The structure of the nicotinic acetylcholine receptor (nAChR) has been studied using a novel combination of hydrogen/deuterium exchange and attenuated total reflectance Fourier transform infrared spectroscopy. Fourier transform infrared spectra show marked changes in both the amide I and amide II bands upon exposure of the nAChR to $^2$H$_2$O. The substantial decrease in intensity of the amide II band reflects the exchange of roughly 30% of the peptide hydrogens within seconds of exposure to $^2$H$_2$O, 50% after 30 min, 60% after 12 h, and 75% after prolonged exposure for several days at room temperature or lower temperatures. The 30% of peptide hydrogens that exchange within seconds is highly exposed to solvent and likely involved in random and turn conformations, whereas the 25% of exchange-resistant peptide hydrogens is relatively inaccessible to solvent and likely located in the transmembrane domains of the nAChR. Marked changes occur in the amide I contour within seconds of exposure of the nAChR to $^2$H$_2$O as a result of relatively large downshifts in the frequencies of amide I component bands assigned to turns and random structures. In contrast, only subtle changes occur in the amide I contour between 3 min and 12 h after exposure to $^2$H$_2$O as a result of slight downshifts in the frequencies of $\alpha$-helix and $\beta$-sheet vibrations. It is demonstrated that the time courses and relative magnitudes of the amide I component band shifts can be used both as an aid in the assignment of component bands to specific secondary structures and as a probe of the exchange rates of different types of secondary structures in the nAChR. Significantly, the intensities of the band shifts reflecting the exchange of $\alpha$-helical secondary structures are relatively weak indicating that a large proportion of the 25% exchange resistant peptides adopt an $\alpha$-helical conformation. Conversely, no evidence is found for the existence of a large number of exchange-resistant $\beta$-strands. The exchange kinetics suggest a predominantly $\alpha$-helical secondary structure for the transmembrane domains of the nAChR.

The nicotinic acetylcholine receptor (nAChR) is a large integral membrane protein (~290,000 daltons) that responds to the binding of cholinergic agonists by transiently opening a cation-selective ion channel across the postsynaptic membrane (for recent reviews see Refs. 1–3). The nAChR is composed of four distinct subunits arranged as a pentamer ($\alpha_2$,$\beta_2$), pseudosymmetrically around a central pore that functions as the ion channel. The four subunits all share a high degree of sequence homology including four conserved ~25-amino acid residue-long hydrophobic segments (designated M1–M4) that likely form four transmembrane $\alpha$-helices (4, 5), although transmembrane $\beta$-strands are possible (6–8). The transmembrane M2 segment from each subunit lines the ion channel pore and controls channel selectivity (9, 10). All four may play an important role in both channel gating and the inactivation of channel gating that occurs upon either prolonged exposure to acetylcholine (desensitization) or reconstitution into lipid membranes lacking both cholesterol and anionic lipids (11–13).

The secondary structure of the four putative transmembrane segments identified by hydrophobicity plots has been the subject of considerable controversy (36). Each of the four segments was originally assigned an $\alpha$-helical secondary structure based largely on the $\alpha$-helical transmembrane segments identified in other integral membrane proteins. An $\alpha$-helical secondary structure of M2 is suggested by site-directed mutagenesis and photoaffinity labeling with noncompetitive channel blockers, which both reveal an $\alpha$-helical periodicity in the exposure of M2 to the ion channel pore (14–16; however, see Ref. 17). Similarly, the labeling pattern by hydrophobic photoaffinity probes suggests an $\alpha$-helical periodicity in the exposure of both M3 and M4 to the hydrophobic core of the lipid bilayer. The labeling of M1 is consistent with an $\alpha$-helix that is slightly distorted due to the presence of a proline residue in the C-terminal half of the hydrophobic region (18, 19). The presence of five cylindrical rods of electron density in the structure of the nAChR probed at 9-Å resolution by electron microscopy has also been interpreted in terms of an $\alpha$-helical secondary structure of the pore-lining M2 segment from each nAChR subunit (7).

However, the lack of similar cylindrical regions of electron density in the periphery of the transmembrane domains of the nAChR in the 9-Å resolution electron density map raises questions concerning the $\alpha$-helical secondary structures assigned to M1, M3, and M4 (7). If these three transmembrane segments do not adopt an $\alpha$-helical conformation, between five and seven transmembrane $\beta$-strands are required for each subunit to account for the 25–30% of the nAChR located within the hydrophobic region of the lipid bilayer. The presence of a large number of transmembrane $\beta$-strands was recently suggested by the appearance of bands characteristic of $\beta$-sheet structures in FTIR spectra of the nAChR pretreated with proteinase K to remove the extramembranous domains of the protein (8). In addition, some studies of the secondary structure of the nAChR...
suggest an α-helical content of between 18 and 23% (12, 13, 20). The roughly 20% α-helix is not sufficient to account for both the four putative transmembrane α-helices proposed for each subunit (20–25% of the total protein) and the α-helical structures detected by electron microscopy in the extramembranous regions of the nAChR, and thus implies the existence of non-α-helical transmembrane segments (13, 20).

The possibility of transmembrane β-strands in the nAChR has profound implications for the mechanisms of both channel gating and desensitization and calls into question the validity of hydrophobicity plots for predicting the transmembrane topology of integral membrane proteins in general. In this paper, we have examined the structure of the nAChR using a combination of FTIR spectroscopy and hydrogen/deuterium exchange. Spectra recorded after prolonged exposure of the nAChR to H2O are characteristic of a mixed α/β protein and suggest a predominance of α-helical secondary structures. The hydrogen/deuterium exchange spectra indicate that a large percentage of the α-helical peptide hydrogens are resistant to exchange with deuterium and likely exist within the hydrophobic, relatively solvent inaccessible region of the lipid bilayer. The large percentage of exchange-resistant α-helical peptides provides strong support for a predominantly α-helical secondary structure of the nAChR transmembrane domains. The results also demonstrate several novel features in hydrogen/deuterium exchange spectra that can be exploited for probing integral membrane protein structure and function.

**EXPERIMENTAL PROCEDURES**

**Materials—**Cholesterol was purchased from Sigma. Egg lecithin (referred to as DOPC) and dioleoyl phosphatidic acid (DOPA) were from Avanti Polar lipids, Inc. (Alabaster, AL). Deuterium oxide was from Aldrich. Frozen Torpedo californica electric tissue (Marinus, Long Beach, CA) was transported to the laboratory in dry ice and stored at −80°C.

**Sample Preparation—**The nAChR was affinity-purified on a bromoacetylcholine bromide-derivatized Bio-Rad (Richmond, CA) Affi-Gel 20 column and then reconstituted into lipid vesicles composed of DOPC/DOPA/cholesterol at a molar ratio of 3:1:1 (17, 24). In DOPC/DOPA/cholesterol, the nAChR is functional and retains the ability to undergo channel gating and ligand-induced desensitization (Refs. 21, 22, and references within). The specific activity of the affinity-purified membranes averaged 7.49 nmol of 125I-labeled α-bungarotoxin binding sites/mg of protein. Samples were stored at −80°C as 100-μl aliquots in phosphate buffer, pH 7.0, containing 250 μg of nAChR protein.

**FTIR Spectroscopy**—FTIR spectra of affinity-purified nAChR reconstituted into membranes composed of DOPC/DOPA/cholesterol were recorded as a function of time from 5 s to 12 h after exposure to H2O and reveal a number of spectral changes that reflect the exchange of peptide N-H for N-D (Fig. 1). These include a marked change in the shape of the amide I band between 1600 and 1700 cm−1 and a substantial decrease in the intensity of the amide II band, which is centered near 1547 cm−1 in H2O but shifts down in frequency in H2O to near 1450 cm−1 (amide II band). The rates of the spectral changes reflect the rates at which the various peptide hydrogens of the nAChR exchange for deuterium in the bulk solvent and provide a sensitive measure of nAChR structure and conformational change.

**Rate and Extent of Peptide 1H/2H Exchange—**The rates and extent to which the nAChR peptide hydrogens exchange for deuterium can be followed by monitoring the residual intensity of the amide II band (primarily peptide N-H bending vibration) near 1547 cm−1 as a function of time after exposure of the nAChR to H2O. The decrease in intensity of the amide II band (plotted as a change in the amide II/amide I ratio in Fig. 1C) is dramatic within the first 30 min of exposure, indicating a rapid exchange of a large number of peptide hydrogens for deuterium, and is followed by a relatively low level of exchange over the next 11 h. At pH 7.0, 22.5°C, model peptides hydrogens bonded to bulk solvent exchange with half-times of less than 1 s, whereas those involved in ordered secondary structures exchange with half-times of up to 10 orders of magnitude longer, depending upon solvent accessibility and the rates at which the secondary structures undergo unfolding/refolding reactions (26).

The large population of rapidly exchanging peptides includes those hydrogen bonded to bulk solvent in random coil and turn structures as well as those involved in highly solvent accessible α-helix and β-sheet secondary structures. The peptides that exchange between 30 min and 12 h are involved in less solvent-accessible secondary structures.

The percentage of nAChR peptides that have exchanged at any time can be calculated by comparing the residual amide II band intensity at 1547 cm−1 with the corresponding intensities in spectra recorded under conditions of complete or no peptide 1H/2H exchange. The amide II band intensity corresponding to the fully protonated nAChR (no exchange) was estimated from spectra recorded in H2O buffer (Figs. 1 and 2, dashed lines) after subtraction of the broad, overlapping 1H–O–1H bending vibration centered near 1640 cm−1. The end point of the subtraction was judged by a flat base line in the 2000–2500 cm−1 region (see also “Discussion”). The amide II intensity at 1547 cm−1, corresponding to 100% exchange of peptide hydrogens for

2 S. E. Reid and J. E. Baenziger, submitted for publication.
deuterium was determined by incubating the nAChR in 2H2O
buffer for extended periods of time (Fig. 2). Complete peptide
1H/2H exchange was achieved after 1 h at pH 11.0 and 95°C,
which resulted in a significant structural alteration of the
nAChR, as judged by a marked change in the shape of the
resulting amide I band contour. The spectra of the structurally
altered or possibly denatured receptor were recorded after re-
adjusting the pH back to 7.0 (Fig. 2A, long dashed line). Spectra
were then recorded after 38, 94, 196, 298, and 364 s (solid lines
from second top to bottom at 1547 cm⁻¹). Spectra are normalized, as the
addition of 2H₂O to a dry film leads to the expansion of the nAChR film
on the surface of the ATR crystal and thus a reduction in the absolute
intensity of the protein signal.

Comparison of either the intensity or area of the residual
amide II band in the various spectra presented in Figs. 1 and 2
indicates that roughly 30% of the nAChR peptides exchange
with deuterium within 5 s of exposure to 2H₂O at 22.5°C. Roughly 50%
have exchanged after 30 min and 60% after 12 h. Prolonged exposure of the nAChR to 2H₂O for several days at
either 4°C or room temperature leads to the exchange of 75%
of the peptide hydrogens, whereas 95% exchange after subse-
quent incubation for up to 24 h at 60°C (Fig. 2). The 30% of
peptides that exchange after 5 s of exposure to 2H₂O includes a
large proportion that are hydrogen-bonded to bulk solvent and is
consistent with the roughly 20% random coil and 6% turn
structures predicted for the nAChR by spectral deconvolution
and curve fitting the amide I band (21). The 25% of exchange-

resistant peptides is similar to both the 25–30% of the nAChR found within the lipid bilayer by electron microscopy (7) and proteinase K digestion (8) and the 20–25% of peptides predicted using hydrophobicity plots to form transmembrane α-helices (4, 5). A large proportion of the exchange-resistant peptides is likely located within the hydrophobic, relatively solvent inaccessible region of the lipid bilayer (see below and "Discussion"). Note that the 5% of peptides that remain protected after incubation of the nAChR for 24 h at 60 °C are unusually resistant to exchange, even for transmembrane α-helices. In comparison, complete exchange of all α-helical transmembrane peptide hydrogens occurs after a 6-h incubation of the integral membrane protein rhodopsin in 2H2O at 60 °C (28, 29). The 5% of extremely exchange-resistant peptide hydrogens is sufficient to form five transmembrane α-helices and may reflect five highly ordered α-helices lining the ion channel pore. The unusually high degree of order of these pore-lining α-helices suggested by Blanton and Cohen (19) may be the cause of their high resistance to peptide 1H/2H exchange and may be required to maintain a consistently closed conformation of the ion channel in the absence of bound acetylcholine.

Secondary Structure-sensitive Amide I Band—The amide I band between 1600 and 1700 cm−1 reflects primarily the peptide C=O stretching vibration coupled to N–H in plane bending and C–N stretching and is very sensitive to hydrogen bonding and thus both protein secondary structure and peptide 1H/2H exchange. In 1H2O, the amide I band exhibits an intense maximum near 1655 cm−1 with shoulders near 1635 cm−1 and between 1670 and 1700 cm−1 (Fig. 3A). After exposure of the nAChR to 2H2O for 3 days at 4 °C, there is a large decrease in intensity above 1650 cm−1 concomitant with an increase in intensity near 1640 cm−1, leading to a symmetric amide I band with a relatively broad maximum between 1630 and 1660 cm−1 (Fig. 3B, top trace). Both the amide I (1H2O) and amide I (2H2O) contours are the summation of several underlying component bands whose frequencies reflect different conformations of the nAChR polypeptide backbone. These “hidden” bands can be visualized by narrowing the individual component band line widths using resolution enhancement techniques and can be assigned to specific secondary structures as discussed in more detail by Méthot et al. (21). The resolution enhanced spectra also provide insight into the component band shifts that result in the overall change in shape of the amide I contour upon exposure to 2H2O.

The deconvolved and fourth derivative spectra reveal a relatively intense component band centered at 1655 cm−1 in spectra recorded in both 1H2O and 2H2O buffer (Fig. 3). The relative intensity of the band decreases upon peptide 1H/2H exchange, which is the main cause of the change in the shape of the amide I band contour in 1H2O. The majority of the loss of intensity can be attributed to a characteristic shift in the frequency of vibrations due to polypeptide segments in random coil conformations from between 1650 cm−1 and 1660 cm−1 in 1H2O down to near 1640 cm−1 in 2H2O. The residual intensity remaining between 1650 and 1655 cm−1 is due to polypeptide chain in the α-helical conformation. This assignment is based on both normal mode calculations (30), which predict a band near this frequency for the α-helix, and empirical observations, which show that an intense band is observed near 1655 cm−1 in the resolution enhanced spectra of numerous proteins exhibiting predominantly α-helical conformations (31, 32). α-Helical vibrations downshift in frequency by only a few wave numbers upon peptide 1H/2H exchange (Ref. 33; see Fig. 4). The strong intensity of the band remaining at 1655 cm−1 is likely due to unexchanged α-helical peptides.

Resolution enhancement resolves the high frequency shoulder of the amide I band into three bands at 1691 cm−1, 1680 cm−1, and 1672 cm−1. Bands near 1670 cm−1 are often attributed to β-sheet and those near 1690 cm−1 and 1680 cm−1 to turn structures. The relatively intense shoulder near 1635 cm−1 in both the amide I and amide I’ bands is resolved into two main component bands centered near 1630 cm−1 and 1625 cm−1. These bands have been detected in numerous proteins and are highly diagnostic of β-sheet structures. In 2H2O, the 1690-cm−1 band partially shifts down in frequency to near 1680 cm−1 contributing to the marked change in shape of the amide I contour upon exposure of the nAChR to 2H2O. Several other relatively minor band shifts upon exposure of the nAChR to 2H2O are discussed in more detail below.

Note that the symmetric amide I band contour with a relatively broad maximum between 1630 and 1660 cm−1 observed in spectra acquired after several days’ exposure to 2H2O (Fig. 3B, top trace) differs from the amide I band contours observed for both predominantly α-helical and predominantly β-sheet proteins and is consistent with a mixed α/β protein. A mixed α/β structure is supported by the strong intensities of the amide I component bands due to peptides in α-helix and β-sheet conformations in the resolution-enhanced spectra, although the greater intensity of the α-helix band near 1655 cm−1 suggests a predominance of α-helical secondary structures in the nAChR. The α-helix amide I component band is relatively less intense in spectra recorded here using ATR than in spectra recorded previously using transmission techniques (21). The reduction in intensity is likely due to a predominant orientation of the α-helices perpendicular to the bilayer surface (7), and thus the plane of the intrinsically dichroic ATR crystal (27). The spectra recorded using ATR slightly underestimate the relative contribution of α-helices to the secondary structure of the nAChR.

The qualitative interpretation of the FTIR spectra is in agreement with recent secondary structure estimates from both FTIR and CD that suggest roughly 40% α-helix and between 20 and 35% β-sheet (21, 35) but contrasts with other reports, which have suggested a predominantly β-sheet protein with only 20% α-helix and closer to 42% β-sheet (13, 20). Significantly, the roughly 40% α-helix confirmed here in the qualitative analysis of the spectra is sufficient to account for the four putative transmembrane α-helices predicted for each nAChR subunit (20–25% of the protein) by hydrophobicity plots (4, 5) as well as a substantial portion of the extramembranous domains (see Ref. 21 for a detailed discussion).

Rate and Extent of Exchange of α-Helix, β-Sheet, Random, and Turn Structures—The rates of exchange of α-helix, β-sheet, random, and turn structures can be monitored by following changes in the shape of the amide I band (which reflect the downshifts in frequency of the individual amide I component bands discussed above) as a function of time after exposure of the nAChR to 2H2O. Surprisingly, the time course of the changes in the amide I band seem to differ from the time course of the decrease in intensity of the amide II band, which directly reflects the rates of peptide 1H/2H exchange. 30% of the nAChR peptides exchange within 5 s of exposure of the nAChR to 2H2O, an additional 20% within the next 30 min, and an additional 10% within the next 12 h. In contrast, the majority of the spectral changes observed in the amide I band of spectra recorded after 3 days in 2H2O are already complete within 5 s of the addition of 2H2O to a dry nAChR film (Fig. 1B), and the subsequent changes over the next 12 h are relatively minor.

The rapidity of the major spectral changes that occur after the addition of 2H2O indicates that the 1H/2H exchange-sensitive peptide carbonyls whose downshifts in frequency are responsible for the majority of the spectral changes in 2H2O must
be highly solvent-accessible, and likely hydrogen bonded to bulk solvent rather than to peptide hydrogens in ordered secondary structures. The solvent accessibility of the peptide carbonyls suggests that the bands near 1680 and 1640 cm\(^{-1}\) in \(^2\)H\(_2\)O (1690 and 1655 cm\(^{-1}\) in \(^1\)H\(_2\)O, respectively) are involved in either turn or random coil conformations, in agreement with the frequency based assignment of these two component bands discussed above. The magnitude of the rapid spectral changes also suggests that the downshifts in frequency of solvent exposed peptide carbonyls upon exposure to \(^2\)H\(_2\)O are much larger than the band shifts observed for \(\alpha\)-helix and \(\beta\)-sheet secondary structures. The lack of a dramatic change in frequency of \(\alpha\)-helical peptides upon exposure to \(^2\)H\(_2\)O may be due to compensating effects of \(^2\)H\(_2\)O on the amide I vibration coupling to the nearest neighbors across hydrogen bonds (33). The differences in the magnitudes of the band shifts of peptides in ordered secondary structures relative to those exposed to aqueous solvent accounts for the apparent differences between the time courses of the changes in the amide I and amide II bands.

The relatively minor amide I component band shifts that occur upon the exchange of \(\alpha\)-helix and \(\beta\)-sheet secondary structures are seen more clearly upon resolution enhancement of the spectra recorded over the time course of the experiment (Fig. 4A) and in “exchange difference spectra” calculated by subtracting the spectrum recorded after 3 min of exposure to \(^2\)H\(_2\)O from those recorded at the various time points indicated in Fig. 4B. Note that the downshift in frequency of vibrations due to random coil structures hydrogen-bonded to bulk solvent should be complete within 1 s of exposure of the nAChR to \(^2\)H\(_2\)O (see above) and therefore do not contribute to the changes observed in spectra recorded over this time period (26).

The effects of peptide \(^1\)H/\(^2\)H exchange on the vibrational frequencies of \(\beta\)-sheet structures are revealed in the resolution-enhanced spectra by a very slight downshift in intensity of bands above 1660 cm\(^{-1}\) and near 1635 cm\(^{-1}\) and in the exchange difference spectra by a negative and a positive band near 1680 and 1630 cm\(^{-1}\) (Fig. 5A). The weak negative band near 1680 cm\(^{-1}\) in the exchange difference spectra could reflect a slight downshift of a high frequency \(\beta\)-sheet component band with the corresponding positive band masked by a negative \(\alpha\)-helical band near 1660 cm\(^{-1}\) (see below). The positive maximum near 1630 cm\(^{-1}\) could reflect the downshift of the low frequency \(\beta\)-sheet vibration with the corresponding negative band masked by a positive \(\alpha\)-helical band near 1645 cm\(^{-1}\).

Alternatively, the \(\beta\)-sheet minimum and maximum near 1680 and 1630 cm\(^{-1}\) could conceivably reflect a change in the intensities of either \(\beta\)-sheet vibrations upon exposure to \(^2\)H\(_2\)O.

The resolution-enhanced spectra reveal a gradual decrease in the intensity of the \(\alpha\)-helical amide I component band near 1655 cm\(^{-1}\) over the first 12 (Fig. 4A) and up to 72 (Fig. 3B) hours of peptide \(^1\)H/\(^2\)H exchange that is due to a slight downshift in frequency of the \(\alpha\)-helix vibration. The downshift in frequency is reflected in the exchange difference spectra by a negative band near 1660 cm\(^{-1}\) coupled with a positive band near 1645 cm\(^{-1}\). The latter band is partially obscured by the bands to the exchange of \(\beta\)-sheet structures but is more evident in exchange difference spectra recorded from the nAChR reconstituted into DOPC, where there is an enhanced exchange of \(\alpha\)-helical secondary structures. Spectral simulations show that a slight downshift in frequency of a broad amide I band centered near 1655 cm\(^{-1}\) can give rise to exchange difference spectra with a negative and a positive band at frequencies near 1660 cm\(^{-1}\) and 1645 cm\(^{-1}\), respectively (data not shown). Exchange difference spectra recently reported for the water-soluble protein, cytochrome c, also exhibit similar bands characteristic of the exchange of \(\alpha\)-helix and \(\beta\)-sheet secondary structures (41).

The time course of the spectral changes in both the resolution-enhanced and exchange difference spectra suggests that most of the \(\beta\)-sheet peptides exchange within the first 2 h of exposure of the nAChR to \(^2\)H\(_2\)O, whereas a large percentage of the \(\alpha\)-helical structures exchange between 2 and 12 h after the addition of \(^2\)H\(_2\)O. The resolution-enhanced spectra also show that the decrease in intensity of the \(\alpha\)-helix vibration near 1655 cm\(^{-1}\) is not complete even after several days of exposure of the nAChR to \(^2\)H\(_2\)O (Figs. 3B and 4A). In addition, considering the predominant intensity of the \(\alpha\)-helix vibration in resolved spectra of the nAChR (Fig. 3), the bands reflecting the exchange of \(\alpha\)-helical peptides near 1660 and 1645 cm\(^{-1}\) in the exchange difference spectra are very weak relative to those due to the exchange of peptides involved in \(\beta\)-sheet structures. For example, the \(\alpha\)-helical amide I component band near 1655 cm\(^{-1}\) dominates the resolution-enhanced spectrum of the nAChR and is several times more intense than the \(\beta\)-sheet component band near 1670 cm\(^{-1}\) (see Ref. 21), whereas the ratio of the corresponding \(\alpha\)-helix and \(\beta\)-sheet difference bands near 1660 and 1685 cm\(^{-1}\), respectively, (referred to as \(I_{\alpha\text{-helix}}/I_{\beta\text{-sheet}}\)) is only 1.2 after 12 h and 1.4 after 3 days of exposure to \(^2\)H\(_2\)O (data not shown). In contrast, the \(I_{\alpha\text{-helix}}/I_{\beta\text{-sheet}}\) of the same two bands in the exchange difference spectra recorded from cytochrome c is roughly 10, which is similar to the ratio of the corresponding amide I component bands in the resolution-enhanced spectra of cytochrome c (41). Both the resolution-enhanced and exchange difference spectra strongly suggest that a large majority of the 25% of peptides suggested above to be resistant to exchange and to exist within the hydrophobic environment of the lipid bilayer adopt an \(\alpha\)-helical conformation. Conversely, no evidence is detected for the existence of a large number of exchange-resistant \(\beta\)-sheets. These results suggest that at least a majority of the transmembrane domains of the nAChR adopt an \(\alpha\)-helical secondary structure.

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3. Méthot, N., Demers, C. N., and Baenziger, J. E., Biochemistry, in press.
DISCUSSION

The rates at which the different peptide hydrogens in a protein tertiary structure exchange with protons (or in this case deuterium) in the bulk solvent vary over several orders of magnitude, depending on hydrogen bonding and solvent accessibility. Peptide hydrogen exchange rates are thus very sensitive to protein secondary structure and conformational change. They are also strongly influenced by the transmembrane topology of an integral membrane protein that governs the proportion of peptides that reside within the hydrophobic, relatively solvent inaccessible region of the lipid bilayer. We have for the first time monitored the rates of exchange of affinity-purified nAChR peptide hydrogens by following changes in ATR FTIR spectra recorded as a function of time of exposure of the nAChR to $^2$H$_2$O. The resulting hydrogen/deuterium exchange spectra provide new insight into the exchange kinetics and thus structure of the nAChR. The important novel features of the exchange data are described in the following paragraphs.

(i) The hydrogen/deuterium exchange spectra reveal a rapid decrease in the intensity of the amide II band corresponding to the exchange of 30% of the peptide hydrogens for deuterium within 5 s after exposure to $^2$H$_2$O, 50% after 30 min, 60% after 12 h, and 75% after days at room temperature or lower temperatures. The roughly biphasic nature of the changes in amide II band intensity are indicative of the existence of both fast and slow exchanging populations of peptide hydrogens similar to the at least two populations of exchanging peptide hydrogens that have been observed for numerous water-soluble and integral membrane proteins, including native nAChR membranes (35). However, for the nAChR the percentage of slowly exchanging peptides is larger, and the exchange times are longer than the exchange times of many water-soluble proteins, despite the location of a substantial number (~75%) of the nAChR peptides in the extramembranous domains of the nAChR. In addition, a relatively large percentage (~25%) of the peptide hydrogens are resistant to exchange even after prolonged exposure of the nAChR to $^2$H$_2$O. It appears that the close association and/or anchoring of the nAChR within the lipid bilayer attenuates the internal motions and/or reduces the accessibility of the peptide hydrogens to $^2$H$_2$O.

(ii) The rapid decrease in amide II band intensity within seconds of exposure to $^2$H$_2$O is accompanied by a marked change in shape of the amide I contour. The rapidity of both spectral changes indicates that the majority of the affected peptide N–H (amide II) and C=O (amide I) groups are hydrogen-bonded to bulk solvent, rather than to other peptides in ordered secondary structures, and are thus involved in random and turn conformations. The 30% extremely fast exchanging peptide hydrogens is consistent with the roughly 20% random coil and 8% turns suggested by a previous curve-fitting analysis of the amide I band contour (21). Similarly, the rapid changes in the amide I contour, which are due predominantly to shifts in frequency of an intense band near 1655 cm$^{-1}$ in $^2$H$_2$O down to near 1640 cm$^{-1}$ in $^2$H$_2$O and a relatively weak band from near 1690 cm$^{-1}$ down to near 1680 cm$^{-1}$, are consistent with the previous frequency-based assignment of these $^2$H/PH exchange-sensitive component bands to random and turn structures, respectively (21). The relatively large magnitude of the rapid spectral changes in the amide I band confirm that the downshifts in frequency of random and turn vibrations upon $^2$H/PH exchange are much larger than those due to the exchange of peptide hydrogens involved in ordered secondary structures (see below and Ref. 33). The rapidity of the band shifts provides the first direct experimental evidence that bands typically assigned to random and turn structures exhibit the exchange kinetics expected for highly solvent exposed peptides.

(iii) The very subtle changes in amide I band contour between 3 min and 12 h after exposure of the nAChR to $^2$H$_2$O reflect the slight amide I component band shifts that occur as a result of the exchange of ordered secondary structures and provide insight into the individual exchange kinetics of $\alpha$-helices and $\beta$-sheet. The relatively small downshifts in frequency of the $\alpha$-helical amide I vibrations are reflected in the resolution-enhanced amide I band by a gradual decrease in the intensity near 1655 cm$^{-1}$, whereas the exchange of $\beta$-sheet leads to slight downshifts in intensity of bands above 1660 cm$^{-1}$ and near 1635 cm$^{-1}$. The downshifts in frequency of $\alpha$-helix and $\beta$-sheet component bands can also be followed in exchange difference spectra and yield bands near 1660 and 1645 cm$^{-1}$, and near 1680 and 1630 cm$^{-1}$, respectively, of both positive and negative intensity. Similar bands due to the exchange of $\alpha$-helix and $\beta$-sheet structures have been reported in exchange difference spectra recorded from the water-soluble protein, cytochrome c (41).

Significantly, the time courses of the various amide I component band shifts reflected in the exchange difference spectra indicate that most $\beta$-sheet secondary structures undergo a relatively rapid exchange within the first 2 h of exposure of the nAChR to $^2$H$_2$O, whereas the $\alpha$-helical secondary structures exchange over a much slower time scale. In contrast, the exchange difference spectra recorded from cytochrome C suggest very similar exchange rates for both $\alpha$-helix and $\beta$-sheet (41). The relatively slow exchange of $\alpha$-helical secondary structures in the nAChR appears to be the cause of the generally slow peptide $^1$H/$^2$H exchange rates that are evident by following the spectral changes in the amide II band intensity.

In addition, both the residual intensity remaining at 1655 cm$^{-1}$ in the resolution-enhanced spectra recorded after 3 days’ exposure of the nAChR to $^2$H$_2$O and the weak intensities of bands in the exchange difference spectra reflecting the $^1$H/$^2$H exchange of $\alpha$-helical peptide hydrogens relative to those reflecting the exchange of $\beta$-sheet strongly suggest the existence of a large population of exchange-resistant $\alpha$-helical peptide hydrogens (this interpretation is discussed in more detail below). Conversely, no features suggesting the existence of a large population of exchange-resistant $\beta$-strands are detected in either the resolution-enhanced or exchange difference spectra of the nAChR. A large proportion of the 25% of peptide hydrogens that are resistant to exchange likely exist in an $\alpha$-helical conformation.

The existence of 25% exchange-resistant peptide hydrogens coupled with both the strong evidence for the existence of a large number of exchange-resistant $\alpha$-helical peptide hydrogens and lack of evidence for the existence of a corresponding population of exchange-resistant $\beta$-strands suggests that the majority of the peptides in the transmembrane domains of the nAChR adopt an $\alpha$-helical secondary structure. A predominately $\alpha$-helical secondary structure of the putative exchange-resistant transmembrane domains of the nAChR is consistent with the four hydrophobic transmembrane segments predicted for each nAChR subunit using hydrophobicity plots (4, 5) and by the $\alpha$-helical periodicity in chemical labeling of M2, M3, and M4 by either chemically reactive noncompetitive channel blockers or hydrophobic photoactive probes (14, 16, 18, 19). The labeling pattern of M1 is consistent with a slightly distorted $\alpha$-helix (19). Five rods of density are observed lining the ion channel pore in the 9-Å resolution electron density map of the nAChR, consistent with five pore-lining M2 $\alpha$-helical segments (one per subunit; Ref. 7). However, the lack of well defined electron density at the periphery of the transmembrane domains of the nAChR is suggestive of the existence of transmem-
brane β-strands (7). Infrared bands characteristic of extended β-sheet structures are also observed in FTIR spectra recorded from the nAChR after treatment with proteinase K to remove the extramembranous domains of the nAChR (8).

There is currently a consensus that the five pore-lining transmembrane segments, which of any of the transmembrane segments are likely to be accessible to aqueous solvent and thus undergo relatively rapid 2H/H exchange, are α-helical in nature. If lipid-exposed M1, M3, and M4 are not α-helices, as possibly suggested by the electron microscopy data, between five and seven β-strands are required to fulfill the 25–30% of nAChR peptides that exist within the lipid membrane (7). Although our data do not completely rule out the existence of transmembrane β-strands, no spectral features indicative of a large population of exchange-resistant transmembrane β-structures are observed in the spectra recorded as a function of time after exposure of the nAChR to 2H2O as would be expected if there were five or seven transmembrane β-strands per nAChR subunit. However, a more detailed analysis of the exchange data is warranted in order to clarify the discussion.

The 25% of exchange-resistant peptide hydrogens was determined by comparing the residual amide II band intensity remaining after 3 days' exposure of the nAChR to 2H2O with the amide II band intensity determined under conditions of both no (100% N–H) and complete exchange (100% N–2H). The amide II band intensities corresponding to 100% N–H were obtained from spectra recorded in 1H2O after subtraction of the overlapping 1H–O–1H bending vibration. Although difficult, the subtraction is facilitated because the amide I and II bands are generally more intense relative to the overlapping absorbance of water in spectra recorded using ATR than in spectra recorded using conventional transmission techniques (27). The ATR technique also offers an internal control in that both the 1H2O and 2H2O spectra are recorded from the same sample, which is deposited on the surface of a germanium ATR crystal. The resulting intensity of the amide I band after 2H2O subtraction is similar to the intensity of the same band immediately after exchanging the 1H2O buffer in the ATR sample compartment for 2H2O, illustrating the accuracy of the spectral subtraction (the 1H–O–2H bending vibration absorbs near 1200 cm⁻¹). In addition, the shape and relative intensities of the amide I and amide II bands in the 1H2O-subtracted spectra (Fig. 1A, dashed line) are similar to the shape and relative intensities of the same bands in spectra of dry films of the nAChR, where no subtraction of the 2H–O–2H bending vibration is required (Fig. 1B, dashed line).

The base line corresponding to the amide II band intensity after complete exchange was determined after incubation of the nAChR at pH 11.0 and 95 °C in spectra recorded at both pH 7.0 and pH 2.0. At pH 2.0, the vibrations of acidic side chains are shifted out of the amide II spectral region, thus providing an unobstructed view of any residual amide II band intensity. Based on the rigorous methods used to determine the amide II band intensities corresponding to 100% N–H and 100% N–2H and an analysis of the factors affecting the quantitative analysis of the data, we believe that the percentage of exchange-resistant peptides in the nAChR is accurate well within a ±10% limit (i.e. 25 ± 10%).

The assignment of the 25% exchange-resistant peptides to those in the transmembrane domains is based on a large body of evidence indicating that transmembrane peptide hydrogens, in general, are resistant to hydrogen exchange. The relative solvent inaccessibility of the hydrophobic environment of the lipid bilayer should inhibit the hydrogen exchange rates of the transmembrane domains. The transient folding/unfolding motions of ordered secondary structures that are necessary for rapid exchange are also likely to be restricted in transmembrane structures because such motions would expose the highly polar peptide N–H and C–O groups to the hydrophobic lipid acyl chains. The exchange resistance of transmembrane peptide hydrogens is borne out experimentally by the very large percentage of exchange-resistant peptide hydrogens that appear to be related to the number of peptides found within the hydrophobic core of the lipid bilayer in both bacteriorhodopsin and rhodopsin (28, 29, 37, 38). In the case of bacteriorhodopsin, the transmembrane secondary structures have been shown directly to be resistant to exchange. An unusually large number of exchange-resistant peptides have also been reported for the multisubunit photosynthetic reaction center, the transmembrane β-barrel pore, porin, as well as other integral membrane proteins of less defined tertiary structure (25, 34, 42, 43).

In analogy to the exchange kinetics of the transmembrane peptide hydrogens observed for other integral membrane proteins, it is concluded that the exchange-resistant peptides detected here likely include those found within the transmembrane domains of the nAChR. As the roughly 25% of 1H/H exchange-resistant peptides observed for the nAChR is similar to the roughly 20–25% of peptides predicted to exist as transmembrane α-helices based on the four transmembrane α-helices per nAChR subunit predicted by hydrophobicity plots (4, 5) and the 25–30% of the nAChR found within the lipid bilayer by both electron microscopy (7) and proteinase K digestion of the extramembranous portions of the nAChR (8), a majority of the 25% exchange-resistant peptides in the nAChR are likely involved in transmembrane structures (however, see below).

A more quantitative analysis of the possible numbers of exchange-resistant α-helices and β-sheet can also be obtained if it is assumed that the nAChR exhibits transmembrane β-strands corresponding to roughly 3/4 of the transmembrane domains (five to seven β-strands/subunit) and that 25% of the nAChR is located in the membrane (see above). In this case, 19% of the total nAChR peptide hydrogens would be found as β-strands and 6% as transmembrane α-helices (five pore-lining α-helices). If all of the transmembrane structures are resistant to exchange, the 19% exchange-resistant β-strands would still be greater in number than the total number of exchange-resistant α-helical structures, regardless of whether 25% (6% exchange-resistant α-helical intramembranous peptides) or even 35% (16% exchange resistant intra- plus extramembranous helical peptides) of the total number of nAChR peptides are resistant to exchange. In either scenario, the relative intensities of bands in the exchange difference spectra reflecting the exchange of β-sheet structures should be reduced in intensity relative to those due to the exchange of α-helices, when the relative intensities are compared with the relative intensities of the corresponding α-helix and β-sheet amide I component bands in the resolution-enhanced spectra of the nAChR. In contrast, the exchange difference spectra recorded for the nAChR reveal a dramatic enhancement (as opposed to a reduction) in the intensities of the β-sheet bands relative to those due to the exchange of α-helical secondary structures indicating far fewer than 19%, if any, exchange-resistant β-strands in the nAChR. Note that for cytochrome c, the exchange rates of both α-helix and β-sheet bands appear to be similar, leading to exchange difference spectra where the relative intensities of the α-helix and β-sheet bands in the exchange difference spectra are similar to the relative intensities of the corresponding bands in the resolution-enhanced amide I band. It is clear that the nAChR does not have sufficient exchange-resistant β-strands, if any, to account for the 5–7 transmembrane β-strands suggested for each nAChR subunit. Therefore, either the transmembrane structures of the nAChR are resistant to
exchange and adopt a predominantly α-helical conformation, as indicated above, or the nAChR exhibits a substantial population of transmembrane β-strands that exchange rapidly within the first few hours of exposure of the nAChR to 2H2O (see above). Given the well established inaccessibility of transmembrane structures to 2H2O and their restricted ability to undergo the transient folding/unfolding reactions necessary for relatively rapid exchange, the possibility of fast exchanging transmembrane β-strands in the nAChR seems unlikely. Transient folding/unfolding of transmembrane β-strands would also require compensatory motions of the extramembranous domains; yet the overall exchange rates of the nAChR are relatively slow, indicating a relatively ordered protein structure. In addition, it is difficult to imagine how the transmembrane structures of the nAChR could undergo the rapid folding/unfolding motions required for relatively fast exchange while still maintaining a closed ion channel pore against a substantial electrochemical gradient. While the possibility of relatively dynamic solvent-accessible transmembrane structures has important structural implications for the nAChR, given the points raised above it seems unlikely that there exists a large number of relatively fast exchanging transmembrane β-strands. Consequently, the most plausible conclusion is that at least a majority of the transmembrane domains of the nAChR are α-helical in nature. This interpretation of our data questions the possible interpretation of the diffuse electron dense bands in the low resolution electron density map of the nAChR in terms of β-strands and supports an α-helical secondary structure of all the transmembrane domains.

Note that the above analysis depends entirely on both the exchange kinetics measured by following the changes in intensity of the amide II vibration and the qualitative analysis of the band shifts that occur in the amide I region upon exposure of the nAChR to 2H2O. The changes in the amide I band were monitored in both resolution-enhanced and exchange difference spectra. Both methods of data analysis provide a consistent picture of the shifts in band intensity that occur over the time course of the exchange. Moreover, the spectra were stringently examined for the presence of water vapor in order to eliminate potential artifacts that can arise upon resolution enhancement. Our conclusions are not dependent upon the secondary structure of the nAChR that has been estimated from the amide I band shape using curve-fitting techniques. We have assumed that the molar absorptivities of the amide I and II bands are the same for both α-helix and β-sheet conformers. However, the close correlation for most proteins between the relative areas of the α-helix and β-sheet amide I component bands and the relative α-helix and β-sheet contents as determined by x-ray crystallography suggest that potential variations are relatively minor. Slight variations in the relative molar absorptivities of different secondary structures should have no bearing on the interpretations of our data.

Furthermore, a recent study in our lab shows that upon reconstitution of the nAChR into a more fluid lipid membrane composed solely of DOPC, there is an enhanced exchange of the peptide hydrogens for deuterium that results predominantly from an increased exchange of α-helical secondary structures. The enhanced exchange of α-helical secondary structures in DOPC is suggested by a further decrease in intensity of the α-helical amide I component band near 1655 cm−1 in resolution-enhanced spectra of the nAChR recorded after 3 days in 2H2O and by an increased intensity of the two bands reflecting the exchange of α-helices near 1660 and 1645 cm−1 in exchange difference spectra of the nAChR, but it does not result from a change in the secondary structure (see Footnote 3 for a detailed discussion). The increased exchange of the nAChR α-helical peptides in DOPC may arise from an increase in the overall dynamics of the nAChR or an increased permeability of the bilayer and thus accessibility of the transmembrane structures to 2H2O, both as a result of a more fluid lipid bilayer (39, 40). As one would expect changes in membrane fluidity to have a predominant affect on the transmembrane domains of the nAChR, the enhanced exchange of α-helical secondary structures in DOPC provides additional support not only for the existence of exchange-resistant α-helices but also for the existence of a significant proportion of the exchange-resistant α-helical structures within the lipid bilayer. In this regard, it is interesting to note that the photosynthetic reaction center exchange to a greater extent in the presence of detergents, which likely increase the “fluidity” of the surrounding lipid bilayer (compare the residual amide II band in the FTIR spectra of Ref. 42 versus Ref. 43).

Blanton and Cohen have suggested that the electron dense cylinders interpreted by Unwin as transmembrane α-helices may reflect highly ordered α-helices, whereas the lack of well defined electron density profiles at the nAChR periphery may be due to less “highly ordered” α-helical conformations at the lipid-protein interface (19). The roughly 5% of the nAChR peptide hydrogens that are extremely 1H/2H exchange-resistant, even after treatment for 24 h at 60 °C (Fig. 2), is consistent with this interpretation. The high order of the pore-lining α-helical structures may be the cause of the high resistance of these peptides to exchange relative to the other transmembrane α-helices in the nAChR and to transmembrane α-helices in other integral membrane proteins, such as rhodopsin. The electron density maps may also have been influenced by the assumption of pentahedral symmetry used in the averaging of the data. While one might expect the pore-lining structures to adopt a well defined and reproducible position relative to the pore axis in each subunit, the location of the transmembrane structures distal to the pore-lining α-helices may vary slightly from subunit to subunit, resulting in a smearing of electron density upon averaging of the data. In addition, the observed infrared bands assigned to transmembrane β-like structures in FTIR spectra of proteinase K-treated nAChR could reflect short unordered polypeptides that remain at the surface of the membrane after incomplete enzymatic digestion of the extramembranous domains. The bands could also reflect a disruption in the structure of the transmembrane domains upon extensive proteinase K treatment (36).

(iv) Finally, the hydrogen exchange spectra presented here reveal several novel features that can be exploited for probing integral membrane protein structure and function. FTIR techniques, in general, offer important advantages over conventional 1H/2H exchange measurements in that the exchange of peptide hydrogens for deuterium can be monitored directly without interference from the exchange of labile side chain protons and without prior exchange-in of 2H by incubation of the sample in 2H2O. This is an important consideration for integral membrane proteins that generally exhibit a large number of exchange-resistant peptide hydrogens whose exchange kinetics would not be monitored using the 1H/2H exchange technique.

We demonstrate that ATR FTIR is particularly advantageous in that it allows a very accurate quantitative interpretation of the exchange data in terms of the number of exchanged protons at a given time after exposure to 2H2O. The ATR technique can be used to acquire exchange spectra within seconds of exposure of a protein to 2H2O, which may be the fastest that hydrogen exchange data have ever been obtained for any protein. The result illustrates the potential of the ATR tech-
nite for examining the exchange kinetics of highly solvent exposed peptide hydrogens that are directly involved in ligand binding and/or protein action, especially if data are recorded at lower temperatures and pH where exchange times are markedly reduced. In addition, the ability to monitor the exchange kinetics of individual secondary structures is unique to FTIR spectroscopy but has not been exploited previously for monitoring the exchange kinetics of integral membrane proteins. We demonstrate for the first time that both the time scales and relative magnitudes of the downshifts in frequency of amide I component bands can be used as an aid in the assignment of amide I component bands to specific secondary structures. The ability to probe the exchange kinetics of different secondary structure can provide unique insight into the structure and function of integral membrane proteins.

CONCLUSIONS

FTIR spectra have been recorded as a function of time of exposure of the nAChR to $^{2}H_{2}O$. The resulting hydrogen/deuterium exchange spectra reveal a number of changes that reflect the exchange of peptide hydrogens for deuterium and provide insight into the secondary structure of the transmembrane domains of the nAChR. Roughly 25% of the peptide hydrogens are found to be resistant to peptide $^{1}H/^{2}H$ exchange. A large majority of the exchange-resistant peptides exist in an $\alpha$-helical conformation, likely within the hydrophobic, relatively solvent inaccessible region of the lipid bilayer. The results provide strong evidence for an exchange-resistant core of $\alpha$-helical transmembrane peptide hydrogens in the nAChR and illustrate the utility of FTIR spectroscopy coupled with hydrogen/deuterium exchange for probing integral membrane protein structure and function.

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