Role of Glycosylation and Membrane Environment in Nicotinic Acetylcholine Receptor Stability

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ABSTRACT The effects of glycosylation and membrane environment on the structural stability of the nicotinic acetylcholine receptor (nAChR) from *Torpedo* have been investigated to improve our understanding of factors that influence eukaryotic membrane protein crystallization. Gel shift assays and carbohydrate-specific staining show that the deglycosylation enzyme, Endo F1, removes at least 50% of membrane-reconstituted nAChR glycosylation. The extent of deglycosylation with Endo F1 increases upon detergent solubilization. Removal of between 60–100% of high mannose moieties from the nAChR has no effect on nAChR secondary structure, stability, or flexibility. Deglycosylation does not influence either agonist binding or the ability of the nAChR to undergo agonist-induced conformational change. In contrast, nAChR structural stability, flexibility, and function are all negatively influenced by simple changes in reconstituted membrane lipid composition. Our results suggest that deglycosylation may represent a feasible approach for enhancing the crystallizability of the nAChR. Our data also demonstrate that the dependence of nAChR structural stability on lipid environment may represent a significant obstacle to nAChR crystallization. Some membrane proteins may have evolved complex interactions with their lipid environments. Understanding the complexity of these interactions may be essential for devising an appropriate strategy for the crystallization of some membrane proteins.

INTRODUCTION

Advances in membrane protein crystallography over the past ten years have mainly involved proteins of bacterial as opposed to eukaryotic origin (Doyle et al., 1998; Iwata et al., 1998; Iverson et al., 1999; Dutzler et al., 2002; Bass et al., 2003). Although there are several factors that contribute to this discrepancy, the extensive glycosylation found on the extramembranous surfaces of some eukaryotic membrane proteins may play a significant role. Large and highly flexible carbohydrate could form a conformationally-labile shield that hinders the formation of crystal contacts. Heterogeneity of membrane protein glycosylation is also common and could further act as a detriment to crystallization. Although the presence of carbohydrate does not necessarily prevent crystal formation (Kurumbail et al., 1996; Malkowski et al., 2000; Palczewski et al., 2000; Teller et al., 2001; Rowlinson et al., 2003; Li et al., 2004) carbohydrate removal may, in some cases, increase the likelihood of success.

Removing glycosylation from the surfaces of eukaryotic membrane proteins, however, is problematic. Recombinant approaches, such as the mutagenesis of glycosylation-attachment sites, could in principle be used to completely eliminate carbohydrate. Unfortunately, the expression of recombinant eukaryotic membrane proteins in either bacteria or eukaryotic systems has proven to be either extremely difficult or prohibitively expensive (Loll, 2003).

An alternative approach is to enzymatically remove interfering carbohydrate from naturally abundant eukaryotic membrane proteins. The enzymatic removal of carbohydrate has been successful with soluble proteins (Grueninger-Leitch et al., 1996; Dale et al., 2000; Celie et al., 2004). Before directly testing the effects of deglycosylation on crystallization, however, there are two additional issues that need to be addressed. First, glycosidase cleavage sites in a natively folded membrane protein may be inaccessible due to the surface topology of the protein (Petrescu et al., 2004) and/or steric hindrance from the surrounding membrane. In every case, the feasibility of deglycosylating a native membrane protein needs to be demonstrated. Second, although the role of glycosylation in membrane protein assembly, recognition, and trafficking is well established (Merlie et al., 1982; Rickert and Imperiali, 1995; Ramanathan and Hall, 1999), very little is known with respect to its role in protein stability and function. The presence of large glycans on protein loop or turn structures may restrict overall protein flexibility (Live et al., 1996) and could be important for the structural stability of a folded membrane protein. As protein stability is particularly critical in membrane protein crystallization (Garavito and Rosenbusch, 1980, 2001; Rosenbusch et al., 2001), a legitimate concern is that deglycosylation could increase protein internal dynamics and render a protein less likely to crystallize. The structural and functional ramifications of removing carbohydrate must therefore be assessed.

The nicotinic acetylcholine receptor (nAChR) from *Torpedo californica* is the best characterized member of a superfamily of ligand-gated ion channels, whose members

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represent the major excitatory and inhibitory neurotransmitter receptors found throughout the mammalian central and peripheral nervous systems. Nicotinic receptors play a central role in intercellular communication within the brain and are the sites of action of numerous pharmaceutical agents (Dani, 2001; Quick and Lester, 2002; Hogg et al., 2003). *T. californica* nAChR is a 290 kDa pentameric glycoprotein complex composed of four highly homologous subunits ($\alpha_2\beta\gamma\delta$) arranged pseudosymmetrically around a central pore, which functions as a cation-selective channel (Karlin, 2002; Unwin, 2003). Seventy-five percent of the nAChR by mass is extramembranous (Miyazawa et al., 1999, 2003). This relatively large extramembranous region should provide ample surface for the establishment of crystal contacts and thus render the nAChR relatively amenable to crystallization. Despite intense effort, the nAChR has remained refractory to crystallization (Hertling-Jaweed et al., 1988; Paas et al., 2003), possibly because of its extensive glycosylation (7.5% by wt.). As the composition and structure of the nAChR's carbohydrate has been characterized (Nomoto et al., 1986; Poulter et al., 1989; Shoji et al., 1992; Strecker et al., 1994), the nAChR is an ideal candidate for testing the feasibility of deglycosylation as an aid to membrane protein crystallization.

In this report, we examine the feasibility of deglycosylating native nAChR and have characterized both the structural and functional consequences of nAChR deglycosylation. Our results suggest that a substantial proportion (~50% by wt.) of carbohydrate can be successfully removed from natively folded nAChR and that removal of this carbohydrate has no effect on nAChR structural stability, internal dynamics, or function. In fact, the structural and functional consequences of deglycosylation are insignificant compared to the effects of simple changes in membrane lipid composition. Our results support the feasibility of deglycosylation as an approach for enhancing crystallization. Our data also emphasize the key role played by membrane environment in maintaining membrane protein structure and stability.

EXPERIMENTAL PROCEDURES

Materials

Frozen *T. californica* electroplax tissue was from either Marinus (Long Beach, CA) or Aquatic Research Consultants (San Pedro, CA). Neuraminidase (sialidase) from *Vibrio cholerae* was from Roche Applied Science (Laval, Quebec, Canada). Recombinant (*Escherichia coli*) GST-Peptide-N^4-(acetyl-*beta*-glucosaminyl)-asparagine amidase (PNGase F) and GST-Endo-β-N-acetylglucosaminidase F1 (Endo F1) from *Chryseobacterium meningosepticum* were from Hampton Research (Laguna Niguel, CA). 1-Palmitoyl-2-oleoyl-*SN*-glycero-3-phosphocholine (POPC) was from Avanti Polar Lipids (Alabaster, AL), whereas L-α-phosphatidyl-choline (soybean asolectin, Type II-S) was from Sigma (St. Louis, MO), and n-decyl-β-D-maltopyranoside (decylmaltoside) was from Calbiochem (La Jolla, CA). Low range prestained SDS-PAGE standards were from BioRad (Missassauga, Ontario, Canada). All other reagents were from Sigma or VWR (Missassauga).

nAChR purification

Crude nAChR membranes from ~100 g of *T. californica* electroplax tissue were solubilized for 1 h at 4°C in a total volume of 100 ml of dialysis buffer (DB = 100 mM NaCl, 10 mM Tris-HCl, 0.1 mM EDTA, 0.02% w/v NaN_3, pH = 7.8) containing 1% cholate. Samples for deglycosylation studies were also solubilized in the presence of 100mM β-mercaptoethanol to reduce disulfide linked δ-δ dimers, which leads to the purification of monomeric receptor (i.e., monomers of the $\alpha_2\beta\gamma\delta$ pentamer). Monomerization has no effect on nAChR structure or function (Anholt et al., 1980; C. J. B. daCosta and J. E. Baenziger, unpublished). In all cases, the solubilized nAChR was centrifuged for 30 min at 87,000 $\times$ *g* to pellet insoluble material and the supernatants applied to a 10-ml affinity column at a flow rate of 1 ml/min.

Decylmaltoside-solubilized nAChR samples for deglycosylation studies were prepared by washing the nAChR on the affinity column with 40 ml of 15mM cholate in DB followed by a 7.5 ml linear gradient to 2.2 mM decylmaltoside in DB. The column was washed with a further 10 ml of 2.2 mM decylmaltoside in DB to ensure complete exchange of cholate for decylmaltoside and then eluted in 2.2 mM decylmaltoside DB containing 10 mM Carb. Fractions with an $A280 > 0.05$ were pooled and concentrated to 10–30 mg/ml by ultrafiltration using a 100 kDa MWCO concentrator (Millipore, Ottawa, Ontario, Canada).

nAChR samples for reconstitution into asolectin or POPC were washed on the affinity column with excess, cholate solubilized asolectin, or POPC, respectively and then eluted with 10 mM Carb, as described in detail elsewhere (daCosta et al., 2002). Fractions with an $A280 > 0.05$ were pooled in dialysis bags (12–14 kDa MWCO) and dialyzed five times against 2 liters of dialysis buffer with buffer change once every 12 h.

Deglycosylation

Decylmaltoside-solubilized nAChR at a concentration of ~20 mg/ml was incubated with Endo F1, PNGase F, or sialidase either alone or in various combinations as described in the text. Aliquots were removed at specified times, added directly to SDS-PAGE sample buffer, and stored at −80°C. Each aliquot containing 10–15 μg protein was thawed and run on a discontinuous Tris-HCl gel, with a 12% w/v separating and a 4% w/v stacking acrylamide gel. Protein was stained with Coomassie blue.

The nAChR reconstituted into soybean asolectin (~20 mg/ml in 10 mM Tris-HCl, pH = 7.5) was deglycosylated by incubation on ice for 90 min with recombinant Endo F1 (15:2 v/v, nAChR:Endo F1). After incubation, the samples were stored at −80°C. Deglycosylation was again monitored by SDS-PAGE (see Fig. 2 *A*). In addition, residual oligosaccharides were detected directly on the SDS gels by sugar-specific staining using the GelCode glycoprotein staining kit from Pierce (Rockford, IL). Briefly, the gels were treated with periodic acid to oxidize carbohydrate glycols, and the resulting aldehydes stained with acidic fuchsin sulfite (aldehyde-specific stain).

Fourier transform infrared spectroscopy

Fourier transform infrared (FTIR) spectra were recorded on either an FTS 40 or an FTS-575c spectrometer (Digilab; Randolph, MA), equipped with a DTGS detector. Experimental protocols have been described in detail elsewhere (Baenziger et al., 1992b; Ryan and Baenziger, 1999; daCosta et al., 2002). Briefly, difference spectra were recorded from a nAChR membrane film deposited on the surface of a germanium attenuated total reflectance element. Carb difference spectra are the difference between spectra of the nAChR recorded in *T. californica* ringer buffer (250 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, 3 mM $CaCl_2$, and 10 mM Tris, pH = 7.0) in the presence or absence of 50 μM carbamylcholine (Carb). Transmission spectra were recorded from the nAChR in ^2H_2O *T. californica* ringer buffer after precisely 72 h exposure to ^2H_2O (2 mM Phosphate, pH = 7.0) at 4°C. Spectra were analyzed using GRAMS/AI v.7.01 software (Thermo Galactic,

Salem, NH). Solvent ^2H_2O was subtracted from each spectrum and deconvolution of the amide I was performed, where noted, between 1900 and 1300 cm^{-1} with $\gamma = 7.0$ and a smoothing parameter of 70%. Spectra were analyzed for residual H_2O vapor, which was subtracted if necessary (Reid et al., 1996).

$^1H/^2H$ exchange kinetics

250 μg of reconstituted nAChR in 50 μL of 2 mM ^1H_2O phosphate buffer (pH = 7.0) was dried on a 45°, 50 × 20 × 2 mm germanium total internal reflection element (Harrick Scientific, Ossining, NY). After complete evaporation of ^1H_2O phosphate buffer, as monitored using the OH stretching vibration (~3500 cm^{-1}), the nAChR film was rehydrated with 2.9 ml of ^2H_2O *T. californica* ringer buffer (10 mM Tris, 250 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, and 3 mM $CaCl_2$, pH = 7.0) and 2 cm^{-1} resolution IR spectra (86 scans) acquired every 3 min for ~12 h (250 total spectra). All spectra were offset corrected at 1900 cm^{-1} and the residual amide II intensity (1547 cm^{-1}) ratioed against the maximal intensity of the amide I. The t_0 point was calculated from spectra of the dry nAChR film before rehydration.

Thermal denaturation

250 μg of reconstituted 72 h $^1H/^2H$ exchanged nAChR was placed in a thermostatic transmission cell, the temperature of which was controlled by a circulating water bath. Spectra (2 cm^{-1} resolution and 256 scans) were recorded at 2°C intervals as the sample was heated from 25°C to ~75°C. Five-minute intervals were allowed for the water bath to reach each temperature, and then a further fifteen minutes for sample equilibration. The actual temperature of the sample cell was monitored using an electronic thermometer (Barnant; Barrington, IL) with a Type J thermocouple probe. Spectra were deconvolved between 1800 cm^{-1} and 1300 cm^{-1} with $\gamma = 8.0$ and a smoothing parameter of 80% using GRAMS/AI v.7.01 software (Thermo Galactic). Percent denaturation was monitored by the percent change in intensity at 1681cm^{-1}. Thermal denaturation is best characterized by the relatively small changes in intensity near 1681 cm^{-1} because changes in intensity near 1623 cm^{-1} are influenced by the spectral changes associated with a gradual increase in peptide hydrogen exchange kinetics with increasing temperature.

RESULTS

nAChR deglycosylation

The ability of three different glycosidase enzymes, Endo F1, PNGase F, and sialidase, to independently and cooperatively deglycosylate detergent solubilized nAChR was tested under varying conditions of temperature, reaction time, and glycosidase/nAChR molar ratio. In each case, the extent and homogeneity of deglycosylation was assessed by SDS-PAGE (Figs. 1 and 2). The presented data are representative of the maximal levels of deglycosylation achieved by each enzyme or enzyme combination under all of the conditions tested. Levels of deglycosylation similar to those observed in Fig. 1 were subsequently achieved in <2 h with two- to threefold increases in enzyme concentration, as shown for the membrane-reconstituted samples in Fig. 2 *A*. Deglycosylation of the membrane reconstituted samples was also assessed with a carbohydrate-specific stain (Fig. 2 *B*).

Treatment with Endo F1 led to an increase in the electrophoretic mobility (~2 kDa) of all four nAChR

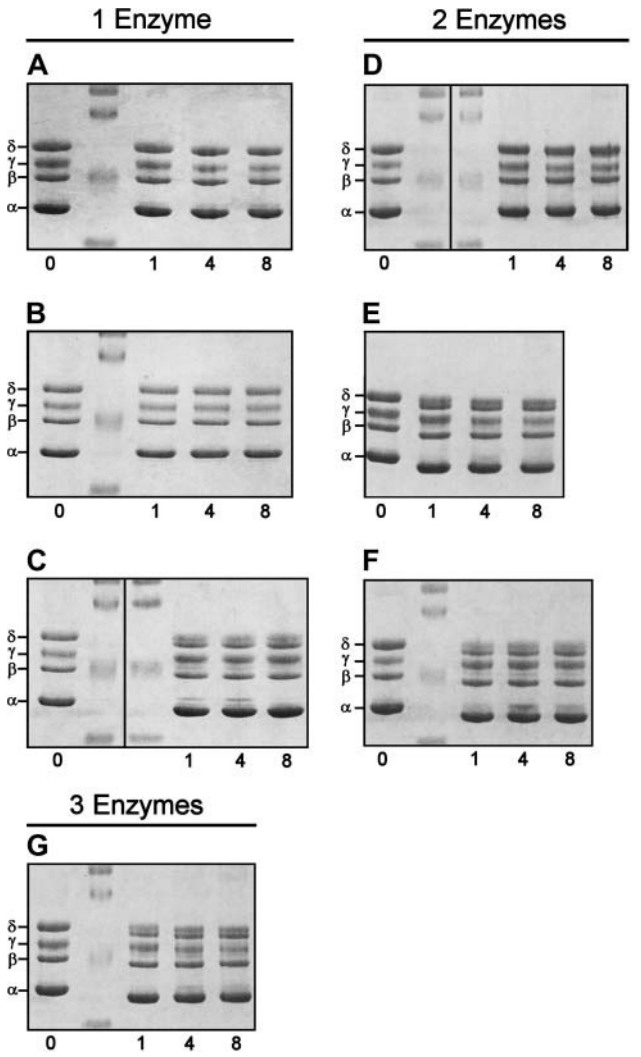

FIGURE 1 SDS-PAGE of decylmaltoside solubilized nAChR incubated with (*A*) sialidase, (*B*) PNGase F, (*C*) Endo F1, (*D*) sialidase and PNGase F, (*E*) sialidase and Endo F1, (*F*) PNGase F and Endo F1, and (*G*) sialidase, PNGase F and Endo F1 for 1, 4 and 8 days. For the individual enzyme experiments, the nAChR (20 mg/ml) was mixed 75:4 (v/v, nAChR/Endo F1 or sialidase) or 75:2 (v/v, nAChR/PNGase F) with each enzyme as supplied by the manufacturer. For the combination experiments, nAChR was mixed 75:2:2 (v/v/v, nAChR/glycosidase 1/glycosidase 2) or 75:2:2:2 (v/v/v/v, nAChR/glycosidase 1/glycosidase 2/glycosidase 3). The controls on the left of each panel have not been incubated with enzyme. The standards shown correspond to MWs of 116, 80, 51.8, and 34.7 kDa from top to bottom, respectively. The positions of the nAChR subunits are indicated on the left.

subunits suggesting deglycosylation (Fig. 1 *C*). Each of the four nAChR subunits has one high mannose carbohydrate moiety that is potentially cleaved by Endo F1. In the case of the two α-subunits and the β-subunit, this high mannose glycan is their only carbohydrate (Poulter et al., 1989). The complete and homogenous deglycosylation of these two subunits indicates that their high mannose moiety is accessible and readily cleaved by Endo F1. Carbohydrate specific staining shows essentially complete removal of carbohydrate from the α- and β-subunits (Fig. 2 *B*).

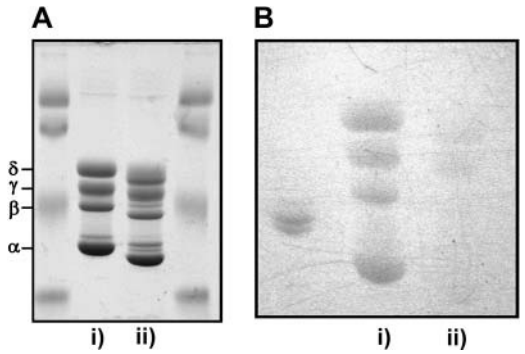

FIGURE 2 Deglycosylation of the nAChR reconstituted into soybean asolectin membranes. (*A*) SDS-PAGE of glycosylated (*i*) and Endo F1 deglycosylated (*ii*) nAChR. (*B*) SDS gel stained with a carbohydrate specific stain. The leftmost lane in (*B*) is a glycoprotein positive control (horse radish peroxidase).

In contrast, the presence of two δ-subunit bands with distinct electrophoretic mobilities indicates that Endo F1 treatment led to heterogeneous deglycosylation of the δ-subunit (Fig. 1 *C*). The γ- and δ-subunits are glycosylated with both high mannose and either one or two complex-type carbohydrates, respectively (Nomoto et al., 1986; Shoji et al., 1992; Strecker et al., 1994). Furthermore, thirty-two different varieties of complex carbohydrates have been identified on these subunits, highlighting their overall heterogeneity in terms of complex carbohydrate structure (Shoji et al., 1992). In some cases, particular complex-type structures may sterically restrict the access of Endo F1 to the γ- and δ-subunit's high mannose moiety. Heterogeneity of the γ- and δ-subunit glycosylation may thus account for the observed inability to homogeneously deglycosylate these subunits.

Unlike Endo F1, PNGase F and sialidase are both capable of removing complex-type carbohydrate, although PNGase F cleaves at the carbohydrate-protein link whereas sialidase cleaves terminal sialic acid residues. Under the conditions tested, treatment with PNGase F had essentially no effect on nAChR subunit mobility and thus carbohydrate content, suggesting that the carbohydrate-protein link is inaccessible to PNGase F in a natively folded nAChR (Fig. 1 *B*). sialidase led to subtle increases in the electrophoretic mobility of both the γ- and δ-subunits, and thus subtle changes in the levels of their complex-type carbohydrate (Fig. 1 *A*). Removal of this small proportion of complex-type carbohydrate did not noticeably enhance the ability of Endo F1 to remove the high mannose glycan on the δ-subunit (Fig. 1, *E* and *G*).

We also compared the ability of Endo F1 to deglycosylate detergent-solubilized versus membrane-reconstituted nAChR. Comparison of Figs. 1 *C* and 2 *A* shows that, although Endo F1 is more effective at deglycosylating detergent solubilized nAChR, it is still able to deglycosylate a substantial proportion of membrane reconstituted receptor. Staining for oligosaccharides suggests that the majority of the receptor's carbohydrate has been removed (Fig. 2 *B*). Consistent with the detergent solubilized nAChR data, the remaining carbohydrate detected by the oligosaccharide stain is located primarily on the γ- and δ-subunits. This ability to partially deglycosylate membrane reconstituted nAChR samples with Endo F1 has allowed us to assess the structural and functional effects of nAChR deglycosylation. All nAChR structural and functional characterization was performed on membrane reconstituted nAChR samples that were deglycosylated with Endo F1 as presented in Fig. 2. Note that it is of particular interest to test whether or not complete deglycosylation of the ligand-binding α-subunits influences nAChR function.

Effect of deglycosylation on nAChR structure

The structural effects of deglycosylation on membrane-reconstituted nAChR were assessed using FTIR spectroscopy. The amide I band (1600–1700cm^{-1}) is due predominantly to the peptide carbonyl stretching vibration and its shape is sensitive to protein secondary structure (Jackson and Mantsch, 1995). The deconvolved amide I band in spectra of glycosylated and Endo F1 deglycosylated nAChR both exhibit the same number of component bands with similar relative intensities, including two major peaks centered near 1655 cm^{-1} and 1639 cm^{-1} due to α-helical and β-sheet secondary structures, respectively (Fig. 3 *A*) (Jackson and Mantsch, 1995). The essentially identical amide I band shapes indicates that extensive deglycosylation has no effect on nAChR secondary structure. The two samples also exhibit essentially identical residual amide II band intensity near 1547 cm^{-1} (primarily N-^{1}H bend) (Fig. 3 *B*). As the amide II vibration shifts down in frequency from 1547 cm^{-1} to 1456 cm^{-1} upon the exchange of peptide N-^{1}H for N-^{2}H (see Fig. 4), the similar residual amide II intensities indicates that glycosylated and deglycosylated nAChR have essentially identical levels of peptide ^{1}H/^{2}H exchange after 72 h exposure to $^{2}H_2O$ at 4°C. Note that the relative levels of peptide ^{1}H/^{2}H exchange in the FTIR spectra can be visually assessed by comparing the residual amide II intensity at 1547 cm^{-1} to that of the adjacent side-chain carboxyl stretching vibration centered near 1580 cm^{-1}.

For comparison, we recorded spectra of glycosylated nAChR reconstituted into POPC as opposed to asolectin membranes. The nAChR undergoes more extensive peptide ^{1}H/^{2}H exchange in POPC, as indicated by the lower residual amide II band intensity near 1547 cm^{-1} (Fig. 3 *B*). Although nAChR secondary structure is not affected by reconstitution into POPC (Methot et al., 1995; daCosta et al., 2002), subtle differences in amide I/I′ band shape of 72 h ^{1}H/^{2}H exchanged samples are observed (Fig. 3 *A*). These differences in band shape reflect an increased number of α-helical peptide hydrogens that have exchanged for deuterium, which results in a shift in α-helical amide I component band

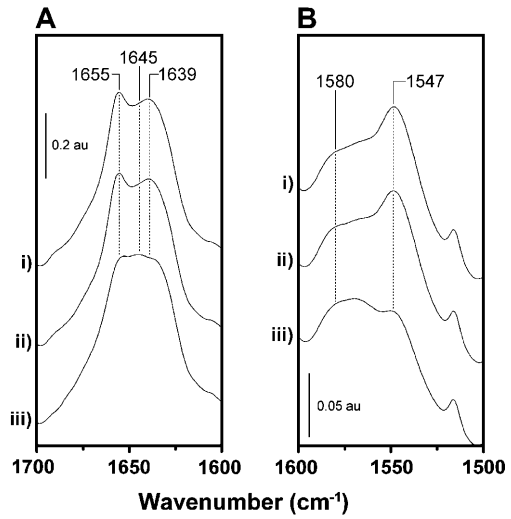

FIGURE 3 Effect of deglycosylation and membrane environment on nAChR structure. (*A*) The deconvolved amide I/I′ band from glycosylated (*i*) and Endo F1 deglycosylated (*ii*) nAChR reconstituted into soybean asolectin membranes, as well as glycosylated nAChR reconstituted into POPC (*iii*) membranes. The deconvolved spectra show the frequencies and relative intensities of amide I (1655 cm^{-1}, α-helix and 1639 cm^{-1}, β-sheet) and amide I′ (1645 cm^{-1}, $^1H/^2H$ exchanged α-helix) component bands, and thus provide a sensitive measure of nAChR secondary structure. (*B*) Nondeconvolved residual amide II band. Note the amide II vibration (1547 cm^{-1}) partially overlaps with broad bands due to side chain asymmetric COO^- stretching vibrations near 1580 and 1560 cm^{-1}. Spectra were recorded at 22.5°C after each sample was exchanged for precisely 72 h at 4°C. Each spectrum is representative of between four and seven spectra recorded from each sample.

intensity from 1655 cm^{-1} down to 1645 cm^{-1} (Methot et al., 1995).

Effects of deglycosylation on nAChR internal dynamics

The rate of peptide $^1H/^2H$ exchange is sensitive to protein internal dynamics. As subtle differences in internal dynamics could potentially have dramatic effects on crystallizability, we examined the effects of deglycosylation on the kinetics of nAChR peptide $^1H/^2H$ exchange. Fig. 4 *A* shows spectra from each of the reconstituted nAChR samples acquired at three different times after exposure to ^2H_2O. The $^1H/^2H$ exchange kinetics, presented as a change in the ratio of amide II/amide I band intensity as a function of time are essentially identical for the glycosylated and deglycosylated forms of the asolectin reconstituted nAChR (Fig. 4 *B*). These results suggest that deglycosylation does not influence nAChR internal dynamics. In contrast, there is a marked increase in the rate of peptide $^1H/^2H$ exchange upon reconstitution of the nAChR into POPC membranes, consistent with the data obtained after 72 h exposure to ^2H_2O at 4°C (Fig. 3, *A* and *B*). Note that the hydrogen exchange data presented in Fig. 4 was recorded at 22.5°C.

Effect of deglycosylation on nAChR thermal stability

The effect of deglycosylation on nAChR thermal stability was examined by recording infrared spectra as a function of increasing temperature. Thermal denaturation of proteins leads to characteristic changes in amide I band intensity at both 1623 cm^{-1} and 1681 cm^{-1} (Fig. 5 *A*). Denaturation also leads to increased exposure of peptide N-^1H groups to ^2H_2O solvent and thus a sharp increase in peptide $^1H/^2H$ exchange. Fig. 5 *B* is a plot of the percent intensity change measured at 1681 cm^{-1} as a function of temperature. The T_d (temperature at 50% denatured) of glycosylated and deglycosylated nAChR are both ~55°C, indicating that deglycosylation does not affect nAChR thermal stability. In contrast, the T_d of the nAChR in POPC is slightly lower (~50°C). The cooperativity of thermal denaturation is also decreased upon reconstitution into POPC suggesting a less compact structure.

Effect of deglycosylation on nAChR function

The effect of deglycosylation on the ability of the nAChR to undergo agonist induced conformational change was probed by infrared difference spectroscopy (Fig. 6), which is a sensitive probe of nAChR conformation and function (Ryan et al., 2001; daCosta et al., 2002). The difference between infrared spectra of the nAChR recorded in the presence and absence of the agonist Carb exhibits a complex pattern of positive and negative bands. Positive intensity in the 1800 cm^{-1} to 1500 cm^{-1} region reflects the vibrational shifts in the polypeptide backbone associated with the resting to desensitized conformational change (Ryan et al., 1996, 2002). The pattern of bands in Carb difference spectra recorded from both glycosylated and deglycosylated nAChR is similar to that observed in native membranes (Baenziger et al., 1992a; Ryan et al., 1996). Based on the FTIR data, we conclude that the nAChR is stabilized primarily in a functional resting conformation that undergoes Carb-induced desensitization (Fig. 6; Ryan et al., 1996). Conversely, intensity is lost in the 1800–1500 cm^{-1} region of difference spectra recorded from the nAChR reconstituted into POPC membranes. The resulting pattern of difference bands is similar to that observed in Carb difference spectra recorded from functional nAChR membranes in which the nAChR is maintained in the desensitized state by a local anesthetic (Ryan and Baenziger, 1999). The difference spectra thus suggest that the nAChR is stabilized in a ''desensitized-like'' conformation in POPC (Baenziger et al., 2000; daCosta et al., 2002).

Note that the intensity of the Carb bands in Carb difference spectra recorded from the nAChR in POPC versus asolectin membranes is similar. Although the affinities of the nAChR for Carb in the two membranes could differ,

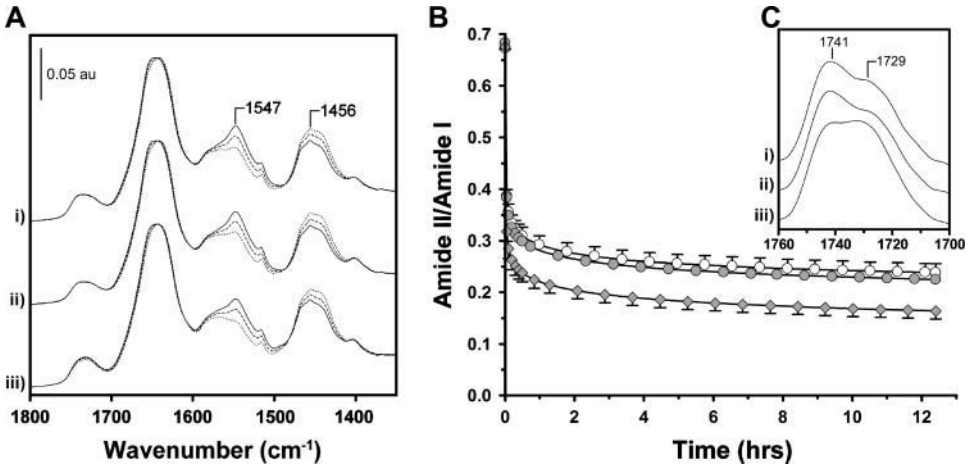

FIGURE 4 Effect of deglycosylation and membrane environment on the kinetics of nAChR peptide $^{1}H/^{2}H$ exchange. (*A*) Nondeconvolved FTIR spectra of glycosylated (*i*) and Endo F1 deglycosylated (*ii*) nAChR reconstituted into asolectin membranes as well as POPC reconstituted nAChR (*iii*). Spectra were recorded after 3 (*solid line, top*), 24 (*dashed line, middle*), and 750 (*dotted line, bottom*) min of exposure to deuterated buffer at 22.5°C. (*B*) The hydrogen exchange kinetics are plotted as the ratio of residual amide II to amide I band intensity as a function of time after exposure to $^{2}H_2O$ *T. californica* ringer buffer for glycosylated (*solid circles*, $n = 5$), Endo F1 deglycosylated (*open circles*, $n = 2$), and glycosylated POPC reconstituted nAChR (*solid diamonds*, $n = 4$). Error bars represent ± SD of the mean. Where error bars are not visible they are smaller than the data points. (*C*) Deconvolved lipid ester carbonyl stretching region. Hydrogen-bonded ester carbonyls (1729 cm^{-1}) and nonhydrogen-bonded ester carbonyls (1741 cm^{-1}) are labeled.

a similar proportion of nAChRs in each is capable of binding Carb. This observation coupled with the fact there are no significant differences in secondary structure between the nAChR reconstituted into POPC and asolectin membranes, suggests that the proportion of folded nAChRs in each membrane sample is similar. The changes in both the hydrogen exchange kinetics and the thermal denaturation profiles observed between the nAChR reconstituted into membranes composed of POPC and asolectin are thus not likely due to the presence of a population of improperly folded proteins in POPC.

DISCUSSION

The general goal of this work was to determine whether or not deglycosylation is a feasible approach for enhancing the crystallizability of glycosylated integral membrane proteins. Specifically, we were interested in testing whether membrane environment sterically hinders membrane protein deglycosylation. We were also interested in assessing whether carbohydrate removal influences membrane protein structure and/or stability. The latter is of concern because there appears to be a critical link between membrane protein stability and crystallizability (Garavito and Rosenbusch,

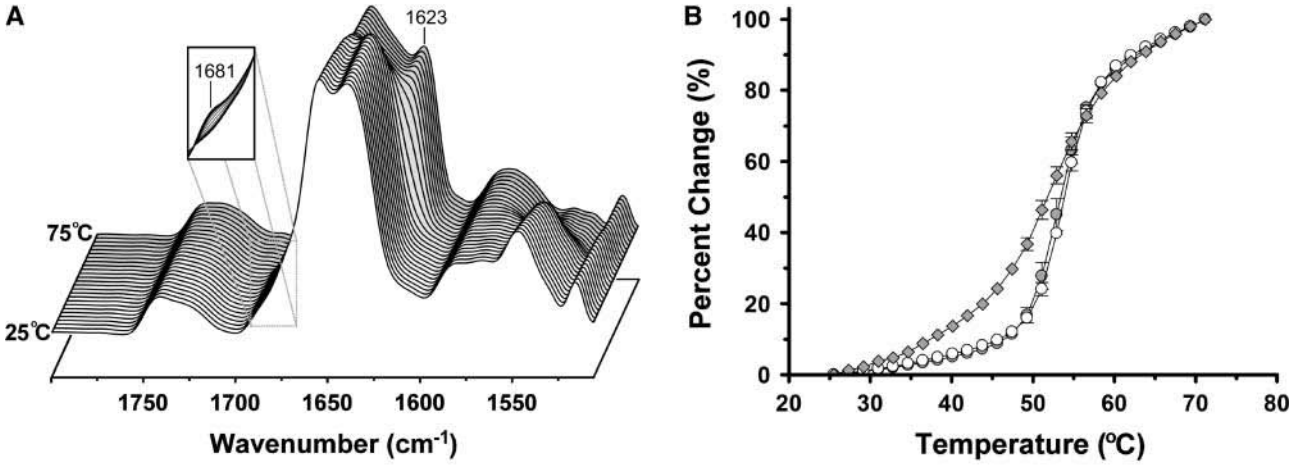

FIGURE 5 Effect of deglycosylation and membrane environment on reconstituted nAChR thermal stability. (*A*) 3D stack plot showing the spectral changes accompanying thermal denaturation of asolectin reconstituted and glycosylated nAChR. Spectra are not $^{2}H_2O$ subtracted. Note: the relative magnitude of change in intensity at 1681 cm^{-1} (and 1623 cm^{-1}) for both the deglycosylated asolectin and POPC samples were similar to those shown above. (*B*) Percent change in intensity at 1681 cm^{-1} as a function of temperature for asolectin reconstituted and glycosylated (*solid circles*, $n = 5$), Endo F1 deglycosylated (*open circles*, $n = 2$) and POPC reconstituted (*solid diamonds*, $n = 5$) nAChR membranes. Error bars represent ± SD of the mean. Where error bars are not visible they are smaller than the data points.

1980; Rosenbusch, 2001; Rosenbusch et al., 2001). Deglycosylation could decrease eukaryotic membrane protein stability in a manner that might preclude crystallization, despite the steric benefits of carbohydrate removal.

Gel shift assays show that of the three deglycosidase enzymes tested, Endo F1 is the only one that leads to profound changes in the overall carbohydrate content of the nAChR. Endo F1 is able to cleave high mannose-type carbohydrate from the α- and β-subunits of natively folded nAChR with relative ease. In the case of the γ- and δ-subunits, it appears that a population of these subunits contains complex-type carbohydrate that sterically restricts Endo F1's access to their high mannose moieties. Consequently, on some receptors it is not possible to remove the high mannose moiety from the γ- and δ-subunits. PNGase F and sialidase were relatively ineffective at removing both high mannose and complex-type carbohydrates from native *T. californica* nAChR. PNGase F cleaves directly at the protein-carbohydrate link. The relative inactivity of PNGase F is likely due to its inability to access substrate cleavage sites as a result of nAChR surface topology.

The five receptor subunits have combined a total of eight carbohydrate moieties, each ~2 kDa in size (Poulter et al., 1989; Shoji et al., 1992). Five of the eight glycans are of the high mannose variety, with there being one high mannose moiety per subunit. The remaining three glycans are of the complex variety, with one chain found on the γ-subunit and two on the δ-subunit. Since Endo F1 is specific for high mannose type carbohydrate, we would expect that at most five of eight glycan chains could be removed with this enzyme (as noted the complex type carbohydrate likely hinders the cleavage of high mannose type carbohydrate from the γ- and δ-subunits). Surprisingly, our carbohydrate-specific staining indicates that more than the expected 60% of the total carbohydrate is removed from the nAChR by Endo F1. This result suggests that either Endo F1 exhibits residual activity for some complex-type carbohydrate or the carbohydrate-specific stain is less effective for complex versus high mannose-type sugars. Another possibility is that some of the so-called ''complex-type'' carbohydrate may not yet have matured from high mannose to complex-type carbohydrate in our receptor samples, and thus may be removed from the nAChR by Endo F1. Regardless, our results suggest that Endo F1 treatment removes more than 60% (three of five chains) of the high mannose type carbohydrate from the nAChR and therefore likely more than 50% of the total nAChR carbohydrate.

Note that deglycosylation is slightly more effective with detergent-solubilized as opposed to membrane-bound nAChR. The reduced efficacy of the enzymes toward reconstituted nAChR could result from steric interference due to the bulky lipid bilayer, and/or adjacent nAChRs in a tightly packed membrane. The glycosylated N-terminal domain of the nAChR extends ~60Å beyond the surface of the membrane (Miyazawa et al., 1999, 2003). Deglycosylation of membrane proteins with relatively small extramembranous domains may be even more sensitive to solubilization. The increased deglycosylation of solubilized samples could also result from increased nAChR internal dynamics brought on by solubilization (see below).

Although only partial deglycosylation of the nAChR was achieved, this may be sufficient to improve the likelihood of crystal formation. Partial removal of carbohydrate from individual linkage sites may decrease or eliminate carbohydrate heterogeneity at that site. Removal of any carbohydrate may also free up protein surfaces for the formation of crystal contacts. For example, Endo F1 deglycosylation improved the diffraction quality of crystals formed from the acetylcholine binding protein (Celie et al., 2004), a soluble protein homologous to the extracellular ligand-binding domain of the *T. californica* nAChR (Brejc et al., 2001).

It should be noted that not all membrane protein carbohydrate is necessarily detrimental to crystallization. Although the large, complex sugars typically found on many membrane proteins (including the nAChR) are more likely to hinder crystallization, relatively small carbohydrate moieties have been shown to participate in membrane protein crystal contacts (Fukuda et al., 1979; Teller et al., 2001). Clearly, the steric benefits of deglycosylation for membrane protein crystallization need to be assessed on a case by case basis.

A significant result of our study is the demonstration that complete deglycosylation of the α- and β-subunits, and partial deglycosylation of some of the γ- and δ-subunits had

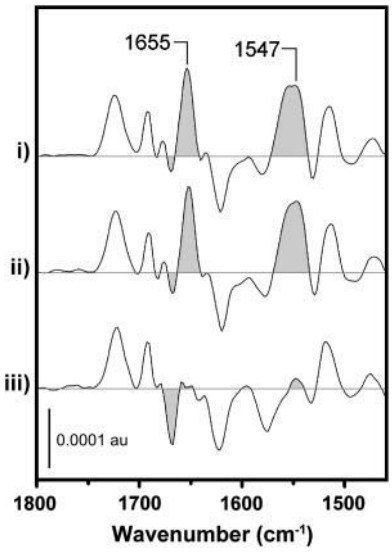

FIGURE 6 Effect of deglycosylation and membrane environment on nAChR function. FTIR difference spectra showing the vibrational shifts occurring in the nAChR upon carbamylcholine binding and subsequent desensitization. The difference spectra were acquired from glycosylated (*i*) and Endo F1 deglycosylated (*ii*) nAChR reconstituted into soybean asolectin membranes, as well as glycosylated nAChR reconstituted into POPC membranes (*iii*). Each presented difference spectrum is the average of at least 30 individual difference measurements.

no measurable effect on the physical properties of the nAChR. FTIR spectroscopy could not detect any changes in nAChR secondary structure upon deglycosylation. Both the cooperativity and the temperature of thermal denaturation for the nAChR were identical before and after removal of protein-linked carbohydrate. Peptide hydrogen exchange kinetics, which are sensitive to nAChR internal dynamics and thus protein flexibility, were unaffected by deglycosylation. Carb difference spectra recorded from both glycosylated and deglycosylated nAChR were also essentially identical., Given the above data and the exquisite sensitivity of Carb difference spectra to the nature of both nAChR-agonist interactions and agonist-induced conformational change (Ryan et al., 1996, 2002), it can be concluded that removal of high mannose-type glycosylation has essentially no effect on nAChR structure or function. More specifically, complete deglycosylation of both the agonist binding α-subunits as well as the β-subunit has no effect on the ability of the nAChR to bind agonist and undergo conformational change. In general, carbohydrate removal from the nAChR, and possibly other integral membrane proteins, may have no detrimental structural/stability effects which could hinder crystallization.

An interesting feature of our study is the demonstration that simple changes in lipid bilayer composition, in contrast to carbohydrate removal, have substantial effects on nAChR structure, function, stability, and flexibility. Reconstitution of the nAChR into POPC versus soybean asolectin membranes leads to the formation of a channel inactive conformation that has both a lower temperature and a decreased cooperativity of thermal denaturation, as well as an increased rate of peptide hydrogen exchange. These physical changes suggest a less compact, more flexible structure. Our data highlight the intimate relationship between lipid bilayer composition and nAChR structure, stability, and function.

The dependence of nAChR structure/stability on membrane environment is significant from a crystallization perspective. The nAChR may require a specific lipid cofactor, which is present in asolectin membranes, to maintain structural integrity. Several membrane protein structures have been solved in which the protein is complexed with a specific lipid (Belrhali et al., 1999; McAuley et al., 1999; Valiyaveetil et al., 2002; Kurisu et al., 2003; Lee, 2003; Zhang et al., 2003). Identifying the specific lipids required for structural integrity may be a prerequisite for crystallization of many membrane proteins. Previous studies have shown that the nAChR requires phosphatidic acid and possibly cholesterol to adopt a functional resting conformation (Fong and McNamee, 1987; Baenziger et al., 2000; daCosta et al., 2002). It is tempting to speculate that these lipids may also be required for nAChR structural integrity and thus crystallization.

Alternatively, nAChR structural stability may be sensitive to a general physical property of the lipid bilayer. Although we have not characterized the physical properties in detail, the lipid ester carbonyl stretching vibration provides qualitative insight into the physical packing of the reconstituted POPC and asolectin membranes (Fig. 4 *C*). The ester carbonyl stretching band consists of two peaks, one due to hydrogen-bonded (1729 cm^{-1}) and the other due to nonhydrogen-bonded lipid ester carbonyls (1741 cm^{-1})(Blume et al., 1988). The relative intensities of these two peaks suggest that the reconstituted nAChR/POPC membranes exhibit a greater degree of water penetration into the bilayer interfacial region than in the reconstituted nAChR/asolectin membranes. The increased solvent penetration in POPC is likely a result of decreased lateral bilayer pressure (Hubner et al., 1994; daCosta et al., 2002; Soderlund et al., 2003). Both the decrease in structural stability and increase in internal dynamics could result from an increase in the fluidity of the reconstituted POPC membranes.

It is important to note that changes in the physical environment surrounding the nAChR upon reconstitution into POPC versus asolectin membranes are relatively small in comparison to the changes in physical environment expected from detergent solubilization. Detergent solubilization should lead to even greater exposure of the transmembrane domain to water and thus an increase in local dielectric constant. The increased dielectric field may reduce the strength of weak polar interactions, such as C_α hydrogen bonds, which may be important for stabilizing transmembrane domain structure (Popot and Engelman, 2000). Lateral bilayer pressure will also be absent with a solubilized nAChR. Detergent solubilization may thus have substantial detrimental effects on nAChR structure/stability.

Rosenbusch suggested that current membrane protein crystallization techniques select for membrane proteins that are particularly stable in the detergent-solubilized state (Rosenbusch, 2001; Rosenbusch et al., 2001). Our previous work has shown an extremely rich complexity to nAChR-lipid interactions (Baenziger et al., 1999; daCosta et al., 2002, 2004). It is possible that the nAChR, and some other membrane proteins, have evolved complex interactions with lipid bilayers. These complex lipid-protein interactions may in some cases be essential for membrane protein structure and function, and could underlie the difficulties associated with solubilization and crystallization of these proteins.

CONCLUSIONS

Our data show that it is possible to enzymatically remove substantial amounts of carbohydrate from a natively-folded integral membrane protein complex, and that extensive removal of protein carbohydrate has no adverse effect on membrane protein structure, function, thermal stability, or flexibility. We show that the overall effects of deglycosylation on nAChR structure and function are insignificant in comparison to those observed upon reconstitution of the nAChR into membranes with a different lipid composition. Although deglycosylation may provide an avenue for in-

creasing nAChR crystallizability, the dependence of nAChR on membrane environment for structural integrity may represent the most significant obstacle in nAChR crystallization.

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