented not parallel but in a 90° orientation to the crystal normal. This would orient amid carbonyls perpendicular to the field around the clusters. As a consequence field enhancement should be observed. The enhancement of the high frequency part of the β-sheet vibration is due to the stronger interaction of the

interaction of the in-phase vibration (1678 cm^{-1}) with the field than the out-of-phase vibration (1636 cm^{-1}) [20]. The proposed orientation of the plane of β-sheet substructure perpendicular to the membrane normal supports findings by cryo electron microscopy [9]. From these studies it is evident that a bent β-sheet structure is formed in one of the subunits.

4.3. Contribution of side chains

In the present quantitative assessment the contribution of the side chains on the amide I band envelope [21,22] has not been taken into account. A detailed study of this contribution has been only done in transmission mode and for samples recorded in D_2O [21–23]. Such conditions do not apply in this work. However, in case of the analysis of complex proteins using the enhancement effect a detailed knowledge of the contribution of the side chains needs to be addressed and clarified in the future.

5. Conclusion

The adsorption of protein on the surface of Ag-cluster leads to the detection of high content of helical structure in the extra membranous parts of the nAChR. In addition β-sheet structure is found perpendicular to the crystal plane. The β-sheet bands are influenced by the enhancement effect due to the Ag-cluster. For future applications of the SEIRA spectroscopy the enhancement effect of the amino acid side chains and their contribution to the overall shape of the amide I band has to be considered.

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