Desensitization of the Nicotinic Acetylcholine Receptor Mainly Involves a Structural Change in Solvent-Accessible Regions of the Polypeptide Backbone†

John E. Baenziger* and Jennifer P. Chew

Department of Biochemistry, University of Ottawa, Ottawa, Ontario, Canada, K1H 8M5

Received November 18, 1996; Revised Manuscript Received January 22, 1997⊗

ABSTRACT: The difference between infrared spectra of the nicotinic acetylcholine receptor (nAChR) recorded using the attenuated total reflectance technique in the presence and absence of carbamylcholine exhibits a complex pattern of positive and negative bands that provides a spectral map of the structural changes that occur in the nAChR upon agonist binding and subsequent desensitization. Two relatively intense bands are observed in the amide I region of the difference spectra recorded in ^1H_2O buffer near 1655 cm^{-1} and 1620 cm^{-1} that were previously interpreted in terms of either a net conversion of β-sheet to α-helix or a reorientation of transmembrane α-helix accompanied by a change in structure of β-sheet and/or turn [Baenziger, J. E., Miller, K. W., & Rothschild, K. J. (1993) *Biochemistry 32*, 5448−5454]. However, difference spectra recorded in ^2H_2O buffer reveal that these and other difference bands in the amide I region undergo downshifts in frequency upon peptide $^1H/^2H$ exchange that are much larger than the downshifts in frequency that are typically observed for the amide I vibrations of either α-helix or β-sheet. Difference spectra recorded in ^2H_2O buffer within either minutes or hours of prior exposure of the nAChR to ^2H_2O exhibit the same amide I difference band shifts that are observed in difference spectra recorded after 3 days prior exposure of the nAChR to ^2H_2O. Most of the peptides that are involved in both ligand binding and the resting to desensitized conformational change and that give rise to bands in the difference spectra therefore exchange their hydrogens for deuterium on the seconds to minutes time scale. The frequencies of the difference bands, the magnitudes of the difference band shifts upon peptide $^1H/^2H$ exchange, and the rapidity of the hydrogen deuterium exchange kinetics of those structures that give rise to amide I bands in the difference spectra all suggest that the formation of a channel-inactive desensitized state results predominantly from a conformational change in solvent-accessible extramembranous regions of the polypeptide backbone as opposed to a large structural perturbation near the ion channel gate. A conformational change in the agonist binding site may be primarily responsible for channel inactivation upon desensitization.

The nicotinic acetylcholine receptor (nAChR)[1] from *Torpedo* is the most intensively studied member of a homologous superfamily of ligand-gated ion channels that are found both at the neuromuscular junction and in the brain. The nAChR is a large integral membrane protein composed of five subunits ($\alpha_2\beta\gamma\delta$) arranged pseudo-symmetrically about a central pore that functions as a cation-selective ion channel. Each subunit has 4 hydrophobic stretches (M1−M4) of roughly 25 amino acid residues that may form 4 transmembrane α-helices (Blanton & Cohen, 1992, 1994; Baenziger & Méthot, 1995), although transmembrane β-strands are possible (Unwin, 1993; Görne-Tschelnokow et al., 1994). The M2 transmembrane segment from each subunit lines the ion channel pore and performs a central role in both ion selectivity and channel gating. The ion channel transiently gates open in response to the binding of two molecules of the neurotransmitter, acetylcholine. Prolonged exposure to acetylcholine converts the nAChR into a high-affinity channel-inactive desensitized (D) state [see Galzi et al. (1991b), Lester (1992), and Karlin (1993)].

The structural changes that occur upon the binding of acetylcholine and other cholinergic agonists, such as carbamylcholine (Carb), to the resting (R) state of the nAChR have been investigated using a variety of physical and biochemical methods. Although detailed models of the mechanisms of both channel gating and desensitization have yet to emerge, electron microscopic images of the nAChR trapped in a channel open conformation by rapid freezing suggest a slight tilting and twisting of the M2 transmembrane α-helices accompanied by subtle structural alterations near the neurotransmitter binding site (Unwin, 1995). More substantial displacements in the structures of both domains have been detected upon desensitization (Unwin, 1995). The latter are consistent with the variable reactivities of both the neurotransmitter binding site and the pore lining M2 amino acid side chains to photoactivatable probes in the R and D states (White & Cohen, 1988; White et al., 1991; Galzi et al., 1991a). In contrast, circular dichroism, FTIR, Raman spectroscopy, and hydrogen exchange studies indicate that the secondary structure and solvent accessibility of hydrogen-bonded secondary structures are not affected by desensitization [see Méthot et al. (1995) and references cited within]. The covalent labeling pattern of the lipid exposed transmembrane surfaces of the nAChR by lipid-soluble photoactivatable probes is also essentially unchanged upon the

† This work was supported by a grant from the Medical Research Council of Canada to J.E.B.

* To whom correspondence should be addressed.

⊗ Abstract published in *Advance ACS Abstracts*, March 1, 1997.

[1] Abbreviations: nAChR, nicotinic acetylcholine receptor; ATR, attenuated total reflectance; Carb, carbamylcholine; Chol, cholesterol; D, desensitized; DOPA, dioleoylphosphatidic acid; EPC, egg phosphatidylcholine; FTIR, Fourier transform infrared; R, resting.

S0006-2960(96)02845-0 CCC: $14.00

binding of Carb and subsequent desensitization (Blanton & Cohen, 1994).

FTIR *difference* spectroscopy was recently developed as a tool for probing structural changes that occur upon the binding of Carb to the nAChR (Baenziger et al., 1992, 1993). FTIR spectra are repetitively recorded while alternately flowing buffer either with or without Carb past an nAChR film deposited on the surface of a germanium internal reflection element. After extensive signal averaging, the difference between spectra of the nAChR recorded in the R and D states (referred to as the R→D difference spectrum) exhibits a complex, but highly reproducible pattern of positive and negative bands that provides a "spectral map" of the structural changes that occur in the nAChR upon ligand binding and subsequent desensitization. The vibrational bands in this spectral map reflect changes in the structure and/or environments surrounding individual amino acid side chains as well as alterations in the conformation and/or orientation of the polypeptide backbone.

In order to gain further insight into the nature of the changes in the conformation of the polypeptide backbone that occur upon desensitization, we have recorded R→D difference spectra in ^2H_2O as a function of the time of prior exposure of the nAChR to ^2H_2O. Surprisingly, the majority of the difference bands in the amide I region of the R→D difference spectra undergo relatively large downshifts in frequency within minutes of exposure of the nAChR to ^2H_2O, consistent with their assignment to a structural perturbation upon desensitization of solvent-accessible secondary structures and/or amino acid side chain vibrations. Both the vibrational frequencies and the rapid exchange kinetics of the affected structures suggest that the formation of a channel-inactive desensitized state may result predominantly from structural changes in extramembranous regions of the polypeptide backbone as opposed to a large structural perturbation near the ion channel gate. A conformational change in the agonist binding pocket may be responsible for channel inactivation upon desensitization.

MATERIALS AND METHODS

FTIR Difference Spectroscopy. FTIR difference spectra were recorded as described previously using the attenuated total reflectance (ATR) technique on a Digilab FTS 40 spectrometer equipped with a DTGS detector (Baenziger et al., 1993; Ryan et al., 1996). Briefly, affinity-purified nAChR reconstituted into membranes composed of either egg phosphatidylcholine (EPC)/dioleoylphosphatidic acid (DOPA)/cholesterol (Chol) (molar ratio of 3:1:1) or simply EPC was deposited on the surface of a germanium internal reflection crystal. Two spectra (512 scans each) of the R state were recorded while flowing buffer (250 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, 3 mM $CaCl_2$, and 5 mM Na_2HPO_4, pH 7.0) continuously past the surface of the nAChR film. The flowing buffer was then switched to one containing 50 μM Carb. After 1 min, a third spectrum was recorded of the D state. The differences between the two R state spectra (control, not shown) and between the R and D state spectra were calculated and stored, and the flowing solution was switched back to buffer without agonist. The entire process was repeated many times, and the successive difference spectra were averaged. All difference spectra were recorded at a resolution of 8 cm^{-1} but were interpolated for presentation to a resolution of 4 cm^{-1}.

$^1H/^2H$ Exchange Difference Spectroscopy. R→D difference spectra were recorded as outlined above, but in ^2H_2O buffer after between 2 h and 3 days (the latter at 4 °C) prior exposure of the nAChR to ^2H_2O. To record R→D difference spectra after brief periods of ^2H_2O exposure, films of the nAChR were deposited on the surface of an ATR crystal from ^1H_2O and were then exposed to flowing ^2H_2O buffer. After either 5 or 15 min in ^2H_2O, spectra of either 128 or 256 scans were recorded of the R and D states, respectively. The nAChR film was then washed with ^1H_2O buffer for either 23 or 60 min, respectively, in order to remove the Carb from the nAChR membrane film and back-exchange the peptides to a protiated form. The $^1H/^2H$ exchange was then reinitiated and the data acquisition protocol repeated several times. Note that the nAChR was reexposed to ^1H_2O for at least twice the length of time of initial exposure to ^2H_2O. Complete back-exchange of the peptides to the protiated form was assessed by recording R→D difference spectra in ^1H_2O after the ^1H_2O wash.

FTIR Spectra of "Model Proteins". FTIR spectra of myoglobin, lysozyme, and trypsinogen (Sigma, St. Louis, MO) were recorded in a dehydrated state as well as in both ^1H_2O and ^2H_2O at a resolution of 2 cm^{-1} using conventional transmission techniques and an MCT detector. Dehydrated films of each protein were prepared by drying an aqueous (^1H_2O) solution containing between 200 and 300 μg of each protein on a CaF_2 window. Spectra of each protein in both ^1H_2O and ^2H_2O were recorded from 10−20 mg/mL solutions placed between CaF_2 windows separated by either a 6 or a 50 μm Teflon spacer, respectively. The latter were recorded as a function of time after the addition of ^2H_2O to a dry powder of each protein. Spectra of the nAChR in the dehydrated state as well as in both ^1H_2O and ^2H_2O buffer were recorded in a similar manner except using the ATR technique as described in detail previously (Baenziger & Méthot, 1995).

RESULTS

R→D Difference Spectra in ^1H_2O and ^2H_2O. The difference between FTIR spectra recorded in ^1H_2O in the presence and absence of Carb from the nAChR reconstituted into EPC/DOPA/Chol exhibits a number of positive and negative bands in both the amide I (1600−1700 cm^{-1}; predominantly peptide C═O stretch) and amide II (1520−1580 cm^{-1}; predominantly peptide N−H bend) regions that likely reflect changes in the structure of the polypeptide backbone upon desensitization (Figure 1A, trace a). The predominant features in these two spectral regions are two relatively intense positive bands near 1655 cm^{-1} and 1544 cm^{-1} as well as a relatively intense negative band near 1620 cm^{-1}. The frequencies of the two positive bands are characteristic of α-helix whereas the latter is characteristic of β-sheet. A previous study suggested the possible interpretation of these three bands in terms of a net conversion of β-sheet to α-helix upon desensitization (Baenziger et al., 1993). As the nAChR films exhibit a net orientation parallel to the plane of the ATR crystal, a net change in the orientation of transmembrane α-helices coupled with a perturbation in the structure of β-sheet and/or turns (positive and negative bands in the 1660−1700 cm^{-1} region) is also possible. However, an unequivocal assignment of these and other bands in the amide I region to a structural perturbation of peptides that exist in specific types of secondary structures is difficult, especially with spectra recorded in ^1H_2O buffer. In ^1H_2O, the amide I vibrations of

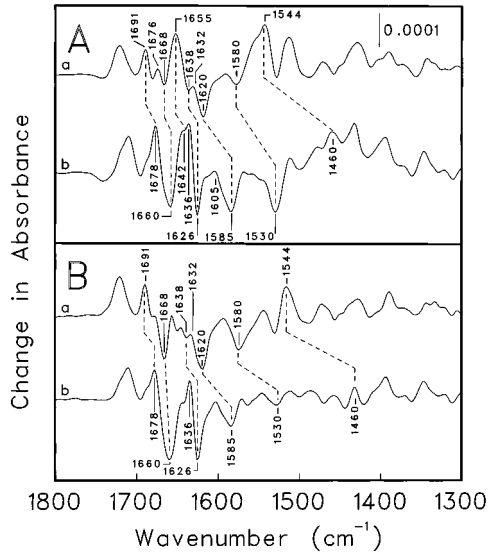

FIGURE 1: R→D difference spectra recorded from the nAChR reconstituted in membranes composed of (panel A) EPC/DOPA/Chol (molar ratio of 3:1:1) and (panel B) EPC. In each case, the R→D difference spectra were recorded in either ^1H_2O (trace a) or ^2H_2O buffer after 3 days prior exposure of the nAChR to ^2H_2O (trace b). Figure 1A, trace a is the average of 57 differences; Figure 1A, trace b the average of 10 differences; Figure 1B, trace a the average of 26 differences; and Figure 1B, trace b the average of 10 differences.

"random coil" absorb near 1655 cm^{-1} and overlap with those bands normally assigned to polypeptides in an α-helical conformation (Jackson & Mantsch, 1995). Several amino acid side chains also absorb in the 1500−1700 cm^{-1} region.

To help clarify the band assignments and thus probe the potential involvement of the transmembrane domains in desensitization, the downshifts in frequency of bands in the amide I and II regions were monitored in R→D difference spectra recorded in ^2H_2O buffer after prior exposure of the nAChR to ^2H_2O for 3 days at 4 °C (Figure 1A, trace b). The band shifts were compared to the downshifts in the frequencies of amide I component bands that are detected in absorbance spectra of the nAChR and several other "model" proteins upon exposure to ^2H_2O (see below). Difference spectra were also recorded in both ^1H_2O and ^2H_2O buffer from the nAChR reconstituted into EPC membranes lacking DOPA and Chol (Figure 1B, traces a and b) to help clarify the frequencies of some of the amide I difference band shifts (summarized in Table 1).

The 1655 cm^{-1} Band. The relatively intense positive band near 1655 cm^{-1} in the ^1H_2O R→D difference spectra that was previously attributed to a possible alteration in the structure of peptides in an α-helical conformation clearly shifts down in frequency to near 1642 cm^{-1} in ^2H_2O. This unexpected band shift is consistent with the lack of intensity near either 1655 cm^{-1} or 1642 cm^{-1} in the R→D difference spectra recorded from the nAChR in EPC membranes in ^1H_2O and ^2H_2O, respectively, and is highly characteristic of the downshift in frequency of amide I vibrations that have been observed for polypeptides in random coil conformations (Jackson & Mantsch, 1995). The downshift in frequency also correlates with the observed shift in frequency of amide I band intensity from near 1655 cm^{-1} down to near 1640 cm^{-1} in absorbance spectra recorded within *seconds* of exposure of the nAChR to ^2H_2O (Baenziger & Méthot, 1995). In contrast, ordered secondary structures such as α-helix and β-sheet typically undergo relatively minor downshifts in frequency of less than 5 cm^{-1} upon peptide $^1H/^2H$ exchange [see below and Susi et al. (1967) and Goormaghtigh et al. (1994)]. These results all support the assignment of the intense 1655 cm^{-1} band in ^1H_2O to a structural perturbation of solvent-accessible peptides in "random coil" conformations upon desensitization as opposed to a change in structure and/or orientation of the transmembrane α-helix.

Table 1: Frequencies, Exchange Kinetics, and Tentative Assignments of Bands in the Amide I and Amide II Regions of the R→D Difference Spectra Recorded in ^1H_2O and ^2H_2O Buffer

frequency (cm^{-1})				
^1H_2O	^2H_2O	intensity	exchange kinetics	band assignment
1991	1678	+	fast[a]	turn
1676	ND	+	ND[b]	β-sheet or other
1668	1660	−	fast	turn
1655	1642	+	fast	random
1638	1626	−	fast	turn
1632	1636	+	ND	β-sheet
1620	1585	−	fast	nonpeptide
1580	1530	−	fast	nonpeptide
1544	1460	+	fast	amide II

[a] Peptide or side chain $^1H/^2H$ exchange complete within 7 min of exposure to ^2H_2O. [b] Not determined: no definitive downshift in frequency is observed after 3 days exposure to ^2H_2O. Either the peptide hydrogens responsible for the bands do not exchange their peptide hydrogens over this time frame or the resulting downshifts in frequency are not detected.

The 1700−1655 cm^{-1} Spectral Region. The positive and negative bands near 1691 and 1668 cm^{-1}, respectively, in the ^1H_2O R→D difference spectra recorded from the nAChR in EPC/DOPA/Chol shift down in frequency to near 1678 and 1660 cm^{-1}, respectively, in ^2H_2O. The assigned shift in negative intensity from near 1668 cm^{-1} in ^1H_2O down to near 1660 cm^{-1} in ^2H_2O is consistent with the increase in negative intensity of both bands in the respective spectra recorded from the nAChR in EPC membranes. The downshifted 1691 cm^{-1} band likely overlaps in the ^2H_2O R→D difference spectrum with the weak positive band near 1676 cm^{-1} (the latter is only observed as a distinct band in ^1H_2O), leading to the increased intensity near 1678 cm^{-1}. This assignment suggests that the vibrational frequency of the weak 1676 cm^{-1} band (^1H_2O) is not affected by 3 days exposure of the nAChR to ^2H_2O at 4 °C. Alternately, the weak 1676 cm^{-1} band either could shift down in frequency to 1605 cm^{-1} in ^2H_2O or could represent the edge of a relatively broad 1655 cm^{-1} band (^1H_2O) superimposed upon a sharp negative band near 1668 cm^{-1}.

Bands in the 1668−1700 cm^{-1} region have been attributed to the amide I vibrations of a variety of secondary structures including turns, β-sheet, and 3_{10} helices (Jackson & Mantsch, 1995) as well as to the side chain vibrations of Asn, Gln, and Arg (Chirgadze et al., 1975; Venyaminov & Kalnin, 1990). The frequency shifts of the bands from near 1691 and 1668 cm^{-1} in ^1H_2O down to near 1678 and 1660 cm^{-1} in ^2H_2O, respectively, are smaller than those reported for either Asn/Gln (25−30 cm^{-1}) or Arg (∼60 cm^{-1}), but are relatively large for the amide I vibrations of polypeptides in ordered secondary structures, such as α-helix and β-sheet. In contrast, relatively large changes are observed in the shapes of the amide I bands, particularly in the 1650−1700 cm^{-1} region, within minutes exposure of the nAChR and several other proteins to ^2H_2O. The rapid and relatively large changes in amide I band shape suggest that highly solvent-accessible peptides (likely turn and/or random coil conforma-

tions) undergo relatively large downshifts in frequency upon peptide $^1H/^2H$ exchange (see below and Discussion). The two bands near 1691 cm^{-1} and 1668 cm^{-1} in ^1H_2O are therefore tentatively assigned to *solvent-accessible* turn-like structures.

A peptide $^1H/^2H$ exchange-insensitive band near 1676 cm^{-1} could reflect either β-sheet or another type of exchange-resistant secondary structure. Alternately, the possible shift in frequency of the band down to near 1605 cm^{-1} in ^2H_2O would be consistent with the vibrations of Arg. The band could also reflect an extension of the 1655 cm^{-1} band that shifts down in frequency to near 1642 cm^{-1} and would thus be due to peptides in random conformations.

The 1650−1500 cm^{-1} Spectral Region. The relatively intense positive band near 1550 cm^{-1} in ^1H_2O appears to shift down in frequency to near 1460 cm^{-1} in ^2H_2O. Both frequencies are highly characteristic of amide II vibrations (Baenziger & Méthot, 1995). The negative band near 1577 cm^{-1}, that is partially obscured in ^1H_2O by the overlapping positive amide II vibration, appears to shift down to near 1530 cm^{-1} in ^2H_2O whereas the negative band near 1620 cm^{-1} in ^1H_2O, that was previously attributed to β-sheet (Baenziger et al., 1993), appears to shift down to a frequency near 1585 cm^{-1} in ^2H_2O. The frequencies of the latter two bands in ^2H_2O are both too low for amide I vibrations, suggesting their assignment to the vibrations of amino acid side chains.

The weak negative band near 1638 cm^{-1} appears to shift down to near 1626 cm^{-1} in ^2H_2O, whereas the overlapping positive band near 1632 cm^{-1} in ^1H_2O appears to be insensitive to ^2H_2O. The overlap of these two bands in ^1H_2O coupled with the lack of overlap in ^2H_2O could be the cause of the apparent increase in intensity of both bands upon peptide $^1H/^2H$ exchange. The weak band near 1632 cm^{-1} in ^1H_2O that appears unaffected by deuteration is tentatively assigned to β-sheet, but other assignments are possible. The frequency and relatively large shift in frequency of the 1638 cm^{-1} band in ^1H_2O down to near 1626 cm^{-1} in ^2H_2O suggest the tentative assignment of the band to some type of solvent-accessible secondary structure, such as turns.

$^1H/^2H$ Exchange Kinetics of the Amide I Difference Bands. The relatively large downshifts in the frequencies of many of the difference bands in the amide I region of the R→D difference spectra recorded in ^2H_2O suggest that the peptides that give rise to these bands exist in solvent-accessible turn and random conformations as opposed to ordered α-helix and β-sheet. The solvent accessibilities of these peptides were examined directly by recording R→D difference spectra as a function of the time of prior exposure of the nAChR to ^2H_2O. R→D difference spectra recorded after between 2 and 5 h prior exposure of the nAChR to ^2H_2O are presented in Figure 2. In support of the amide I band assignments, the band shifts observed from near 1691, 1668, 1655, 1638, 1620, and 1577 cm^{-1} in ^1H_2O down to near 1678, 1660, 1642, 1626, 1585, and 1530 cm^{-1}, respectively, in R→D difference spectra recorded after 3 days prior exposure to ^2H_2O are clearly evident in difference spectra recorded at all times after between 2 and 5 h of peptide $^1H/^2H$ exchange. The labile hydrogens of most of the peptides and/or amino acid side chains that give rise to bands in the amide I and II regions of the R→D difference spectra therefore have completely exchanged for deuterium within 2 h of exposure of the nAChR to ^2H_2O. Note that subtle negative variations in intensity are observed near 1550 cm^{-1} in some of the

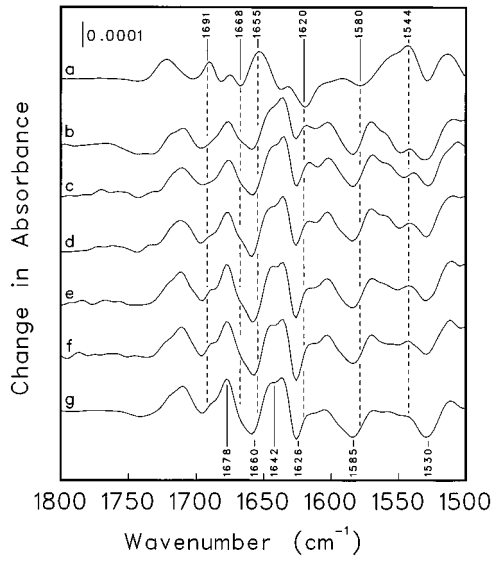

FIGURE 2: R→D difference spectra recorded from the nAChR reconstituted in membranes composed of EPC/DOPA/Chol at a molar ratio of 3:1:1 in either ^1H_2O buffer (trace a) or ^2H_2O buffer after trace b, 2 h; trace c, 2.5 h; trace d, 3 h; trace e, 4 h; trace f, 5.5 h; and trace g, 3 days prior exposure of the nAChR to ^2H_2O. Each trace from b to f represents one difference spectrum.

difference spectra due to the shift in amide II band intensity from near 1550 cm^{-1} down to near 1460 cm^{-1} with peptide $^1H/^2H$ exchange.

R→D difference spectra recorded within minutes of exposure of the nAChR to ^2H_2O (Figure 3A, trace a) also exhibit features similar to those observed in the R→D difference spectra recorded after 3 days in ^2H_2O, but superimposed upon a more intense, broad background that reflects the typical changes in both the amide I and amide II bands that occur as a result of general peptide $^1H/^2H$ exchange over the time course of the difference measurements [see Baenziger and Méthot (1995) and Méthot et al. (1995)]. Roughly 20% of the nAChR peptide hydrogens exchange between 5 s and 30 min exposure of the nAChR to ^2H_2O (Baenziger & Méthot, 1995). After subtraction of the spectral features reflecting general peptide $^1H/^2H$ exchange (Figure 3B, traces b and d), it is evident that the R→D difference spectra recorded after either 7 or 20 min prior exposure of the nAChR to ^2H_2O are both similar to those recorded after 3 days prior exposure to ^2H_2O. The peptides and/or side chains that give rise to the $^1H/^2H$ exchange-sensitive bands in the amide I region of the R→D difference spectra therefore have completely exchanged their labile hydrogens for deuterium within 7 min of exposure to ^2H_2O. The rapid exchange kinetics of the affected structures confirm their high accessibility to solvent and are consistent with the assignment of the majority of the bands in the amide I region to a structural perturbation of either solvent-accessible turn and random structures or amino acid side chain vibrations (Table 1). Note that control difference spectra recorded after 7 min prior exposure of α-bungarotoxin-treated nAChR to ^2H_2O exhibit a flat line as shown in Figure 3B, trace c.

FTIR Spectra of "Model" Proteins in ^1H_2O and ^2H_2O. The downshifts in frequency of several bands in the amide I region of the R→D difference spectra upon exposure of the nAChR to ^2H_2O are smaller than the downshifts in frequency of the amino acid side chain vibrations that typically absorb in the 1600−1700 cm^{-1} region, but are

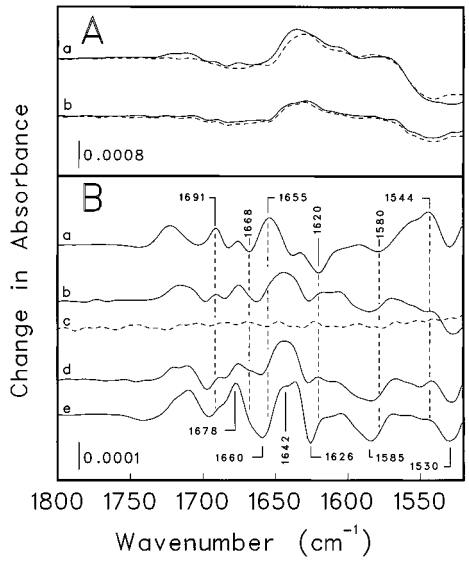

FIGURE 3: R→D difference spectra recorded from the nAChR reconstituted into membranes composed of EPC/DOPA/Chol after various times prior exposure to ^2H_2O buffer. Panel A: Difference spectra are shown for traces a, nAChR reconstituted in EPC/DOPA/Chol membranes; and traces b, nAChR reconstituted in EPC/DOPC/Chol membranes, but pretreated with the essentially irreversible competitive inhibitor α-bungarotoxin (control difference spectrum). For both traces a and b, the solid line represents the difference between spectra recorded in the presence and absence of Carb whereas the dashed line represents the difference between two spectra both recorded in the absence of Carb. The dashed line difference spectra reflect those spectral changes that occur as a result of the downshift in frequencies of amide I component bands upon general peptide $^1H/^2H$ exchange. The spectra were recorded after roughly 7 min prior exposure of the nAChR to ^2H_2O. Panel B: R→D difference spectra recorded in trace a, ^1H_2O; trace b, ^2H_2O after 7 min prior exposure to ^2H_2O; trace c, ^2H_2O after 7 min prior exposure to ^2H_2O from nAChR membranes pretreated with α-bungarotoxin (control difference spectrum); trace d, ^2H_2O after 20 min prior exposure to ^2H_2O; and trace e, ^2H_2O after 3 days prior exposure to ^2H_2O. Traces b−d are double difference spectra generated by subtracting the general peptide $^1H/^2H$ exchange background (dashed lines in panel A) from the difference spectra which exhibit the R→D differences superimposed on the peptide $^1H/^2H$ exchange background (solid lines in panel A). Traces b, c, and d are the average of 16, 9, and 7 differences respectively.

larger than the downshifts in frequency of amide I bands typically assigned to polypeptides in α-helix and β-sheet conformations. We examined in more detail the downshifts in frequency of amide I bands upon peptide $^1H/^2H$ exchange by recording absorbance spectra of a predominantly α-helical protein, myoglobin, a predominantly β-sheet protein, trypsinogen, and two mixed α/β proteins, lysozyme and the nAChR, in a dehydrated state, in ^1H_2O buffer, and at different times after exposure of each protein to ^2H_2O buffer (Figure 4). For each protein, the amide I contour is the summation of several underlying component bands whose frequencies reflect the different conformations of the polypeptide backbone. These individual amide I component bands are better visualized upon resolution enhancement of the spectra (not shown). Resolution enhancement shows that the frequencies and relative intensities of the individual amide I component bands differ for all four proteins according to their respective secondary structural features (Byler & Susi, 1986; Goormaghtigh et al., 1990). However, all four proteins exhibit two common characteristics regarding the spectral changes that occur in the amide I contour upon exposure to ^2H_2O.

The first notable feature is that the amide I peak maxima corresponding to peptides in either α-helix (1650−1660 cm^{-1} region) or β-sheet (1620−1640 cm^{-1} region) conformations appear to be relatively insensitive to the exposure of the corresponding protein to ^2H_2O over the minutes to hours time scale where ordered secondary structures are expected to exchange their labile protons for deuterium. Note that substantial exchange of peptide hydrogens for deuterium occurs for each protein over this time frame as indicated by a decrease in the intensity of the amide II band centered near 1550 cm^{-1} (not shown). Resolution enhancement of the spectra of myoglobin and lysozyme confirms that the vibrations of α-helical structures between 1650 and 1660 cm^{-1} in ^1H_2O undergo a relatively small downshift in frequency of less than 5 cm^{-1} upon exposure to ^2H_2O [see also Susi et al. (1967)]. The vibrations of peptides in a β-sheet conformation appear to be even less sensitive to ^2H_2O, as is illustrated by the minor changes in the β-sheet region of the absorption spectra of both lysozyme and trypsinogen with peptide $^1H/^2H$ exchange.

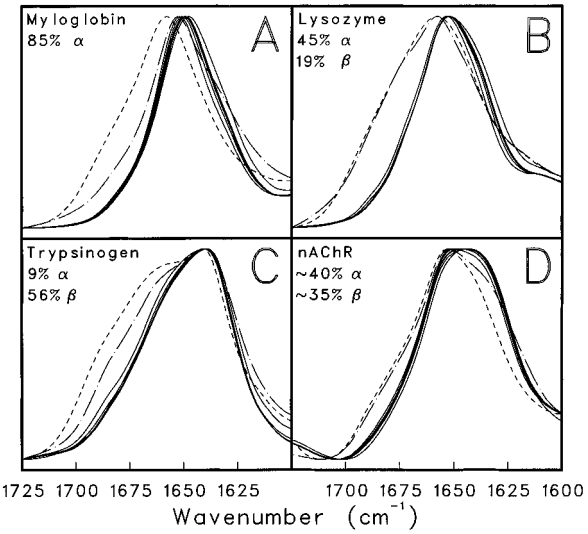

FIGURE 4: FTIR spectra of (A) myoglobin, (B) lysozyme, (C) trypsinogen, and (D) the nAChR recorded in the dehydrated state (short dashed lines), ^1H_2O (long dash−dot lines), and ^2H_2O (solid lines) after, from left to right, roughly 1, 15, 40, 80, 160, and >1500 min prior exposure of each protein to ^2H_2O. Spectra of myoglobin, lysozyme, and trypsinogen were recorded using the transmission technique whereas the spectra of the nAChR were recorded using ATR spectroscopy [see Baenziger and Méthot (1995)].

In contrast, relatively large changes in the shapes of the amide I bands, particularly in the 1650−1700 cm^{-1} region, are observed upon initial exposure (i.e. within roughly 1 min) of each protein to ^2H_2O. The rapid and relatively large changes in amide I band shape suggest that highly solvent-accessible peptides that likely exist in turn and/or random coil conformations undergo relatively large downshifts in frequency upon peptide $^1H/^2H$ exchange. In agreement, the spectral changes in the amide I band contour upon initial exposure to ^2H_2O are more dramatic for lysozyme, trypsinogen, and the nAChR which have a much larger percentage of peptides in nonordered secondary structures than does myoglobin. Furthermore, the relatively large downshift in amide I band intensity from near 1655 cm^{-1} down to near 1640 cm^{-1} that is observed within minutes of exposure of the nAChR to ^2H_2O (Figure 4A) has previously been shown to occur within *seconds* of exposure, consistent with the expected peptide $^1H/^2H$ exchange kinetics for solvent-exposed polypeptides (Baenziger & Méthot, 1995). In summary, these observations suggest that peptides involved in solvent-accessible turn and "random" secondary structures

undergo relatively large downshifts in frequency upon exposure to ^2H_2O whereas the vibrational frequencies of those peptides involved in ordered less solvent-accessible secondary structures are relatively insensitive to peptide $^1H/^2H$ exchange.

DISCUSSION

The goal of the present work was to examine the nature of the changes in structure of the polypeptide backbone that occur upon desensitization of the nAChR with a particular focus on determining whether any of the bands in the amide I region of the R→D difference spectrum reflect and thus provide insight into possible changes in the structure and/or orientation of either transmembrane α-helix or β-sheet. Note that FTIR difference spectroscopy has provided detailed insight into the structural changes that occur upon the absorption of light by light-activated proteins, such as bacteriorhodopsin and the photosynthetic reaction center (Braiman & Rothschild, 1988; Rothschild, 1992). Similarly, the positive and negative bands observed in the difference between FTIR spectra of the nAChR recorded in the presence and absence of Carb should reflect the structural changes induced in the nAChR upon the binding of Carb. The specificity of the difference bands to Carb-induced structural changes in the nAChR has been demonstrated by the absence of bands in control difference spectra recorded from nAChR films pretreated with the essentially irreversible competitive antagonist α-bungarotoxin (Baenziger et al., 1993) and by the increasing intensities of bands in difference spectra recorded from nAChR samples with increasing levels of purity (Ryan et al., 1996). Fluorescence spectroscopy has also shown that nAChR films similar to those used in the ATR difference measurements undergo the resting to desensitized affinity state transition under parallel experimental conditions (Baenziger et al., 1992). Based on these control experiments, it can be concluded that the complex pattern of positive and negative bands in the R→D difference spectra reflects the structural changes that occur upon the binding of Carb to and subsequent desensitization of the nAChR.

A previous analysis of the R→D difference spectra recorded in ^1H_2O had suggested the possible assignment of the two relatively intense amide I bands near 1655 and 1620 cm^{-1} to either a net change in structure from β-sheet to α-helix or a change in orientation of the transmembrane α-helix with a concomitant change in the structure of the β-sheet and/or turns (Baenziger et al., 1993). The possible assignment of the 1655 cm^{-1} difference band to a net change in orientation of transmembrane α-helices is significant because transmembrane α-helices form the ion channel pore and perform a central role in both channel selectivity. A slight tilting and twisting of the M2 transmembrane α-helices is responsible for channel gating (Unwin, 1995). A structural perturbation of the pore-lining transmembrane α-helices could also play a role in the inactivation of the ion channel gate upon desensitization. However, the data presented here suggest an alternate interpretation of these and other bands in the amide I region of the R→D difference spectrum and indicate that desensitization may result predominantly from a structural perturbation of solvent-accessible extramembranous regions of the polypeptide backbone as opposed to a large structural perturbation near the ion channel gate. This conclusion is based upon the following observations.

(1) The majority of the positive and negative bands in the amide I region of the R→D difference spectrum that reflect a conformational change in the polypeptide backbone upon desensitization undergo relatively large downshifts in frequency of at least 8 cm^{-1} upon peptide $^1H/^2H$ exchange. In general, the frequencies of peptides in ordered secondary structures are relatively insensitive to ^2H_2O, possibly as a result of different couplings between groups linked across intermolecular peptide hydrogen bonds in the protiated and deuterated states (Susi et al., 1967). As shown here for myoglobin, lysozyme, and trypsinogen, the amide I frequencies of α-helical peptides in globular proteins typically undergo downshifts in frequency of less than 5 cm^{-1} upon peptide $^1H/^2H$ exchange. The amide I frequencies of peptides in β-sheet conformations are even less sensitive to ^2H_2O. In contrast, the amide I bands of solvent-accessible secondary structures appear to undergo much larger downshifts in frequency as illustrated by the relatively dramatic changes in the shape of the amide I band contours of myoglobin, lysozyme, trypsinogen, and the nAChR upon initial exposure of each protein to ^2H_2O. Although these correlations are not definitive, they suggest that the majority of the bands in the amide I region of the R→D difference spectrum reflect a structural perturbation upon desensitization of solvent-accessible secondary structures, such as turns and random coil (or in some cases amino acid side chains), as opposed to a structural perturbation of ordered secondary structures, such as α-helix and β-sheet.

(2) The intense positive band near 1655 cm^{-1} in the R→D difference spectra recorded in ^1H_2O, that was previously attributed to a possible change in orientation of transmembrane α-helix, shifts down to a frequency near 1642 cm^{-1} in ^2H_2O. This downshift in frequency is highly characteristic of the amide I band shifts observed for peptides in random coil conformations (Jackson & Mantsch, 1995) and correlates with the downshift in frequency of amide I band intensity that is observed in absorbance spectra recorded within *seconds* of exposure of the nAChR to ^2H_2O (Baenziger & Méthot, 1995; see also Figure 4). In contrast, the frequency of an amide I difference band that has been attributed to a structural perturbation of transmembrane α-helix during the bacteriorhodopsin photocycle is insensitive to prolonged exposure to ^2H_2O, consistent with the exchange-resistant nature of the transmembrane α-helices of this integral membrane protein (Rothschild et al., 1993). The large downshift in frequency of the 1655 cm^{-1} difference band upon exposure of the nAChR to ^2H_2O strongly suggests the assignment of the band to a structural perturbation of peptides in solvent-accessible random conformations.

(3) No clear bands are observed in the 1650−1655 cm^{-1} region of the ^2H_2O R→D difference spectra that can be attributed to a structural perturbation of transmembrane α-helix. The nAChR exhibits a core of exchange-resistant α-helical peptides that give rise to an amide I band centered near 1655 cm^{-1} in absorbance spectra recorded after 3 days exposure of the nAChR to ^2H_2O (Baenziger & Méthot, 1995). A net change in either the orientation or the structure of transmembrane α-helices that are resistant to peptide $^1H/^2H$ exchange should give rise to a band in the R→D difference spectrum near 1655 cm^{-1} in both ^1H_2O and ^2H_2O buffer (Susi et al., 1967; Surewicz et al., 1993; Jackson & Mantsch, 1995; Baenziger & Méthot, 1995). Alternately, a net change in orientation or structure of transmembrane channel pore-lining segments that might be more accessible to solvent and that might exchange during prolonged exposure to ^2H_2O (however, see below) should give rise to a difference band near

1655 cm^{-1} in ^1H_2O that shifts down to near 1650 cm^{-1} in ^2H_2O upon peptide $^1H/^2H$ exchange. The lack of intensity between 1650 cm^{-1} and 1660 cm^{-1} in the R→D difference spectra recorded in ^2H_2O suggests that there are no large net structural perturbations of transmembrane α-helical secondary structures upon desensitization, regardless of whether or not the transmembrane structures are resistant to peptide $^1H/^2H$ exchange.

Note that Ludlum et al. (1995) recently showed that the ATR technique is sufficiently sensitive to detect a change in amide I band intensity arising from a structural reorientation of a *single* amino acid residue located in a transmembrane α-helix. The ATR FTIR difference technique should thus be sufficiently sensitive to detect any net change in the orientation of transmembrane α-helical structures upon desensitization of the nAChR.

(4) Most of the downshifts in frequency of amide I vibrations, including the downshift in frequency of the 1655 cm^{-1} band, that are observed in difference spectra recorded in ^2H_2O are evident within 7 min prior exposure, indicating that the majority of the polypeptides that are involved in both ligand binding and the R→D conformational change and that give rise to bands in the amide I region of the R→D difference spectra exchange their labile hydrogens for deuterium on the seconds to minutes time scale. The rapid exchange kinetics confirm the high accessibility of most of the affected structures to bulk aqueous solvent and thus place restrictions on the possible locations of those regions of the polypeptide backbone that are affected by desensitization.

Model peptides hydrogen bonded with bulk solvent exchange within seconds of exposure to ^2H_2O. Those involved in ordered secondary structures exchange on a time scale from minutes to days or longer depending on their accessibility to solvent and the rates and amplitudes of local peptide motions (Englander et al., 1972). Transmembrane peptide hydrogens are particularly resistant to exchange due to the relative solvent inaccessibility of the hydrophobic environment of the lipid bilayer. The transient folding/unfolding motions of ordered secondary structures that are necessary for hydrogen exchange are also likely to be restricted in transmembrane structures because such motions would expose the polar N−H and C═O groups to the hydrophobic lipid acyl chains. The resistance of transmembrane secondary structures to peptide $^1H/^2H$ exchange has been observed experimentally for numerous integral membrane proteins including bacteriorhodopsin and rhodopsin (Osborne & Nabedryk-Viala, 1977; Konishi et al., 1977; Englander et al., 1982; Earnest et al., 1990). An unusually large number of exchange-resistant peptide hydrogens has also been noted for the photosynthetic reaction center, the transmembrane β-barrel pore, and porin, as well as other transmembrane proteins (Kleffel et al., 1985; Nabedryk et al., 1988; He et al., 1991; Sonveaux et al., 1994).

For the nAChR, roughly 30% of the peptide hydrogens exchange within seconds of exposure of the nAChR to ^2H_2O, 50% after 30 min, 65% after 12 h, and 75% after 3 days at 4 °C (Baenziger & Méthot, 1995). The 30% rapidly exchanging peptides is similar to the 25−30% of peptides in turn and random conformations suggested by a curve fit analysis of the amide I band (Méthot et al., 1994). The roughly 25% peptide hydrogens that are resistant to exchange after 3 days in ^2H_2O likely include the 20−30% of peptide hydrogens both predicted (Claudio et al., 1993) and found experimentally (Unwin, 1993; Görne-Tschelnokow, 1994) within the lipid bilayer. The exchange-resistant peptides in the nAChR have been tentatively assigned to polypeptides in the lipid bilayer based on the expected resistance of such structures to peptide $^1H/^2H$ exchange and the strong correlation observed for other integral membrane proteins of known structure between the number of exchange-resistant polypeptides and the proportion of polypeptides within the lipid bilayer, as noted above.

Based upon both the expected resistance of the transmembrane segments to peptide $^1H/^2H$ exchange and the experimentally observed existence of an exchange-resistant core in the nAChR, one would not expect any of the amide I bands in the R→D difference spectra that reflect a net change in structure and/or orientation of transmembrane peptides to undergo a downshift in frequency (i.e., no peptide $^1H/^2H$ exchange) after 3 days exposure of the nAChR to ^2H_2O, let alone within either minutes or hours of exposure. Even the peptide hydrogens involved in the pore-lining transmembrane α-helices are likely to be resistant to exchange as the transient folding/unfolding motions necessary for rapid exchange are likely incompatible with the maintenance of a consistently closed ion channel in the absence of cholinergic agonists. The rapidity of the amide I difference band shifts within minutes of exposure of the nAChR to ^2H_2O strongly suggests that the majority of the changes in the conformation of the polypeptide backbone upon desensitization detected by FTIR difference spectroscopy involve solvent-accessible extramembranous structures as opposed to those found within the hydrophobic environment of the lipid bilayer.

In conclusion, our results suggest that the formation of the channel-inactive desensitized state results predominantly from a structural perturbation of extramembranous regions of the nAChR as opposed to a large structural perturbation near the ion channel gate. One possible interpretation of these data is that the structure of the ligand binding site may be altered in the desensitized state in such a way that it is no longer capable of initiating channel gating upon the binding of Carb or other cholinergic ligands. Structural alterations have been detected in the ligand binding site upon desensitization (Galzi et al., 1991a). The affinity of the nAChR for agonists increases dramatically upon desensitization (Boyd & Cohen, 1980), consistent with a change in structure of the ligand binding site. The tentative assignment of many of the bands in the R→D difference spectra to turn and random conformations is also consistent with a structural perturbation near the ligand binding site, as the active sites of many proteins often involve solvent-accessible turn and unordered regions of the polypeptide backbone.

However, our data do not completely rule out a structural change in the transmembrane domains upon desensitization. The weak potentially exchange-resistant structures that give rise to bands near 1676 and 1632 cm^{-1} in the ^1H_2O R→D difference spectra could reflect a change in structure and/or orientation of the transmembrane β-sheet, although other interpretations of these bands are possible. The existence of transmembrane β-strands has been suggested by both electron microscopy and FTIR studies of the transmembrane domains of the nAChR generated by proteinase K treatment (Unwin, 1993; Görne-Tschelnokow, 1994). In contrast, site-directed mutagenesis, chemical labeling, and FTIR/hydrogen exchange studies suggest that the transmembrane segments are α-helical in nature (Giraudat et al., 1987; Villarroel et

al., 1992; Charnet et al., 1990; Blanton & Cohen, 1992, 1994; Baenziger & Méthot, 1995). The latter experiments argue against the assignment of the two bands at 1676 and 1638 cm^{-1} to a structural perturbation of exchange-resistant transmembrane β-sheet. A definitive assignment and thus interpretation of these bands will require a more detailed study including linear dichroism difference measurements.

Both a twisting of transmembrane segments about their long axes and/or compensatory changes in the orientation of transmembrane segments (i.e., no net change in orientation of transmembrane peptide carbonyls) could also occur, but would not give rise to bands in the R→D difference spectra. Such subtle changes in structure would likely involve the pore-lining α-helices as opposed to the nonpore-lining transmembrane segments as the surface exposure of the latter to the hydrophobic environment of the lipid bilayer does not change in the presence of Carb (Blanton & Cohen, 1994). Subtle changes in the structure of the pore-lining α-helices might account for both the conformational sensitivity of the nAChR to the composition of the lipid membrane [see Ryan et al. (1996) and references cited within] and the sensitivity of the rates of nAChR desensitization on the phosphorylation state of side chains in the cytoplasmic domains (Huganir & Miles, 1989). In addition, the affinities of the nAChR for several noncompetitive antagonists that bind to the ion channel pore are affected by desensitization (Krodel et al., 1979; White et al., 1991). It is also interesting that subtle mutations in leucine residues at the midpoint of the pore-lining transmembrane α-helices not only affect channel gating but also influence the affinity of the nAChR for acetylcholine (Revah et al., 1991; Labarca et al., 1995).

It is possible that subtle changes in the structure of the pore-lining α-helices, such as those caused by site-directed mutagenesis (Revah et al., 1991; Labarca et al., 1995), may lead to more substantial changes in the structure of the extramembranous domains. Conversely, "relatively" large changes in the structure of the solvent-accessible regions of the polypeptide backbone in the extramembranous domains of the nAChR upon agonist binding may be required to initiate channel gating and desensitization, but may only lead to subtle changes in the structure of the pore-lining transmembrane segments.

ACKNOWLEDGMENT

We thank Robin Reiber for the preparation of the figures and both Nathalie Méthot and Stephen Ryan for technical assistance.

REFERENCES

Baenziger, J. E., & Méthot, N (1995) *J. Biol. Chem.* 270, 29129−29137.
Baenziger, J. E., Miller, K. W., & Rothschild, K. J. (1992) *Biophys. J. 61*, 983−992.
Baenziger, J. E., Miller, K. W., & Rothschild, K. J. (1993) *Biochemistry 32*, 5448−5454.
Blanton, M. P., & Cohen, J. B. (1992) *Biochemistry 31*, 3738−3750.
Blanton, M. P., & Cohen, J. B. (1994) *Biochemistry 33*, 2859−2872.
Braiman, M. S., & Rothschild, K. J. (1988) *Annu. Rev. Biophys. Biophys. Chem. 17*, 541−570.
Charnet, P., Labarca, C., Leonard, R. J., Vogelaar, N. J., Czyzyk, L., Gouin, A., Davidson, N., & Lester, H. A. (1990) *Neuron 4*, 87−95.
Chirgadze, Y. N., Fedorov, O. V., & Trushina, N. P. (1975) *Biopolymers 14*, 679−694.
Claudio, T., Balliver, M., Patrick, J., & Heinemann, S. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 1111−1115.
Englander, J. J., Downer, N. W., & Teitelbaum, (1972) *Annu. Rev. Biochem. 41*, 903−924.
Englander, J. J., Downer, N. W., & Englander, S. W. (1982) *J. Biol. Chem. 257*, 7982−7986.
Galzi, J.-L., Revah, F., Bouet, F., Ménez, A., Goeldner, M., Hirth, C., & Changeux, J.-P. (1991a) *Proc. Natl. Acad. Sci. U.S.A. 88*, 5051−5055.
Galzi, J.-L., Revah, F., Bessis, A., & Changeux, J.-P. (1991b) *Annu. Rev. Pharmacol. 31*, 37−72.
Giraudat, J., Dennis, M., Heidmann, T. H., Maumont, P. Y., Lederer, F., & Changeux, J.-P. (1987) *Biochemistry 26*, 2410−2418.
Goormaghtigh, E., Cabiaux, V., & Ruysschaert, J.-M. (1994) *Subcell. Biochem. 23*, 405−450.
Görne-Tschelnokow, U., Strecker, A., Kaduk, C., Naumann, D., & Hucho, F. (1994) *EMBO J. 13*, 338−341.
He, W.-Z., Newell, W. R., Haris, P. I., Chapman, D., & Barber, J. (1991) *Biochemistry 30*, 4552−4559.
Huganir, R. L., & Miles, K. (1989) *Crit. Rev. Biochem. Mol. Biol. 24*, 183−215.
Jackson, M., & Mantsch, H. H. (1995) *Crit. Rev. Biochem. Mol. Biol. 30*, 95−120.
Karlin, A. (1993) *Curr. Opin. Neurobiol. 3*, 299−309.
Kleffel, B., Garavito, R. M., Baumeister, W., & Rosenbusch, J. P. (1985) *EMBO J. 4*, 1589−1592.
Konishi, T., & Packer, L. (1977) *FEBS Lett. 80*, 455−458.
Krodel, E. K., Beckman, R. A., & Cohen, J. B. (1979) *Mol. Pharmacol. 15*, 294−312.
Labarca, C., Nowak, M. W., Zhang, H., Tang, L., Deshpande, P., & Lester, H. A. (1995) *Nature 376*, 514−516.
Lester, H. A. A. (1992) *Annu. Rev. Biophys. Biomol. Struct. 21*, 267−292.
Ludlam, C. F. C., Sonar, S., Lee, C.-P., Coleman, M., Herzfeld, J., RajBhandary, U. L., & Rothschild, K. J. (1995) *Biochemistry 34*, 2−6.
McCarthy, M. P., & Moore, M. A. (1992) *J. Biol. Chem 267*, 7655−7663.
Méthot, N., Demers, C. N., & Baenziger, J. E. (1995) *Biochemistry 34*, 15142−15149.
Nabedryk, E., Andrianambinintsoa, S., Mäntele, W., & Breton, J. (1988) *NATO ASI Ser., Ser. A 168*, 237−250.
Osborne, H. B., & Nabedryk-Viala, E. (1977) *FEBS Lett. 84*, 217−220.
Revah, F., Bertrand, D., Galzi, J.-L., Devillers-Thiéry, A., Mulle, C., Hussy, N., Bertrand, S., Ballivet, M., & Changeux, J.-P. (1991) *Nature 353*, 846−849.
Rothschild, K. J. (1992) *J. Bioenerg. Biomembr. 24*, 147−167.
Rothschild, K. J., Marti, T., Sonar, S., He., Y.-W., Rath, P., Fischer, W., & Khorana, H. G. (1993) *J. Biol. Chem. 268*, 27046−27052.
Ryan, S. E., Demers, C. N., Chew, J. P., & Baenziger, J. E. (1996) *J. Biol. Chem. 271*, 24590−24597.
Sonveaux, N., Conrath, K., Capiau, C., Brasseur, R., Goormaghtigh, E., & Ruysschaert, J.-M. (1994) *J. Biol. Chem. 269*, 25637−25645.
Susi, H., Timasheff, S. N., & Stevens, L. (1967) *J. Biol. Chem. 242*, 5460−5466.
Unwin, N. (1993) *J. Mol. Biol. 229*, 1101−1124.
Unwin, N. (1995) *Nature 373*, 37−43.
Venyaminov, S. Y., & Kalnin, N. N. (1990) *Biopolymers 30*, 1243−1257.
Villarroel, A., & Sakmann, B. (1992) *Biophys. J. 62*, 196−205.
White, B. H., & Cohen, J. B. (1988) *Biochemistry 27*, 8741−8751.
White, B. H., Howard, S., Cohen, S. G., & Cohen, J. B. (1991) *J. Biol. Chem. 266*, 21595−21607.

BI962845M