Dissecting the Chemistry of Nicotinic Receptor-Ligand Interactions with Infrared Difference Spectroscopy*

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The physical interactions that occur between the nicotinic acetylcholine receptor from Torpedo and the agonists carbamylcholine and tetramethylamine have been studied using both conventional infrared difference spectroscopy and a novel double-ligand difference technique. The latter was developed to isolate vibrational bands from residues in a membrane receptor that interact with individual functional groups on a small molecule ligand. The binding of either agonist leads to an increase in vibrational intensity at frequencies centered near 1663, 1655, 1547, 1430, and 1059 cm\(^{-1}\) indicating that both induce a conformational change from the resting to the desensitized state. Vibrational shifts near 1580, 1516, 1455, and 1334, and between 1300 and 1400 cm\(^{-1}\) are assigned to structural perturbations of tyrosine and possibly both tryptophan and charged carboxylic acid residues upon the formation of receptor-quinary amine interactions, with the relatively intense feature near 1516 cm\(^{-1}\) indicating a key role for tyrosine. Other vibrational bands suggest the involvement of additional side chains in agonist binding. Two side-chain vibrational shifts from 1668 and 1605 cm\(^{-1}\) to 1690 and 1620 cm\(^{-1}\), respectively, could reflect the formation of a hydrogen bond between the ester carbonyl of carbamylcholine and an arginine residue. The results demonstrate the potential of the double-ligand difference technique for dissecting the chemistry of membrane receptor-ligand interactions and provide new insight into the nature of nicotinic receptor-agonist interactions.

The binding of a signaling molecule to an integral membrane receptor is a key event in many biological processes, including cell growth, intercellular communication, sensory perception, etc. Understanding the chemistry of membrane receptor-ligand interactions is thus central to understanding many biological phenomena. Because membrane receptors are the targets of pharmaceutical products, membrane receptor-ligand interactions are also of particular interest to the pharmaceutical industry. Unfortunately, the modern physical methodologies commonly used to probe the structural features responsible for receptor-ligand interactions are still limited in their application to membrane-imbedded receptors.

The chemical nature of receptor-ligand interactions at the post-synaptic membrane have been studied intensively for the nicotinic acetylcholine receptor (nAChR) from Torpedo. Each of the two acetylcholine (ACh) binding sites on the nAChR consists of two subsites, an esterophilic subsite that binds the ester functional group of ACh and an anionic subsite that binds the quaternary ammonium cation (1, 2). Sequence analysis, affinity labeling, and site-directed mutagenesis identify both aromatic and negatively charged residues in the anionic subsite that likely interact with the charged nitrogen of ACh via cation-π electron interactions and/or hydrogen bonding (3-9). The crystal structure of the homologous ACh binding protein confirms the essential role for tyrosine and tryptophan residues in the anionic subsite (10). In contrast, the nature of the chemical interactions that occur between ACh and the esterophilic subsite remain poorly understood.

Infrared difference spectroscopy is a technique that has been used extensively to probe the subtle changes in chemical structure and/or local environment that are associated with protein conformational change (reviewed in Ref. 11). The difference between spectra of the nAChR recorded in the presence and absence of the agonist carbamylcholine (Carb) exhibits a complex pattern of positive and negative bands that reflects shifts in the intensities and/or frequencies of vibrations from those amino acid residues whose structures are altered upon Carb binding (12, 13). Difference spectroscopy has been used to map the conformational states of the nAChR stabilized by both a variety of ligands and upon reconstitution into lipid bilayers of varying lipid compositions (14-17). The difference spectra also contain vibrational information pertaining to the physical interactions that occur between Carb and nAChR binding site residues. Extensive band overlap, however, has thus far limited interpretation of the latter in terms of either the types of amino acid side chains involved or the precise nature of the physical interactions that occur between protein side chains and Carb.

In this report we present the first analysis of nAChR-ligand interactions as studied by infrared difference spectroscopy. To circumvent the problems of extensive band overlap, we developed a new “double ligand difference” method that can be used to isolate the vibrational features from protein side chains that interact directly with individual functional groups on a small molecule ligand. This new double ligand approach allows one to map individual ligand-receptor contacts and simplifies the difference spectra allowing for a more detailed interpretation of the data. We show here that the frequencies of two main protein vibrations are altered when the nAChR interacts with the carbonyl oxygen of Carb. The frequencies of the detected

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1 The abbreviations used are: nAChR, nicotinic acetylcholine receptor; ACh, acetylcholine; FTIR, Fourier transform infrared; Carb, carba-mylcholine; TMA, tetramethylamine; R→D, resting to desensitized; D→D, desensitized to desensitized.
The wash and trigger buffers. In the Carb-minus-TMA difference spectrum, for example, the trigger buffer contained 50 μM Carb and both the wash and trigger buffers contained 1 mM TMA. Two spectra of the nAChR with TMA bound to the neurotransmitter site were recorded. The flowing wash buffer was then switched to the trigger buffer, which contains sufficient Carb to displace TMA from the neurotransmitter binding site. The differences between both the two spectra recorded with TMA bound and the consecutive spectra recorded with first TMA and then Carb bound to the nAChR were calculated and stored, and the process was repeated many times. For the Carb-minus-choline difference, the trigger buffer contained both 50 μM Carb and 10 mM choline, and the wash buffer contained 10 mM choline. For the ACh-minus-TMA difference, the trigger buffer contained both 100 μM ACh and 1 mM TMA, and the wash buffer contained 1 mM TMA. For the ACh-minus-choline difference, the trigger buffer contained both 100 μM ACh and 10 mM choline, and the wash buffer 10 mM choline.

RESULTS

The difference between spectra of the nAChR recorded in the presence and absence of the agonist Carb (referred to as a Carb R→D difference spectrum) exhibits a pattern of positive and negative bands (Fig. 2, middle trace) that is absent in control difference spectra recorded from α-bungarotoxin-treated nAChR membranes (13). These bands reflect three related phenomena: 1) vibrations of Carb bound to the nAChR, 2) vibrational changes in the nAChR that occur upon the formation of physical interactions, such as hydrogen bonds, cation–π electron interactions, etc., between Carb and neurotransmitter binding site residues, and 3) vibrational changes in the nAChR that result from the Carb-induced R→D conformational transition.

To interpret the Carb R→D difference spectrum, bands were first assigned to each of the three related phenomena noted above. Bands due to nAChR-bound Carb were identified by comparing band frequencies in the difference spectrum with those observed in solution spectra of Carb (light shading in Fig. 2), as well as by similar comparisons of difference and solution spectra recorded using ACh and an isotopically labeled derivative (13). Bands that reflect the structural changes associated with the R→D conformational change were identified by recording Carb difference spectra from nAChR membranes that were maintained continuously in the desensitized state prior to (and after) Carb addition (referred to as a Carb D→D difference spectrum; Fig. 2, bottom trace). The resulting Carb D→D difference spectra consistently lack positive intensity centered near 1668, 1655, 1547, 1430, and 1059 cm⁻¹ that is observed in the Carb R→D difference spectrum (14–17). These five positive bands in the Carb R→D difference spectrum thus reflect the main vibrational changes associated with transition of the nAChR from a resting to the desensitized state (hatched shading in Fig. 2, middle trace). In contrast, bands that are not affected by desensitization and that are not attributable to nAChR-bound Carb must reflect vibrational changes in protein residues that occur upon the formation of physical interactions between Carb and the desensitized nAChR. These features are not shaded in Fig. 2.

The large number of bands not attributable to either nAChR-bound Carb or the R→D conformational transition highlights the fact that the Carb R→D difference spectrum contains substantial vibrational information regarding the nature of Carb–nAChR interactions. Several vibrational features are observed at frequencies consistent with the expected frequencies of side chains known to be located in the neurotransmitter binding site (see Fig. 3). The most notable is a relatively intense band near 1516 cm⁻¹ that is characteristic of tyrosine (19). The relative intensity of the band is indicative of a strong interaction, consistent with mutagenesis data that highlight the importance of tyrosines in agonist binding (5, 6). In addition, weak bands are observed in regions of the spectrum normally

**Nicotinic Receptor-Ligand Interactions Probed by FTIR**

The nAChR from frozen Torpedo californica electric tissue (Marinus) was affinity-purified on a bromoacetylcholine bromide-derivatized Bio-Rad Affi-Gel 201 column and reconstituted into membranes composed of either 3:1:1 egg phosphatidylcholine/dioleoylphosphatidic acid/cholesterol or asolectin (18). Both lipid compositions stabilize the nAChR in a conformation that conducts cations across the membrane and undergoes agonist-induced desensitization (16).

**FTIR Spectroscopy—FTIR spectra were recorded using the attenuated total reflection technique on either an FTIR-40 or an FT/575 spectrometer (Bio-Rad), both equipped with a DTGS detector. A schematic diagram of the attenuated total reflectance cell used to record FTIR difference spectra, as described above, except that the trigger buffer contained 1 mM TMA instead of 50 μM Carb. Carb desensitized-to-desensitized (D→D) difference spectra were recorded as described above for the Carb R→D difference spectra, except that the wash and trigger buffers both contained 200 μM dibucaine so that the nAChR was bathed continuously with dibucaine and thus maintained throughout the experiment in the desensitized state.

**Double Ligand Difference Spectra—**The double ligand difference spectra were recorded as described above for the Carb R→D difference spectra except that one ligand (either Carb or ACh) was included in the trigger buffer while the other ligand (either TMA or choline) was included in both the wash and trigger buffers. In the Carb-minus-TMA difference spectrum, for example, the trigger buffer contained 50 μM Carb and both the wash and trigger buffers contained 1 mM TMA. Two spectra of the nAChR with TMA bound to the neurotransmitter site were recorded. The flowing wash buffer was then switched to the trigger buffer, which contains sufficient Carb to displace TMA from the neurotransmitter binding site. The differences between both the two spectra recorded with TMA bound and the consecutive spectra recorded with first TMA and then Carb bound to the nAChR were calculated and stored, and the process was repeated many times. For the Carb-minus-choline difference, the trigger buffer contained both 50 μM Carb and 10 mM choline, and the wash buffer contained 10 mM choline. For the ACh-minus-TMA difference, the trigger buffer contained both 100 μM ACh and 1 mM TMA, and the wash buffer contained 1 mM TMA. For the ACh-minus-choline difference, the trigger buffer contained both 100 μM ACh and 10 mM choline, and the wash buffer 10 mM choline.

**EXPERIMENTAL PROCEDURES**

**Sample Preparation—**The nAChR from frozen Torpedo californica electric tissue (Marinus) was affinity-purified on a bromoacetylcholine bromide-derivatized Bio-Rad Affi-Gel 201 column and reconstituted into membranes composed of either 3:1:1 egg phosphatidylcholine/dioleoylphosphatidic acid/cholesterol or asolectin (18). Both lipid compositions stabilize the nAChR in a conformation that conducts cations across the membrane and undergoes agonist-induced desensitization (16).

**FTIR Spectroscopy—**FTIR spectra were recorded using the attenuated total reflection technique on either an FTIR-40 or an FT/575 spectrometer (Bio-Rad), both equipped with a DTGS detector. A schematic diagram of the attenuated total reflectance cell used to record the spectra is shown in Fig. 1. For Carb resting-to-desensitized (R→D) difference spectra, two consecutive spectra of the resting nAChR were recorded while flowing wash buffer (250 mM NaCl, 5 mM KCl, 2 mM MgCl₂, 3 mM CaCl₂, and 10 mM Tris, pH 7.0) past the nAChR film present on the surface of a germanium internal reflection element. The flowing solution was switched to an identical one containing 50 μM Carb (trigger buffer) and, after 1 min, a spectrum was recorded of the desensitized state. The differences between both the resting state spectra and the consecutive resting and desensitized state spectra were calculated, stored, and the flowing solution switched back to the wash buffer without Carb. After a 20-min washing period to remove Carb from the film, the process was repeated many times. Each spectrum was recorded at 8-cm⁻¹ resolution using 512 scans. The presented spectra are averages of greater than 30 difference spectra recorded from at least two separate films. All difference spectra were baseline-corrected between 1800 and 1000 cm⁻¹ and were interpolated to an effective resolution of 4 cm⁻¹.

Tetramethylamine (TMA) R→D difference spectra were recorded as described above, except that the trigger buffer contained 1 mM TMA instead of 50 μM Carb. Carb desensitized-to-desensitized (D→D) difference spectra were recorded as described above for the Carb R→D difference spectra, except that the wash and trigger buffers both contained 200 μM dibucaine so that the nAChR was bathed continuously with dibucaine and thus maintained throughout the experiment in the desensitized state.

**Double Ligand Difference Spectra—**The double ligand difference spectra were recorded as described above for the Carb R→D difference spectra except that one ligand (either Carb or ACh) was included in the trigger buffer while the other ligand (either TMA or choline) was included in both

**RESULTS**

The difference between spectra of the nAChR recorded in the presence and absence of the agonist Carb (referred to as a Carb R→D difference spectrum) exhibits a pattern of positive and negative bands (Fig. 2, middle trace) that is absent in control difference spectra recorded from α-bungarotoxin-treated nAChR membranes (13). These bands reflect three related phenomena: 1) vibrations of Carb bound to the nAChR, 2) vibrational changes in the nAChR that occur upon the formation of physical interactions, such as hydrogen bonds, cation–π electron interactions, etc., between Carb and neurotransmitter binding site residues, and 3) vibrational changes in the nAChR that result from the Carb-induced R→D conformational transition.

To interpret the Carb R→D difference spectrum, bands were first assigned to each of the three related phenomena noted above. Bands due to nAChR-bound Carb were identified by comparing band frequencies in the difference spectrum with those observed in solution spectra of Carb (light shading in Fig. 2), as well as by similar comparisons of difference and solution spectra recorded using ACh and an isotopically labeled derivative (13). Bands that reflect the structural changes associated with the R→D conformational change were identified by recording Carb difference spectra from nAChR membranes that were maintained continuously in the desensitized state prior to (and after) Carb addition (referred to as a Carb D→D difference spectrum; Fig. 2, bottom trace). The resulting Carb D→D difference spectra consistently lack positive intensity centered near 1668, 1655, 1547, 1430, and 1059 cm⁻¹ that is observed in the Carb R→D difference spectrum (14–17). These five positive bands in the Carb R→D difference spectrum thus reflect the main vibrational changes associated with transition of the nAChR from a resting to the desensitized state (hatched shading in Fig. 2, middle trace). In contrast, bands that are not affected by desensitization and that are not attributable to nAChR-bound Carb must reflect vibrational changes in protein residues that occur upon the formation of physical interactions between Carb and the desensitized nAChR. These features are not shaded in Fig. 2.

The large number of bands not attributable to either nAChR-bound Carb or the R→D conformational transition highlights the fact that the Carb R→D difference spectrum contains substantial vibrational information regarding the nature of Carb–nAChR interactions. Several vibrational features are observed at frequencies consistent with the expected frequencies of side chains known to be located in the neurotransmitter binding site (see Fig. 3). The most notable is a relatively intense band near 1516 cm⁻¹ that is characteristic of tyrosine (19). The relative intensity of the band is indicative of a strong interaction, consistent with mutagenesis data that highlight the importance of tyrosines in agonist binding (5, 6). In addition, weak bands are observed in regions of the spectrum normally
associated with the vibrations of tryptophan (near 1334 and 1455 cm\(^{-1}\)) and carboxylate (near 1580 cm\(^{-1}\) and between 1300 and 1400 cm\(^{-1}\)) residues. A number of other vibrations, most notably near 1820 cm\(^{-1}\), could reflect interactions between Carb and additional binding site residues (19). The difference spectra thus suggest a rich complexity of interactions between Carb and the nAChR.

To determine which vibrational features reflect interactions that occur between binding site residues and the quaternary amine of Carb, the difference between spectra recorded in the presence and absence of the agonist analog TMA, which lacks the alkyl carbamylester functional moiety, was calculated (third from top trace in Fig. 3). The TMA difference spectrum exhibits many of the same vibrational features observed in the Carb R→D difference spectrum, including bands near 1580, 1516, 1455, 1334, and between 1300 and 1400 cm\(^{-1}\) that are potentially attributable to the aromatic side chains of tyrosine and tryptophan and the carboxylate side chains of aspartic/glutamic acid. In fact, most of the bands in the Carb R→D difference spectrum that are potentially attributable to vibrational changes in residues interacting with Carb are also present in the TMA difference spectrum. This result provides direct experimental evidence that tyrosine and possibly both tryptophan and carboxylate residues interact in some manner with the quaternary amine. The result also shows that the interactions between the nAChR and Carb are dominated by interactions with the quaternary amine functional group.

The TMA difference spectrum also exhibits positive intensity at each of the five frequencies centered near 1668, 1655, 1547, 1430, and 1059 cm\(^{-1}\) that serve as markers of the R→D conformational transition. The positive intensity at each frequency suggests that exposure to TMA leads to a shift of the nAChR from a predominantly resting to a predominantly desensitized state. The ability of TMA to desensitize the nAChR is not surprising given that TMA is an agonist of the receptor (20, 21). The desensitizing capability of TMA was also confirmed in a separate fluorescence spectroscopic study of the interactions between the conformationally sensitive probe, ethidium bro-

**Fig. 2.** A Carb R→D difference spectrum (middle trace) is obtained by subtracting a spectrum recorded from the nAChR in the unliganded resting conformation from a spectrum recorded from the nAChR in the Carb-bound desensitized conformation (see accompanying schematic where the clear round form of the nAChR represents the resting state and the shaded square form represents the desensitized state). Carb R→D difference spectra exhibit positive bands due to nAChR-bound Carb (intensity approximated with light shading) that are evident at frequencies close to those observed for bands in the solution spectrum of Carb (top trace, the background H,O spectrum has been subtracted). Carb R→D difference spectra also exhibit positive bands that reflect the R→D conformational change (hatched shading). These bands are absent in Carb D→D difference spectra (bottom trace). A Carb D→D difference spectrum is calculated by subtracting a spectrum of the nAChR in a Carb-free desensitized conformation from a spectrum of the nAChR in the Carb-bound desensitized conformation (see accompanying schematic). In this case, the nAChR was maintained in a desensitized state by incubating with the desensitizing local anesthetic dibucaine. Note that dibucaine binds to the two neurotransmitter sites as well as to the ion channel pore to induce desensitization. Intensity variations between the Carb D→D and Carb R→D difference spectra that are not discussed in the text reflect displacement of dibucaine from the neurotransmitter sites upon Carb binding and are discussed in detail in Ref. 14. A horizontal line is included in each spectrum to provide a visual reference.

**Fig. 3.** A comparison of the Carb R→D (second from top trace) and the TMA R→D (third from top trace) difference spectra. Bands due to either nAChR-bound Carb or nAChR-bound TMA are approximated with light shading and correspond to similar bands in the solution spectra of Carb and TMA (top and bottom traces, respectively). The vibrational features in the two difference spectra that reflect the R→D conformational transition are approximated with hatched shading. Bands near 1580, 1516, 1455, 1334, and between 1300 and 1400 cm\(^{-1}\) that likely reflect structural perturbations of tyrosine, tryptophan, and charged carboxylic acid residues are noted with the long dashed lines (see text). The two asterisks denote regions in the TMA R→D difference spectrum that exhibit variations in intensity relative to the Carb R→D difference spectrum resulting from differences in how Carb and TMA bind to the nAChR (see text). A horizontal line is included in each spectrum to provide a visual reference.
To simplify the difference spectrum and thus permit a definitive interpretation of the data, we devised a novel double ligand difference method (illustrated schematically in Fig. 4). The idea was to calculate the difference between spectra of the nAChR recorded consecutively with TMA and then Carb bound to the neurotransmitter binding sites. Because both Carb and TMA stabilize the nAChR in a desensitized conformation, the difference between spectra of the nAChR recorded with either TMA or Carb bound to the neurotransmitter binding sites (referred to as a Carb-minus-TMA difference spectrum) should not exhibit vibrational bands reflecting the R→D conformational change. The vibrational features resulting from the formation of physical interactions between the quaternary amine of Carb and the anionic subsite should also be absent from the Carb-minus-TMA difference spectrum, because these interactions are already formed in the TMA-bound state. In fact, the Carb-minus-TMA difference spectrum should only exhibit positive and negative vibrational bands due to the intrinsic vibrations of bound Carb and competitively displaced TMA, respectively, as well as bands from esterophilic subsite residues whose structures and/or environments are altered upon interaction with the alkyl carbamylester portion of Carb.

As expected, the Carb-minus-TMA difference spectrum exhibits very few difference bands compared with either the Carb or TMA R→D difference spectra (Fig. 5). Clearly defined positive and negative bands due to nAChR-bound Carb and competitively displaced TMA are observed throughout the 1800–1000 cm⁻¹ region (Fig. 4, light shading). In contrast, relatively intense protein vibrations are only observed in the 1600–1700 cm⁻¹ region near 1690, 1668, 1635, 1620, and 1605 cm⁻¹. Other weak bands are difficult to assess at the signal-to-noise ratio of these spectra. The lack of protein vibrational features attributable to tyrosine, tryptophan, and carboxylic acid containing residues, etc. confirms that the majority of the strong physical contacts that occur between Carb and the nAChR take place at the anionic subsite. This result further highlights the importance of the quaternary amine in agonist binding.

Two sets of positive/negative protein vibrations located near 1690/1668 cm⁻¹ and 1620/1605 cm⁻¹, as well as a weak positive vibration near 1635 cm⁻¹ reflect vibrational changes in esterophilic subsite residues that occur upon interaction with the alkyl carbamylester portion of Carb. To further define the functional group on Carb that interacts with the esterophilic subsite residues leading to the noted band shifts, we recorded a

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2 J. E. Baenziger, unpublished observations.

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Fig. 4. Schematic diagram of the double ligand difference approach showing the neurotransmitter binding site of the nAChR with either bound TMA or Carb and the chemical structures that give rise to bands in the Carb-minus-TMA difference spectrum. Carb binding to the nAChR leads to interactions between Carb and the esterophilic subsite that are not present in the TMA-bound state. The difference between spectra of the nAChR recorded with either Carb or TMA-bound to the nAChR exhibits vibrational bands predominantly from the alkyl carbamylester portion of Carb as well as from side chains in the esterophilic subsite whose structures are altered upon interaction with the alkyl carbamylester portion of Carb.

Fig. 5. A Carb-minus-TMA difference spectrum (middle trace) is the difference between a spectrum of the desensitized nAChR recorded with bound TMA subtracted from a spectrum of the desensitized nAChR recorded with bound Carb (see Fig. 4). The solution spectra of Carb and TMA are presented for comparison (top and bottom traces, respectively). Light shading denotes bands due to Carb and TMA.
Nicotinic Receptor-Ligand Interactions Probed by FTIR

The bands in the double ligand difference spectra located near 1690/1668 cm\(^{-1}\) and 1620/1605 cm\(^{-1}\) suggest possible candidate residues for vibrational changes that result from the formation of physical interactions between the nAChR and Carb. The former interpretation of the data seems unlikely given the absence of band shifts in the amide II (mainly N–H bending and C–N stretch) region of the spectrum (1520–1580 cm\(^{-1}\)).

Several side chains are also located in the ACh binding pocket of the homologous ACh binding protein that could interact with the carbonyl of Carb (10), although the precise location of agonist binding to the neurotransmitter site has not yet been determined. These residues in the ACh binding protein include tyrosine 192, glutamate 55, arginine 104, valine 106, leucine 112, and methionine 114. The homologous Torpedo nAChR residues are α tyrosine 198, γ glutamate 57 and δ aspartate 59, γ leucine 109 and δ leucine 111, γ tyrosine 111 and δ arginine 113, γ tyrosine 117 and δ threonine 119, and γ leucine 119 and δ leucine 121, respectively (28). The side chains of tyrosine, glutamate, aspartate, leucine, and threonine do not normally contribute strong vibrational intensity exclusively in the 1600–1700 cm\(^{-1}\) region of the infrared spectrum and thus are not likely responsible for the vibrations detected in the Carb-minus-TMA difference spectrum (19). In contrast, the asymmetric and symmetric CN\(_3\)H\(_5\) stretching vibrations of arginine are expected near 1670 and 1630 cm\(^{-1}\), frequencies consistent with the two sets of positive and negative couples near 1690/1668 cm\(^{-1}\) and 1620/1605 cm\(^{-1}\). Although other assignments are possible, a preliminary interpretation of our data is that the ester carbonyl of Carb interacts with an arginine side chain when binding to the desensitized state of the nAChR. Note, however, that the binding affinity of the ACh binding protein for ACh is 4.2 μM (29). In contrast, the desensitized nAChR binds ACh with an affinity of ~2 nM (22) suggesting conformational differences between the binding sites of the ACh binding protein and the desensitized nAChR. Additional candidate residues may thus be possible.

Shifting in vibrational band frequencies are typically observed upon a change in strength of hydrogen bonding. For a stretching vibration, an increase in hydrogen bond strength typically leads to a downshift in vibrational frequency to lower wave-numbers. Surprisingly, the interaction of Carb with the esterophilic subsite leads to shifts from 1668 and 1605 cm\(^{-1}\) upon a change in strength of hydrogen bonding. For a stretch-
other protein vibrations suggest additional complexity to Carb-nAChR interactions.

We have developed a novel double ligand difference method that can be used to isolate the vibrational features from those residues in a large integral membrane receptor that interact physically either directly or indirectly with specific functional groups on a small molecule ligand. The difference between spectra of the nAChR recorded with either Carb or TMA bound to the neurotransmitter binding sites exhibits protein vibrations from only those residues in the esterophilic subsite that interact with the alkyl carbamylester portion of Carb (see below). Additional double ligand difference spectra show that these vibrations reflect residues that interact either directly or indirectly with the ester carbonyl functional group. Two main band shifts from 1690 and 1620 cm\(^{-1}\) to 1668 and 1605 cm\(^{-1}\), respectively, are consistent with the formation of a hydrogen bond between the ester carbonyl of Carb and an arginine side chain. Significantly, these data highlight the utility of the double ligand difference method for dissecting the physical interactions that occur between a large integral membrane receptor and a bound ligand.

The relative simplicity of the resulting double ligand difference spectra is significant from a technical perspective. Band overlap in the Carb R→D difference spectrum prevents interpretation of the data in terms of both the identities of the amino acid side chains that are involved in Carb binding and the nature of the Carb-nAChR contacts. In fact, the two main band shifts observed from 1690 and 1620 cm\(^{-1}\) to 1668 and 1605 cm\(^{-1}\), respectively, that occur upon interaction of the ester carbonyl of Carb with the esterophilic subsite are not discernible in either the Carb or TMA R→D difference spectra. The lack of band overlap in the double ligand difference spectrum permits a precise determination of the band frequencies of the protein side chains that interact with the ester carbonyl in both the Carb bound and TMA bound states. The relative simplicity of the spectrum greatly improves the possibility that Carb-minus-TMA difference spectra recorded in \(^2\text{H}_2\text{O}\) and at different pH values will lead to a definitive assignment of the protein vibrations tentatively attributed here to an arginine residue.

The main conclusions regarding the nature of Carb-nAChR interactions derived from this study are the following: 1) The data show that the main physical interactions between Carb and the nAChR arise from interactions between the quaternary amine and the anionic subsite. This conclusion is based on the observation that the majority of features in the Carb R→D difference spectrum attributable to vibrational changes in residues that interact with Carb are also observed in the TMA R→D difference spectrum. Both the FTIR and fluorescence data show that TMA is capable of stabilizing the nAChR in the desensitized state (see “Results”). The demonstrated importance of the quaternary amine in both agonist-nAChR interactions and agonist-induced conformational change is in agreement with electrophysiological studies, which show that TMA is an agonist of the receptor (20, 21). 2) The difference spectra provide direct evidence that the quaternary amine of Carb interacts with tyrosine residues in the agonist-binding pocket. Carb binding to the nAChR leads to an increase in the vibrational intensity of a band near 1516 cm\(^{-1}\) indicating a change in the structure and/or local environment surrounding one or more tyrosine residues. This increase in tyrosine vibrational intensity is observed upon Carb binding to a desensitized nAChR suggesting that it reflects Carb-tyrosine physical interactions as opposed to a vibrational perturbation associated with the R→D conformational transition. The increase in intensity near 1516 cm\(^{-1}\) is also observed upon TMA binding supporting the assignment of the band to one or more interactions between one or more tyrosines and the quaternary amine functional group. The relative strong intensity of the 1516 cm\(^{-1}\) band firmly establishes a key role for tyrosines in agonist binding in agreement with extensive data obtained using both chemical labeling and site-directed mutagenesis (4–8, 20, 21).

3) Vibrational features that are indicative of the interactions between the quaternary amine and several other amino acid side chains are detected suggesting that a number of residues contribute to the anionic subsite. Bands potentially attributable to tryptophan and the carboxylate groups of aspartate and/or glutamate may reflect changes in structure and/or local environment surrounding both types of residues upon interaction with Carb, as has been proposed elsewhere (9, 23). Other vibrational features, most notably near 1620 cm\(^{-1}\), suggest the involvement of additional amino acid side chains. 4) Finally, positive and negative bands near 1690/1668 cm\(^{-1}\) and 1620/1605 cm\(^{-1}\) detected in the Carb-minus-TMA, the Carb-minus-choline, the ACh-minus-TMA, and ACh-minus-choline difference spectra suggest the formation of a hydrogen bond between an esterophilic subsite residue and the ester carbonyl of Carb while the nAChR is in the desensitized state. Although other band assignments are possible, the frequencies are consistent with an arginine side chain interacting directly with the ester carbonyl. An arginine residue is likely located close to the agonist binding pocket (10).

The ability of the double ligand difference technique to detect specific ligand-receptor contacts highlights the potential of the technique for mapping subtle structural variations that exist between the binding sites of homologous ligand binding proteins. For example, Carb-minus-TMA difference spectra recorded from both the nAChR and the homologous ACh binding protein would reveal similarities and/or differences in amino acid side chains located in the esterphilic subsite. Given that the crystal structure of the ACh binding protein has been solved, such information could prove valuable for modeling the binding site of the nAChR. A similar approach could also be used to investigate the structural basis for the different neuronal nAChR receptor pharmacologies.

The experimental approach used here is readily adaptable to almost any membrane-bound receptor. The main requirement is the ability to form a membrane film that adheres to the surface of an internal reflection element in the presence of flowing buffer. In our experience, most biological membranes adhere to germanium internal reflection elements (see also Refs. 24, 25). A variety of internal reflection element sizes, geometries, and materials allow one to tailor the experimental approach for a given application (26). Novel methods of recording difference spectra are continuously being developed for water-soluble proteins (reviewed in Ref. 11). The approaches described here should be applicable to a variety of both membrane-embedded and water-soluble proteins.

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