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# Protein structural effects of agonist binding to the nicotinic acetylcholine receptor

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The effects on the protein structure produced by binding of cholinergic agonists to purified acetylcholine receptor (AcChR) reconstituted into lipid vesicles, has been studied by Fourier-transform infrared spectroscopy and differential scanning calorimetry. Spectral changes in the conformationally sensitive amide I infrared band indicates that the exposure of the AcChR to the agonist carbamylcholine, under conditions which drive the AcChR into the desensitized state, produces alterations in the protein secondary structure. Quantitative estimation of these agonist-induced alterations by band-fitting analysis of the amide I spectral band reveals no appreciable changes in the percent of α-helix, but a decrease in β-sheet structure, concomitant with an increase in less ordered structures. Additionally, agonist binding results in a concentration-dependent increase in the protein thermal stability, as indicated by the temperature dependence of the protein infrared spectrum and by calorimetric analysis, which further suggest that AcChR desensitization induced by the cholinergic agonist implies significant rearrangements in the protein structure.

*Torpedo* acetylcholine receptor; Fourier-transform infrared spectroscopy; Quantitative estimation of secondary structure; Differential scanning calorimetry; Thermal stability

## 1. INTRODUCTION

The nicotinic acetylcholine receptor (AcChR) from *Torpedo* is a transmembrane glycoprotein composed of four different polypeptide subunits (α, β, γ and δ) in a 2:1:1:1 stoichiometry (see [1–4] for review). Binding of cholinergic agonists to the appropriate binding sites on extracellular domains of the AcChR, elicits the formation of a transient cation channel, responsible for the initiation of postsynaptic membrane depolarization. On continuous exposure to the agonist, however, the channel opening response becomes blocked and the affinity for the agonists increases, a process known as desensitization.

AcChR channel activation and the corresponding functional responses have been described in great detail through the application of biochemical and electrophysiological techniques (see [5] and references therein). On the contrary, the experimental information on the structural differences between the resting, open and desensitized states of the AcChR protein and on the molecular events leading to channel gating or desensitization upon agonist binding, is scarce [6,7]. Fourier-transform infrared spectroscopic (FT-IR) methods have shown great potential to detect structural differences between the various possible conformers of complex membrane proteins [8–11], including the AcChR [12–16]. In this paper, we have made use of the conformational sensitivity of the protein amide I infrared absorbance band [17,18] to probe the effects of agonist binding on structural features of purified AcChR reconstituted into asolectin lipid vesicles. The interference of water infrared absorbance (1645 $cm^{-1}$ [8]) on the protein amide I band has been eliminated by using $D_2O$ instead of $H_2O$ as the solvent. The strong amide I band, comprising the 1600–1700 $cm^{-1}$ spectral region, results primarily from stretching vibrations of C=O groups in peptide bonds [17], the exact frequencies of which depend on the nature of the hydrogen bonding involving the C=O groups which, in turn, is determined by the particular secondary structure adopted by the protein [18]. Thus, the amide I band contours of proteins represent complex composites of spectral components of characteristic frequencies, which have been correlated in $H_2O$ and $D_2O$

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*Abbreviations.* AcChR, acetylcholine receptor; α-Bgt, α-bungarotoxin, FT-IR, Fourier-transform infrared spectroscopy; DSC, differential scanning calorimetry.

with different secondary structures in both, soluble and membrane-bound proteins.

## 2. MATERIALS AND METHODS

Carbamylcholine chloride, deuterium oxide ($D_2O$, 99.9% by atom) and crude extracts of phosphatidylcholine from soybean (type 2-S, asolectin lipids) were purchased from Sigma. [$^{125}$I]α-Bungarotoxin (α-Bgt) was from New England Nuclear.

### 2.1. *Acetylcholine receptor purification and reconstitution*

AcChR-enriched membranes were prepared from the electroplax of *Torpedo marmorata* [19]. The AcChR was purified from cholate extracts of those membranes by affinity chromatography in the presence of asolectin lipids [1]. The purified AcChR had specific activities of approximately 8 nmol of α-Bgt bound per mg of protein.

Plain lipid vesicles used for reconstitution were prepared from asolectin lipids, at ~40 mg/ml, by a CHAPS dialysis procedure [5]. The dried lipids were hydrated for 1 h in 10 mM Tris buffer, pH 7.4, containing 100 mM NaCl and 2% CHAPS, vortexed and sonicated in a probe-type Soniprep 150 apparatus. Samples appeared transparent at the end of sonication. Lipid vesicles were formed by elimination of the detergent by dialysis. The dialyzed samples were resolubilized in 4% sodium cholate and used inmediately for reconstitution.

Reconstituted AcChR samples were prepared by mixing aliquots of purified AcChR with the solubilized lipid vesicles from above. Final concentrations in the reconstitution mixtures were: AcChR, ~1 mg/ml; asolectin phospholipids, ~5 mg/ml; sodium cholate, 1.5%. Under these conditions, the lipid to protein molar ratio ranged from 2000 to 3000. Reconstitution was accomplished by dialysis at 4°C for about 50 h (8 × 1 litre changes in 10 mM Tris, pH 7.4, containing 100 mM NaCl). The reconstituted AcChR retained fully the ability to undergo agonist-induced, affinity transitions between sensitized and desensitized states as well as the characteristic cation flux responses to cholinergic agonists measured by a rapid kinetics, stopped-flow assay of $Tl^+$ influx [20].

### 2.2. *Infrared measurements*

Exchange of water by $D_2O$ in the reconstituted AcChR samples was carried out by submitting the samples to at least two centrifugation–resuspension cycles in $D_2O$ buffers of identical saline composition than the original $H_2O$ media. The final concentration of the samples for FT-IR analysis was adjusted to ~20 mg of protein/ml. FT-IR spectra were taken in a Nicolet 520 instrument equipped with a DTGS detector, as previously described [16]. Standard procedures for Fourier derivation and self-deconvolution of the spectra were carried out [21]. Derivation was performed using a power of 3, breakpoint of 0.3. Self-deconvolution was performed by using a Lorenztian bandwidth of 18 $cm^{-1}$ and a resolution enhancement factor ranging 1.8–2.0.

For quantitative estimation of protein secondary structures, curve-fitting analysis was accomplished by band decomposition of the original amide I band following an improvement of the method previously described [22], whose details have been discussed elsewhere [17]. In brief, after obtaining band positions and an estimation of band widths by Fourier deconvolution and Fourier derivation as described earlier, the iterative process is performed in CURVEFIT running under SpectraCalc (Galactic Industries Corp., Salem, NH), using the height of the original spectrum as the initial intensity estimation. The gaussian fraction is set to 0.5 for all bands and kept fixed, together with band positions, during the first 200 iterations. Then, the gaussian fraction is allowed to change in all bands and another 200 iterations are made. Band positions are also allowed to change for another 20 iterations and the final parameters obtained are used to calculate the percent of band areas, not taking into account the contributions of side chains at 1605 and 1615 $cm^{-1}$. The fits between experimental and theoretical spectra were excellent and exhibited chi-square values ranging $0.7 \times 10^{-5}$ to $4.8 \times 10^{-5}$.

### 2.3. *Differential scanning calorimetry (DSC)*

DSC was performed on a Microcal MC-2 microcalorimeter, as described previously [19]. The difference in the heat capacities between 1 ml aliquots of reconstituted AcChR samples at 1–1.5 mg protein/ml (contained in the 'sample' cell of the instrument) and buffer alone ('reference' cell) were recorded by raising the temperature at a constant rate of 90°C/h. Reported transition temperatures correspond with those at which there is a maximum differential heat capacity, as observed in the original thermograms without any baseline corrections.

## 3. RESULTS AND DISCUSSION

Fig. 1A shows the 1800–1500 $cm^{-1}$ region of the infrared spectra of reconstituted AcChR membranes and plain asolectin lipid vesicles in the $D_2O$ buffer. The absorbance band centered at 1735 $cm^{-1}$ corresponds to vibrations from the carbonyl ester groups of phospholipids, while those centered at 1653 and 1547 $cm^{-1}$ are the amide I band and a residual amide II band, respectively, corresponding mostly to vibrations from the protein's peptide bonds [9,16].

The information provided by the amide I band in the original spectrum is limited by the intrinsic widths of the spectral components contributed by the different protein secondary structures, which are usually larger than their frequency separation and thus, result in spectral overlapping. Nonetheless, these individual components can be visualized after the application of resolution-enhancement, band-narrowing techniques [23–25], such as Fourier self-deconvolution and Fourier derivation (Fig. 1B). On band-narrowing, the amide I region exhibits maxima at 1605, 1615, 1633, 1656, 1680 and 1690

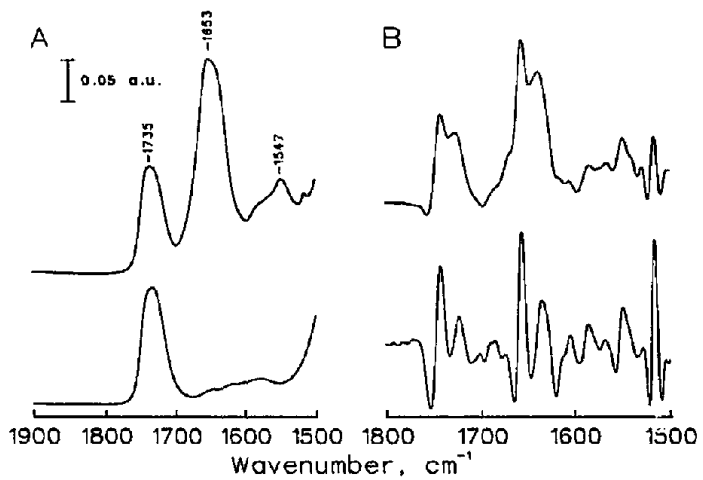


Fig. 1. Infrared absorbance spectrum of purified AcChR reconstituted into asolectin lipid vesicles and resuspended in a $D_2O$ buffer, as indicated under Materials and Methods. The original spectrum of the reconstituted AcChR vesicles and that of plain asolectin lipid vesicles are shown in panel A (upper and lower trace, respectively). Panel B shows the deconvolved and derivative spectra of the reconstituted AcChR vesicles (upper and lower trace, respectively). Bar indicates 0.05 absorbance units. In this and in all the other figures, the spectra of the $D_2O$ buffer alone (without AcChR vesicles) were substracted from those of the vesicle-containing samples and were recorded at 20°C, unless stated otherwise.

$cm^{-1}$. Whereas the 1605 and 1615 $cm^{-1}$ components correspond to amino acid side chain vibration, all the other maxima have been assigned to vibration of the carbonyl group in peptide bonds within different protein secondary substructures [9,16]: the 1656 $cm^{-1}$ band is attributed to α-helix, the 1633 $cm^{-1}$ to β-sheet, the 1690 $cm^{-1}$ to turns and the 1680 $cm^{-1}$ includes contributions from turns as well as from the (0,π) β-sheet vibration band.

Presence of carbamyl holine, a cholinergic agonist, at concentrations ranging $10^{-5}$ to $10^{-2}$ M induces small, reproducible changes in the spectral shape of the amide I band (Fig. 2A). These changes (inset to Fig. 2A) refer to a progressive, concentration-dependent decrease in the absorbance centered at 1653 $cm^{-1}$ and to a concomitant increase in the absorbance at 1642 $cm^{-1}$. On band narrowing, a prominent shoulder appears at 1642 $cm^{-1}$, which partly overlaps with the β-sheet peak at 1633 $cm^{-1}$ and is not detected in the absence of carbamylcholine, along with other spectral changes at the 1700–1670 $cm^{-1}$ region (Fig. 2B). Nevertheless, securing quantitative information on the protein secondary structural changes responsible for the observed alterations in spectral shape, requires the application of quantification methods based either on curve-fitting of the spectra or on the use of calibration sets (for a discussion on quantification methods, see reference [17]). Band-fitting analysis of the amide I spectral band (Table I) indicates that carbamylcholine does not affect the percent of α-helical structure in the AcChR protein, represented by the band at 1656 $cm^{-1}$ and which accounts for ~43% of the secondary structure in all cases. Carbamylcholine, however, produces a significant decrease in extended structures such as the β-pleated sheet at 1633

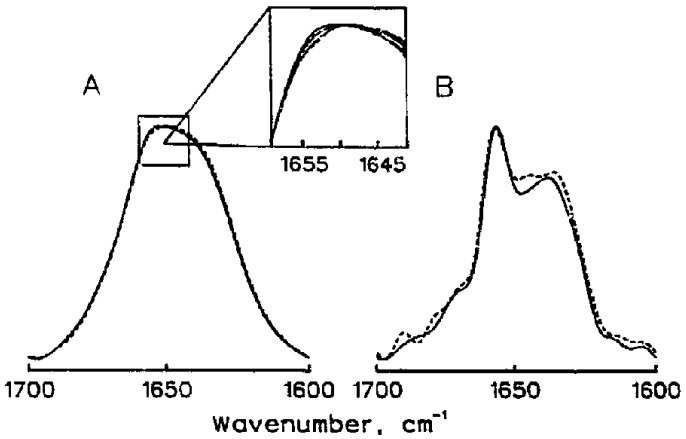


Fig. 2. Infrared amide I band region of the original (A) and deconvolved (B) spectra of reconstituted AcChR vesicles in the absence (control, solid trace) and in the presence of $10^{-2}$ M of carbamylcholine (discontinuous trace). The inset to (A) is a blown-up of the indicated region of the amide I band to illustrate the progressive effects on the protein infrared spectra exerted by concentrations of the cholinergic agonist ranging $10^{-5}$ to $10^{-3}$ M.

$cm^{-1}$, from ~48% in the absence of the agonist to ~24% at $10^{-3}$ M carbamylcholine, concomitant with an increase in the band at 1642 $cm^{-1}$. In spectra taken in $D_2O$, the latter band is usually assigned to non-ordered structures, and is related to the band shifting to approximately 1657 $cm^{-1}$, overlapping with the α-helix, when the spectra are taken in $H_2O$ [9]. Other authors [26], however, have recently described another component band in $H_2O$ spectra at approximately 1640 $cm^{-1}$, which does not shift in $D_2O$ and which they have assigned to 'flexible loops'. In either case, the observations reported here seem to indicate that the interaction of the cholin-

Table I

Quantitative estimation of AcChR secondary structure and fitting parameters obtained for the major components of the protein amide I band in the presence and in the absence of carbamylcholine[a]

| Carbamylcholine (M) | Position of the maxima ($cm^{-1}$) | Percent Structure | Gaussian Fraction | Intensity ratio (1655/1633) | FWHH[b] ratio (1655/1633) |
|---|---|---|---|---|---|
| 0 (Control) | 1655.1 | 43.26 | 0.53 | 0.971 | 0.863 |
| | – | – | – | | |
| | 1639.9 | 48.64 | 0.35 | | |
| $10^{-5}$ | 1655.1 | 43.19 | 0.46 | 1.228 | 0.859 |
| | 1641.1 | 8.10 | 0.47 | | |
| | 1634.1 | 39.75 | 0.43 | | |
| $10^{-4}$ | 1656.0 | 42.23 | 0.44 | 1.430 | 0.900 |
| | 1641.9 | 18.12 | 0.44 | | |
| | 1633.1 | [illegible] | 0.39 | | |
| $10^{-3}$ | 1656.1 | 43.22 | 0.49 | 1.880 | 0.920 |
| | 1640.5 | 28.06 | 0.27 | | |
| | 1633.2 | 24.20 | 0.41 | | |

[a] The minor components at 1680 and 1690 $cm^{-1}$ have been omitted for simplicity.
[b] FWHH, full-width at half-height.

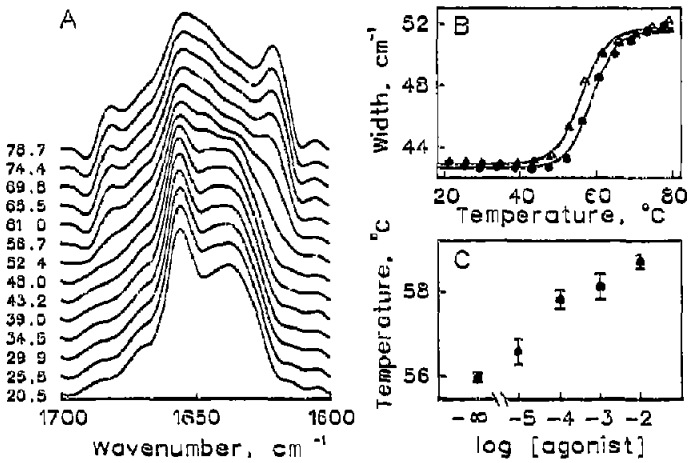


Fig. 3. Temperature dependence of the infrared amide I band of reconstituted AcChR. Panel A shows the deconvolved amide I band region of spectra recorded at the indicated temperatures during a heating cycle. The duration of a heating cycle including data acquisition and storage at each temperature, was approximately 2.5 h. Panel B shows the temperature-induced changes in the full-width at half-height of the infrared amide I band, in the absence (control, triangles) and in the presence of $10^{-2}$ M of carbamylcholine (circles). The inflection points in the sigmoid-like patterns are taken as the protein denaturation temperatures [14]. Panel C illustrates the effects of increasing concentrations of carbamylcholine on the protein denaturation temperatures determined as in (B).

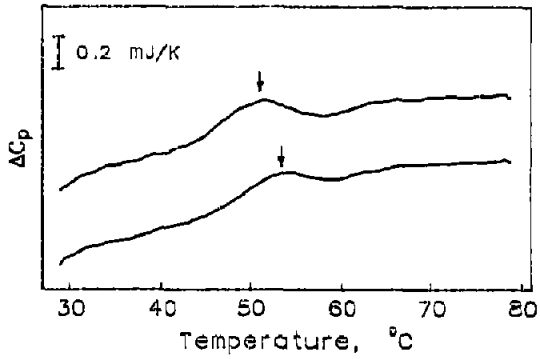


Fig. 4. DSC thermograms of reconstituted AcChR vesicles in 10 mM phosphate buffer, pH 7.4, 100 mM $NaNO_3$, obtained in the absence (upper trace) and in the presence (lower trace) of $10^{-3}$ M carbamycholine. Transition temperatures estimated under these conditions (indicated by arrows) were 51.15 and 53.4°C, respectively. Experimental error was less than 0.4°C.

ergic agonist with the AcChR, results in 'opening' or 'loosening' the $\beta$-sheet structure of the protein which, according to most theoretical models proposed for the AcChR, forms primarily the extracellular portion of the protein [3,27], thus, including the agonist binding sites. This seems consistent with the experimental low-resolution model available on the AcChR [6], which predicts that AcChR desensitization implies structural rearrangements in which the subunits switch to a less symmetrical configuration, with the overall changes being most pronounced in the synaptic and cytoplasmic regions of the protein.

Other authors have used FT-IR difference spectroscopy in $H_2O$ media to assess spectral changes due to agonist binding in AcChR native membranes [13,14] and in purified AcChR reconstituted into lipid vesicles [15]. These reports are somewhat contradictory to each other regarding the sign and relative magnitude of the spectral changes observed in the difference spectra [13,14]. The apparent contradictions could perhaps be attributed to differences in the preparation of their samples, which need to be partly dried prior to the experiment, or to a different degree of water interference. In this regard, we described previously that drying of AcChR samples in the absence of adequate protecting agents may result in a complete loss of the characteristic cation channel function of the AcChR, which is accompanied by a change in protein structure [16]. In any case, it should be noticed that while in difference spectroscopy a qualitative view of the protein conformation changes is obtained, the decomposition of the amide I band components allows that these changes can be quantitatively assigned to the secondary structural motives present in the protein. On the other hand, difference spectroscopy gives a more precise account of what happens to narrow vibrational bands such as those resulting from the agonist molecule [14].

IR spectra were also taken at progressively higher temperatures to monitor AcChR thermal denaturation. The temperature-dependent changes observed in the deconvolved amide I band (Fig. 3A) are similar to those reported previously in native AcChR-rich membranes from *Torpedo* [16], indicating a loss of organized protein secondary substructures, such as the $\alpha$-helix (1656 $cm^{-1}$) or $\beta$-sheets (1633 $cm^{-1}$), and the appearance of two components at 1620 and 1684 $cm^{-1}$, which have been related to aggregation of thermally denatured proteins [28]. Monitoring of thermal denaturation in the presence of carbamylcholine induces a noticeable thermal stabilization of the protein, which is translated in an increase of up to 2–3°C in the temperature corresponding to the inflection point of the sigmoidal curve describing the heat-induced loss of protein structure, with respect to that obtained in the absence of the ligand (Fig. 3B). Such stabilizing effects of carbamylcholine are readily detectable at $10^{-5}$ M and become maximal in the $10^{-3}$–$10^{-2}$ M range (Fig. 3C).

The observations on the protein thermal stability made by FT-IR monitoring of the samples at different temperatures, resemble those that can be obtained by DSC analysis of similar reconstituted AcChR samples (Fig. 4). It should be noted, however, that heating of the samples in the FT-IR and the DSC instruments is achieved differently (step-like jumps in temperature in the former and a continuous linear increase in the latter). This causes that the absolute values of protein denaturation temperatures obtained by those tech-

niques can not be compared since thermal denaturation of complex proteins such as the AcChR is a kinetically controlled, irreversible process highly dependent on the heating rate. Nevertheless, the DSC data in Fig. 4 (higher denaturation temperature in presence of the agonist) lends support to the observations made by FT-IR (Fig. 3B and C) in that desensitization induced by carbamylcholine leads to a different conformation of the AcChR protein and that such conformation has an increased thermal stability.

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