# Effects of periodate oxidation and glycosidases on structural and functional properties of the acetylcholine receptor and the non-receptor, peripheral ?-polypeptide (M(r) 43,000).

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# EFFECTS OF PERIODATE OXIDATION AND GLYCOSIDASES ON STRUCTURAL AND FUNCTIONAL PROPERTIES OF THE ACETYLCHOLINE RECEPTOR AND THE NON-RECEPTOR, PERIPHERAL *v*-POLYPEPTIDE ($M_r$ 43,000)

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**Abstract**—$NaIO_4$ oxidation, exo- and endo-glycosidase treatments and combinations thereof have been applied to acetylcholine receptor from *Torpedo marmorata* in its membrane-bound and detergent-solubilised forms. The effects of these chemical and enzymatic treatments are made apparent in the electrophoretic properties of the four receptor subunits ($\alpha$, $\beta$, $\gamma$ and $\delta$) and of the non-receptor polypeptides, their thermal and proteolytic susceptibility, and the steady-state and kinetic parameters of receptor–toxin complex formation. The electrophoretic pattern of the membrane polypeptides is found to depend on the redox state of the membranes, presence or absence of the non-receptor peripheral *v*-peptide ($M_r$ 43,000), pH and temperature. Very low $NaIO_4$ concentrations (50 $\mu$M) suffice to prevent the penetration of the *v*-peptide into $NaDodSO_4$ polyacrylamide gels. This effect could be abolished by *N*-ethylmaleimide alkylation of free sulphydryl groups, suggesting the involvement of easily oxidizable vicinal thiols in the aggregation of the peptide. Higher reagent concentrations resulted in the altered mobility and subsequent splitting of the receptor subunit carrying the ligand recognition site ($\alpha$, $M_r$ 40,000) into a doublet. In contrast, $NaIO_4$ treatment of the detergent-solubilized receptor aggregated the $\alpha$-subunit, presumably via chemical groups hidden in the membrane but exposed in detergent. Only this subunit underwent such $NaIO_4$-dependent changes within the concentration range in which (a) an increase of the 13-S dimeric receptor species at the expense of the 9-S monomeric form was observed and (b) half-maximal quenching of the intrinsic fluorescence occurred ($\sim$ 2 mM $NaIO_4$).

Neuraminidase digestion affected exclusively the $\gamma$- and $\delta$-subunits of the receptor, suggesting the presence of substantial amounts of sialic acid residues in these subunits. $\beta$-Glucosidase and endoglycosidase D had no effect on the electrophoretic properties of receptor and non-receptor polypeptides. Neither $NaIO_4$ nor neuraminidase treatments had any effect on the thermal sensitivity of the receptor. Similarly, the equilibrium and kinetic properties of receptor–$\alpha$-neurotoxin complex formation were not modified by such treatment nor was the susceptibility to tryptic digestion. The thermal and proteolytic sensitivities were affected by acid pH (5.2) and $\beta$-glucosidase treatments. The latter enzymatic digestion reduced the $\alpha$-toxin binding capacity of the receptor by 35% and increased the equilibrium dissociation constant by 2-fold.

The nicotinic acetylcholine receptor (AChR) is an intrinsic membrane glycoprotein. The intrinsic character of the protein stems from the application of physicochemical criteria for the classification of membrane proteins (Barrantes, 1975; Vandlen, Wu, Eisenach and Raftery, 1979) and from the results of various studies aimed at identifying the topography of the AChR in the membrane by structural (Ross *et al.*, 1977; Klymkowsky, Heuser and Stroud, 1980; Zingsheim, Neugebauer, Barrantes and Frank, 1980), biochemical (Huang, 1979), and immunohistochemical (Tarrab-Hazdai, Geiger, Fuchs and Amsterdam, 1978; Strader, Revel and Raftery, 1979) methods. More recently, these studies have been extended to the topography of individual AChR subunits, and the degree of exposure on either of the membrane faces and their membrane domains are beginning to be unravelled. Thus, lactoperoxidase-catalyzed iodination of sealed AChR vesicles reveals exposure to the external synaptic surface of the $M_r$ 40,000 ($\alpha$), 50,000 ($\beta$) and 65,000 ($\delta$) subunits (Strader *et al.*, 1979); a photolabile $\alpha$-bungatotoxin derivative labels the $\alpha$ and $\beta$ subunits from the outside of the vesicles (Witzemann and Raftery, 1978); a iodo-naphthyl-azide lipophilic label can be attached to the $\alpha$-subunit from the hydrocarbon interior of the membrane bilayer (Tar-

rab-Hazdai, Bercovici, Goldfarb and Gitler, 1980) and similarly a nitrene photogenerated pyrenesulphonyl-azide tags the $\beta$ and $\gamma$ subunits from the lipid bilayer (Sator, Gonzalez-Ros, Calvo-Hernandez and Martinez-Carrion, 1979). The susceptibility to proteolytic attack has also been used as a means to determine the exposure and sidedness of the AChR subunits (Strader and Raftery, 1980; Wennogle and Changeux, 1980). Selection of suitable probes for topographical studies is a difficult task. The availability of region and subunit-specific antibodies is certainly going to accelerate progress in the area, specially in conjunction with chemical modification studies which disclose hidden antigenic determinants in the AChR subunits (Froehner, 1981) or in non-receptor peptides (Froehner, Gullbrandsen, Hyman, Jeng, Neubig and Cohen, 1981; Barrantes, 1982a).

That the AChR is a glycoprotein has been demonstrated in purified *Electrophorus* AChR (Meunier, Sealock, Olsen and Changeux, 1974), denervated rat diaphragm (Brockes and Hall, 1975) and cat skeletal muscle (Dolly and Barnard, 1977), foetal calf muscle (Merlie, Changeux and Gros, 1978), *Torpedo* electric tissue (Mattson and Heilbronn, 1975) and in the BC3H-1 non-fusing cell line (Schubert, Harris, Devine and Heineman, 1974). All individual AChR subunits possess glyco-moieties (Raftery, Vandlen, Reed and Lee, 1976; Vandlen *et al.*, 1979; Lindstrom, Merlie and Yogeeswaran, 1979). However, no advantage has been taken of the fact that the carbohydrate moiety constitutes almost invariably a vectorial marker in membrane glycoproteins, the sugar residues being exposed to the extracellular milieu. Since no topographical relationship appears to exist between the ligand recognition site of the AChR and the carbohydrate moiety (Wonnacott, Harrison and Lunt, 1980a), and the antigenicity of the AChR is not affected by elimination of such moiety (Wonnacott, Harrison, Lunt and Barkas, 1980b), appropriate markers of the sugar domains might complement and topographically/temporally coexist with affinity labels of the recognition site.

In the present work we make use of $NaIO_4$ oxidation in combination with enzymatic digestion of AChR and non-receptor peptides with exo- and endoglycosidases to study some structural and functional characteristics of the receptor protein. A description of the electrophoretic properties of the AChR membrane polypeptides under different redox conditions and/or depletion of non-receptor proteins precedes the characterization of $NaIO_4$/glycosidase effects. The influence of the above reagents on the thermal and protease sensitivities of the AChR $\alpha$-toxin binding parameters and the distribution of AChR oligomeric species is also investigated.

## EXPERIMENTAL PROCEDURES

### *Materials*

Sodium cholate was obtained from Serva, Heidelberg. Dithiothreitol (DTT), *N*-ethylmaleimide, aprotinine, neuraminidase type IX (EC 3.2.1.18) from *Clostridium perfringens*, $\alpha$-methyl-D-mannoside, concanavalin A-Sepharose 4B and bovine serum albumin were from Sigma, Munich. Native $\alpha$-bungarotoxin and its tritium derivative (spec. act. 48 Ci/mmol) were purchased from Miami Serpentarium, Miami, and Amersham Buchler, Braunschweig, respectively. $\beta$-Glucosidase (EC 3.2.1.21) from sweet almonds (1295 U/mg material) was from Miles Laboratories, Frankfurt and endoglycosidase D from *D. pneumoniae* was obtained from Seikagaku Kogyo, Tokyo. Electrophoretically pure molecular weight standards were obtained from LKB Products, Stockholm. All other reagents were analytical grade and purchased from Merck, Darmstadt. *Torpedo marmorata* electric fish were obtained from the Marine Biological Station at Arcachon, France. They were kept in an aquarium with artificial sea water for at least two days before use. Only young specimens of less than 40 cm in length were selected. On average a total of 120 $\pm$ 40 g electric tissue (wet wt) was obtained from each fish.

### *Methods*

The preparation of the AChR membranes was carried out as described by Barrantes (1982b). Alkaline treatment followed the procedure of Neubig, Krodel, Boyd and Cohen (1979) with slight modifications, as given in Barrantes (1982a). Thiol modifications of the AChR membranes were conducted at 20 or 2°C at the concentrations which were previously found to accomplish saturation of the corresponding reaction (Barrantes, 1980).

Periodate oxidation of the membrane-bound or detergent-solubilised AChR (0.5–1 mg protein/ml) was performed for 10 min at 2°C in the dark. $NaIO_4$ was freshly prepared in 100 mM NaCl, 1 mM EDTA, 0.1 mM phenylmethane sulphonyl fluoride (PMSF), 10 mM sodium phosphate buffer, pH 7.2 at the concentrations indicated in the figure legends. The reaction was stopped with excess ethyleneglycol. AChR membranes (0.25–0.5 mg protein/ml) were incubated for 15 min with 0.35 U of neuraminidase in 100 mM citrate–phosphate, pH 5.6, containing 0.3 mg bovine serum albumin/ml at 37°C. When appropriate, this treatment was followed by an incubation with 10 mg/ml $\beta$-glucosidase in 50 mM sodium acetate buffer, pH 5.2 (37°C, 15 min). Endoglucosidase D treatment was carried out at 37°C for 15 h with addition of toluene, in 3 mM EDTA, 1 mM EGTA, 0.1 mM PMSF, 50 mM citrate-phosphate buffer, pH 6.0.

The affinity fractionation with Con A-Sepharose 4B was made in a column (3.5 ml bed volume) using 200 mM NaCl, 30 mM DTT, 50 mM Tris–HCl pH 8.0 for packing and elution. The supernatant of the alkaline-extracted membranes (Neubig *et al.*, 1979) was taken to pH 8.0 with HCl. DTT was added to give a final concentration of 30 mM. Increasing concentrations of $\alpha$-Me-D-mannoside (50–500 mM) were used for elution and samples were analyzed by polyacrylamide gel electrophoresis.

A mild tryptic proteolysis was carried out on membranes with or without prior glycosidase digestion(s) by using 40 nM trypsin in 100 mM NaCl, 10 mM sodium phosphate buffer, pH 7.4, at 25°C for 5 or 35 min. The samples were subsequently centrifuged and washed repeatedly, and electrophoresis was performed as described below.

The thermal stability of $NaIO_4$ or glycosidase-treated AChR was tested by heating the samples at 56°C for 5 min and subsequently measuring the [$^3$H]α-bungarotoxin binding as described below.

The sedimentation behavior of the AChR was followed by using linear sucrose gradients (5.2 ml, 5–20% sucrose in 1% sodium cholate, 30 mM DTT, 3 mM EDTA, 1 mM EGTA, 0.1 mM PMSF, 50 mM Tris–HCl, pH 7.5. Samples were centrifuged at 50,000 r.p.m. in a Beckman SW 50.1 rotor for 5 h at 4°C. Between 25 and 35 samples (120–150 μl each) were collected, applied to Whatman GF/C glass filters, dried, and counted in POPOP–toluene mixtures with 35–40% efficiencies.

Polyacrylamide gel electrophoresis was done according to Laemmli (1970) with the modifications indicated in the text and figure legends. Protein concentration and toxin binding activity of the membranes were determined as described by Schmidt and Raftery (1973) and Barrantes (1978). Fluorescence experiments were carried out as in the latter work.

## RESULTS

### *AChR membrane redox state and polypeptide patterns*

Various factors were found to contribute to the observability, penetrability and relative mobility of the polypeptides present in AChR membranes. The combined effects of the temperature and thiol treatments on $NaDodSO_4$ polyacrylamide patterns is shown in Fig. 1. Differing thermal sensitivities were found for AChR and non-receptor peptides. The δ (or $\delta_2$) bands appeared to be relatively thermal-insensitive, whereas the γ-subunit showed the opposite behaviour (Fig. 1c–j). In the absence of reducing agents only the α and $\delta_2$ AChR subunits penetrated the running gel (Fig. 1a–b). After reduction, the missing β and γ bands were observed (Fig. 1c) together with the non-receptor peptides ν, ϵ, ζ. If reduction was conducted at 100°C, however, the γ-band was totally absent, and the ζ, ϵ and actin bands were also affected (Fig. 1d). Additional effects of thiol modifications and temperature are shown in Fig. 1.

NEM-membranes, consisting mainly of the 13S, dimeric AChR species, differed from the standard membranes in their electrophoretic properties. Firstly, although the ν-peptide was missing from electropherograms of unreduced, standard membranes (Fig. 1), in the case of NEM-membranes it readily penetrated the gels even in the absence of reduction (Fig. 2a). Further, the ν-peptide from NEM-membranes showed a lower mobility than in the controls, and only one component of the doublet (the slowest) was apparent. The thermal sensitivity of this peptide also differed from that of standard membranes (Fig. 2). The most remarkable difference between the two types of membranes was the failure of alkaline treatment to extract the ν-peptide from NEM-membranes (Fig. 2d–e). The lack of extractibility of this peptide explains the lower specific activities of these membranes with respect to the standard ones.

### *Effects of $NaIO_4$ oxidation on the AChR and the ν-peptide*

As shown in Fig. 3, concentration-dependent changes in the electrophoretic mobility of the polypeptides present in AChR membranes occurred upon oxidation with $NaIO_4$. The extent of the electrophoretic alterations differed among the various polypeptides and was also dependent on the availability of free sulphydryl groups. In standard membranes, retardation in the mobility of the AChR α-subunit already occurred at 1 mM $NaIO_4$ (Fig. 3A). The β and γ bands were only slightly affected in the 1–10 mM range. The δ subunit did not undergo any noticeable change. At concentrations higher than those needed to alter the electrophoretic mobility, the α-subunit was split into a doublet, which was more clearly visible in the NEM-protected membranes (Fig. 3B). This α-doublet should not be confused with the doublet of the ν-peptide (Barrantes, Neugebauer and Zingsheim, 1980; Barrantes, 1982a, b). Aside from these changes in the receptor polypeptides, the nonreceptor ν-peptide was more conspicuously altered by the $NaIO_4$ treatment. Even at the lowest concentrations tested (50 μM), the staining intensity of the ν-peptide was significantly diminished in electropherograms of standard membranes, and totally absent above 1 mM $NaIO_4$ (Fig. 3A). On the contrary, if the SH groups of the AChR membranes were previously blocked, the ν-peptide was only *retarded* in its migration above 5 mM $NaIO_4$ (Fig. 3B). None of these electrophoretic changes was affected by previous incubation with excess α-bungarotoxin, and they were all present under conditions in which 'overoxidation' effects are minimised (0°C, in the dark, acid pH).

The effects of $NaIO_4$ on detergent-solubilised membranes markedly differed from those observed in membrane samples. These effects were explored in three different detergents and various pH conditions. As shown in Fig. 4, $NaDodSO_4$ solubilised the same polypeptides at the three pH values tested. Low solubility of the polypeptides was observed in Triton X-100 and sodium cholate at acid pH values. When

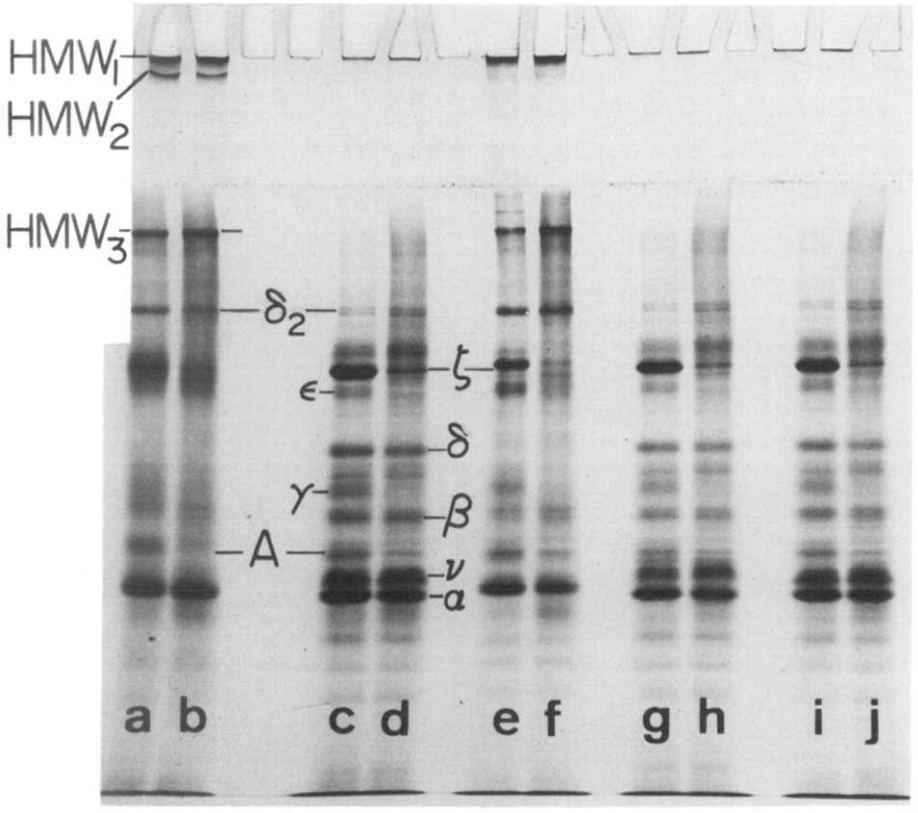


Fig. 1. Effects of reduction, alkylation, reduction + alkylation and temperature on the polypeptide patterns of standard membranes. The paired tracks correspond to AChR processed at 20°C for the thiol treatment and at 12°C for their loading and running in the gels (left in each pair) or heated at 100°C for 3 min (right in each pair) prior to application to the thermostatted slab gel. (a, b) In the absence of reducing agent. (c and d) treated with 100 mM DTT for 30 min at 20°C. (e and f) Alkylated with 30 mM NEM (20°C). (g and h) Alkylated as e, followed by DTT reduction as in c. (i and j) Reduced with 100 mM DTT and subsequently alkylated with 30 mM NEM as above. DTT reduction was carried out in 50 mM Tris–HCl buffer, pH 8.0 for samples g–h. Other treatments were performed in the Laemmli (1970) buffer system. Thirty microliters of each sample (35 μg protein) were loaded in each slab well of 5% acrylamide stacking gel, in the absence of reducing agent. Electrophoresis was performed for 1 h at 35 V and for 4.5 h at increasing voltage (70–160 V) after sample penetration in the 10% running gel.

the membranes were blocked by NEM, the $\nu$-peptide was very poorly solubilised (Fig. 4). The oxidation with 10 mM $NaIO_4$ slightly retarded the electrophoretic mobility of the $\beta$, $\gamma$, $\delta$, $\nu$, $\zeta$-peptides. The most noticeable difference between membrane-bound and solubilized AChR was apparent on the $\alpha$-subunit. At 10 mM $NaIO_4$ this chain was only faintly visible at basic pH (Fig. 4A). When increasing concentrations of $NaIO_4$ were used, the lack of penetration of the $\alpha$-subunit was already evident between 0.5 and 2.5 mM reagent. Similar trends were apparent in the effects of $NaIO_4$ on the sedimentation behaviour of the two main AChR species. As seen in Table 1, the 2:1 usual monomer:dimer ratio was only slightly modified when $NaIO_4$ acted on the membrane, whereas the oxidation was markedly enhanced if the AChR was in solution, a 1:1 monomer:dimer ratio being attained. No effects were apparent on the sedimentation coefficients of the two species.

Treatment of AChR membranes with $NaIO_4$ produced changes in the intrinsic fluorescence occurring in the time-course of seconds to minutes (Fig. 5). Half-saturation of this effect was accomplished at about 2 mM reagent. The kinetics of the fluorescence changes (Fig. 5, inset) were characterized by a faster initial quenching followed by partial recovery. Preincubation of the membranes with carbamoylcholine did not alter the $NaIO_4$ effects.

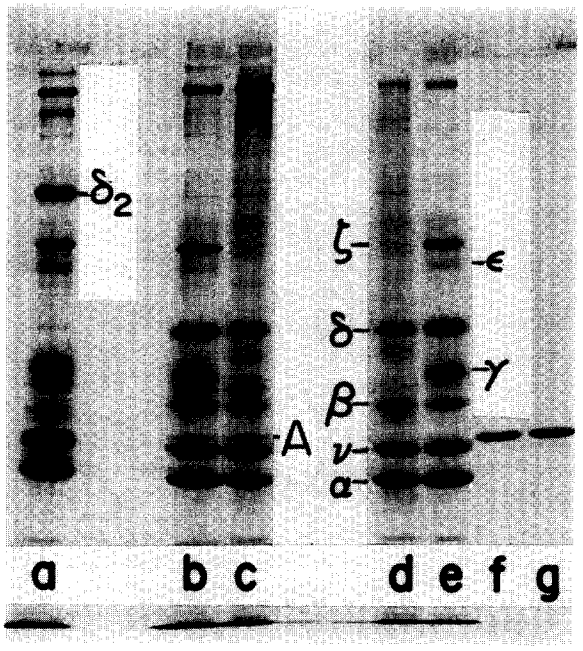


Fig. 2. Effects of NEM alkylation at early preparative stages on the membranes on the electrophoretic patterns. (a) Sample applied without further treatment, in the absence of reduction. Observe the absence of the δ-subunit and the presence of the ν-polypeptide (compare with Fig. 1a). (b) 100 mM DTT reduction, 20°C, 30 min. (c) 100 mM DTT reduction, 3 min, 100°C. The same membranes, but extracted with NaOH for 1 h, and reduced with 100 mM DTT (30 min, 20°C) are shown in (e). (d) The same, but reduction as in (c). Purified *Torpedo* actin, DTT-reduced at 20°C (f) or at 100°C (g) is also shown. Other experimental conditions as in Fig. 1.

*Effects of glycosidases on AChR-rich membranes*

Aside from the amino acid side chains, and in particular the thiol groups certainly involved in the AChR interconversions explored in the preceding sections, other chemical groups could have been affected by the $NaIO_4$ oxidation. Vicinal hydroxyl groups of the glycopeptides present in AChR membranes were candidates of choice. For this reason a series of experiments were undertaken firstly to identify such components and secondly to characterize the effects of glycosidic cleavage on the above properties.

Fig. 6A shows that all the AChR polypeptides react with concanavalin A, as tested by the sensitive blotting technique using peroxidase staining. No concanavalin A-reactive groups were detected in the region of the ν-peptide. The glycoprotein nature of the presumptive Na, K-dependent ATPase heavy chain (ζ, Fig. 6A) could be attested. The lack of concanavalin A reactivity in the ν-peptide was confirmed by affinity chromatography experiments using Sepharose-coupled concanavalin A (Fig. 6B).

Although earlier reports indicate relatively low contents of sialic acid in the total AChR (Vandlen *et al.*, 1979) or in its individual subunits (Lindstrom *et al.*, 1979) cleavage of these residues by neuraminidase treatment enhanced the electrophoretic mobility of (only) the γ- and δ-chains of the AChR (Fig. 7). Another glycosidic enzyme, the almond β-glucosidase, was tested for its potential effects on the AChR membrane polypeptide pattern. In addition to its main β-glucoside substrate specificity, this enzyme preparation (see Experimental Procedures) contains α- and β-galactosidase, β-fucosidase (Kleeman and Whelan, 1966; Conchie, Gelman and Levy, 1967), and α-manosidase activities. Neither this enzyme mixture, however, nor endoglycosidase D treatments produced any effect on the electrophoretic pattern of AChR membranes (Fig. 7). When applied after glycosidase treatments, $NaIO_4$ showed the same effects as those observed in unmodified membranes (see above).

Neuraminidase affected only slightly the sedimentation pattern but not the sedimentation coefficients of the AChR (Table 1). The treatment at pH 5.2, that is the control of β-glycosidase digestion, increased the proportion of higher molecular forms without effects on the sedimentation coefficient. This was not appreciably modified by β-glucosidase treatment (Table 1). There was some degree of insolubilization of the AChR in sodium cholate when the treatment at pH 5.2 was carried out.

*Thermal stability and susceptibility to mild proteolysis of the AChR*

Various treatments are known to alter the thermal stability of the AChR (Saitoh, Wennogle and Changeux, 1979). As a consequence of $NaIO_4$ oxidation there was a roughly 20% reduction in the thermal stability of the AChR (Table 2). Neuraminidase (or its control treatments) had no effects on this property, while treatment at pH 5.2 (a control condition, see Table 2) decreased appreciably the thermal stability of the AChR. β-Glucosidase treatment further enhanced this latter effect (Table 2).

Another criterion used to test the effects of the above treatments was to measure the susceptibility of the polypeptides in AChR membranes to proteolytic attack by trypsin. Neuraminidase treatment did not appear to modify the susceptibility to proteolysis (Fig. 8), whereas incubation at pH 5.2 increased the proteolytic degradation. In contrast, β-glucosidase-treated membranes showed increased resistance to trypsinization (Fig. 8).

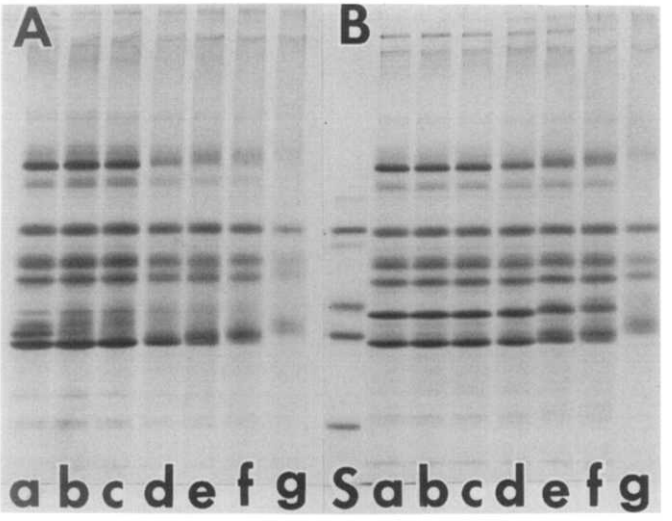


Fig. 3. Effects of $NaIO_4$ on the $NaDodSO_4$-polyacrylamide patterns. AChR-rich membranes (40 $\mu$g protein, 170 pmol $\alpha$-toxin sites/mg protein) were incubated with (B) or without (A) 5 mM NEM for 30 min at 18°C in a buffer containing 50 mM NaCl, 1 mM EDTA, 3 mM $NaN_3$, 10 mM Na phosphate, pH 7.0. Samples were centrifuged in an Airfuge for 5 min (140,000 × *g*) and washed twice with the above buffer with two additional centrifugations. They were then treated with 0.05 mM (b), 0.1 mM (c), 1 mM (d), 5 mM (e), 10 mM (f) or 50 mM (g) $NaIO_4$ respectively as described under Methods. The reaction was stopped with excess ethyleneglycol and samples were centrifuged and washed as above prior to gel electrophoresis. Reduction was carried out with 100 mM DTT (30 min, 20°C). Other conditions as in Fig. 1. The track marked S corresponds to the following standard proteins: bovine serum albumin ($M_r$ 67,000), ovoalbumin ($M_r$ 45,000), aldolase ($M_r$ 40,000) and chymotrypsin ($M_r$ 25,000).

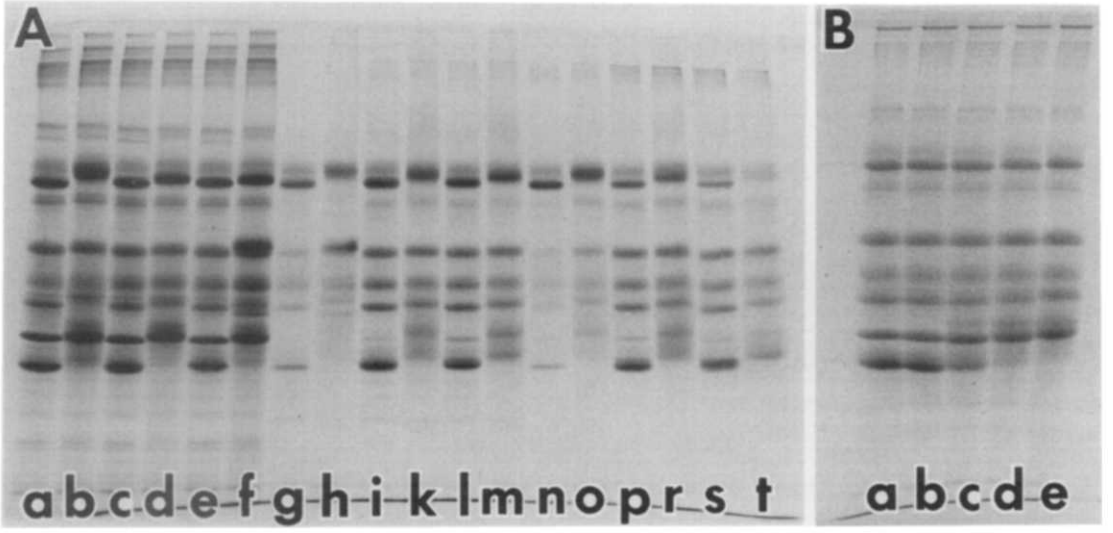


Fig. 4. Periodate oxidation of membranes solubilized in different detergents and at different pH values. (A) Aliquots containing 30 $\mu$g protein and 75 pmol $\alpha$-toxin sites were alkylated with NEM as in Fig. 3. Pellets were resuspended in 2% $NaDodSO_4$ (a–f), 1% Triton X-100 (g–m) or 1% Na cholate (n–t) in 100 mM NaCl, 1 mM EDTA, 0.1 mM PMSF and 10 mM Na phosphate buffer at the following pH values: 4.0 (tracks a, b, g, h, n, o), 7.2 (tracks c, d, i, k, p, r), and 10.7 (tracks e, f, l, m, s, t). Non-soluble material was removed by centrifugation and the supernatants oxidised (b, d, f, h, k, m, o, r, t) or not (a, c, e, g, i, l, n, p, s) with 10 mM $NaIO_4$ as before. Reaction was stopped with 6 $\mu$l glycerol. DTT and $NaDodSO_4$ were added if appropriate to give final concentrations of 0.1 M and 2% just before loading onto the gels. (B) After solubilization in $NaDodSO_4$ as in A, supernatants were treated with $H_2O$ (a), 0.1 mM (b), 0.5 mM (c), 2.5 mM (d) and 10 mM $NaIO_4$ (e) at pH 7.2 as in A.

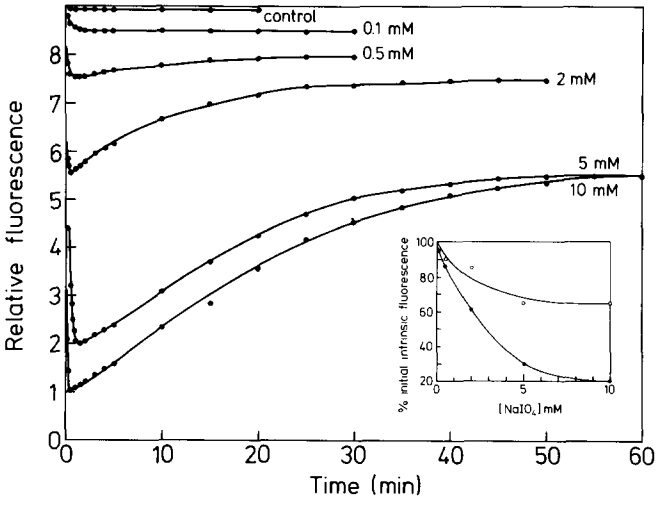


Fig. 5. Steady-state and kinetics of $NaIO_4$ effects on AChR intrinsic fluorescence. Membrane suspensions (60 μg protein/ml, 120 nM in α-toxin sites) were equilibrated in 100 mM NaCl, 10 mM sodium phosphate buffer, pH 7.4 for 20 min at 20°C. A concentrated stock solution of $NaIO_4$ was injected and mixed within 5 s and the fluorescence was measured with phase-sensitive detection at the indicated times. Values are expressed as the percentage of the initial resting fluorescence ($F_0$) at the given times. *Inset*: Amplitude of the fast initial quenching (●——●) and maximal steady-state quenching (○——○) as a function of $NaIO_4$ concentration.

*Effects of $NaIO_4$ and glycosidase treatments on α-toxin binding parameters*

As previously found with the detergent-solubilized AChR (Wonnacott *et al.*, 1980a) $NaIO_4$ had no effect on the maximum binding of α-toxin to the membrane-bound AChR (Table 3). Neuraminidase treatment was also without effects, but its combination with β-glucosidase digestion resulted in diminished binding of the toxin (Table 3). It was therefore of interest to establish whether this alteration reflected concomitant modifications of kinetic binding parameters. As seen in Table 4, a slight diminution of the association and dissociation rates followed β-glucosidase treatment, with a two-fold reduction in the equilibrium dissociation constant.

## DISCUSSION

The redox state of the AChR membranes prior to gel electrophoresis under denaturing conditions, and the temperature at which the thiol treatments and/or electrophoresis are performed bears on the variability of the polypeptide patterns. This applies to the normal and thiol-modified membranes, i.e. to AChR in different oligomeric states. It has recently been found (Barrantes *et al.*, 1980) that heating of the samples prior to gel electrophoresis results in a substantial diminution of the polypeptide staining, as confirmed by Sobel, Heidmann, Cartaud and Changeaux (1980). The thermal lability varied among the individual AChR and non-receptor peptides. The δ band appeared to be relatively thermal-insensitive, whereas the γ-subunit of the AChR exhibited a marked thermal-sensitivity, followed in order by the non-receptor peptide ζ, actin, and ε (Figs. 1 and 2). The γ-chain of the AChR ($M_r$ 57,000) in *Torpedo* appears to be the most sensitive of all subunits to thermal (Barrantes *et al.*, 1980) and proteolytic (Froehner and Rafto, 1979) degradation and/or redox state. Strader and Raftery (1980), however, found the α-subunit to be more drastically affected by prolonged heating, resulting in its degradation to a $M_r$ 35,000 peptide. In the case of the γ-subunit the protective effects of early NEM-alkylation may result both from stabilization of thiol groups in this subunit and inhibition of thiol proteases (Froehner and Rafto, 1979). In *Electrophorus* the γ-subunit also appears to be the most delicate subunit (Lindstrom, Cooper and Tzartos, 1980a). The thermal sensitivity is independent of the redox state (Figs. 1 and 2). Extraction of the non-receptor ν-peptide is also without effect on the thermal lability of the AChR subunits in gel electrophoresis (Fig. 2). However, diminution of the resistance to thermal inactiva-

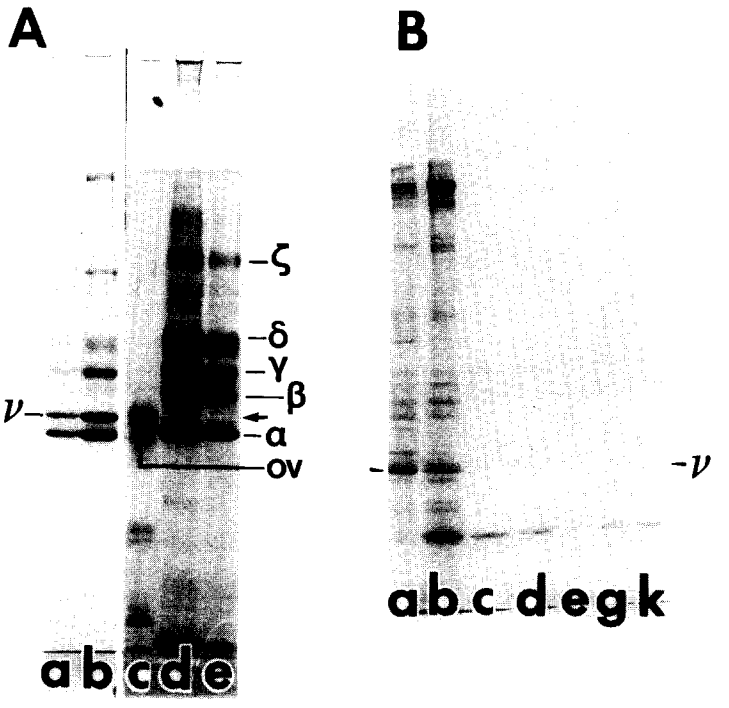


Fig. 6. (A) Glycoprotein nature of the polypeptides in AChR membranes. Tracks a and b, corresponding to two different concentrations of AChR membranes (15 and 25 μg protein/lane respectively), are stained with Coomassie Blue. Track c is ovoalbumin, and d and e the same as in a and b, but incubated with concanavalin A and stained with peroxidase-conjugated anti-lectin antibodies. Notice the positive reaction in all AChR subunits and in the control (ovoalbumin) and the negativity of the ν-peptide. $NaDodSO_4$ polyacrylamide gel under reducing conditions as in Figs. 1 and 2. (B) Lack of binding of the ν-peptide to Con-A-Sepharose 4 B affinity resin. (a) The soluble extract of pH 11 treated membranes (NaOH, 1 h, 2°C) contains several non-receptor, peripheral polypeptides, conspicuously the ν-peptide (arrow). This material was applied to a Con-A-Sepharose 4B column. Track b shows the material eluting with the void volume of the column; the ν-peptide is not retained. Tracks c, d, e, g and k correspond to the polypeptides eluted with 50, 100, 200, 300 and 500 mM α-Me- D-mannoside respectively.

tion of the AChR binding properties has been observed after depletion of the ν-peptide (Saitoh *et al.*, 1979).

*Structural heterogeneity of the $M_r$ 40,000 and 43,000 polypeptides*

Sodium periodate is a water soluble oxidising agent of moderate strength which has found wide applicability in protein chemistry. Yet its effects on proteins are not necessarily specific, and great variability is found in terms of reactive groups affected (see below). In the membrane-bound state, mild $NaIO_4$ oxidation produces two major electrophoretic modifications: Splitting of the α-subunit of the AChR and mobility retardation or disappearance of the ν-peptide. The former of these two effects suggests the existence of two forms of the α-chain reduced and oxidised in equilibrium. Such equilibrium appears to be altered by $NaIO_4$ in favour of the oxidised form without direct involvement of free thiols, as attested by the lack of effects of NEM alkylation (Fig. 3B) in modifying the progressive splitting of the α-chain. This could be a consequence of different environments of the two subunits in the AChR. In fact, it has recently been possible to identify differences in the environment of such subunits by structural methods (Zingsheim, Barrantes, Frank, Haenicke and Neugebauer, 1982). Additional sources of heterogeneity can be detected in the α-subunit (Saitoh *et al.*, 1980), a fact which may also be attributed to differences in the two homologous α-chains.

With respect to the second of the above effects, (i) the substantial protection exerted by NEM alkylation on the $NaIO_4$ oxidation of the ν-peptide, (ii) the rich-

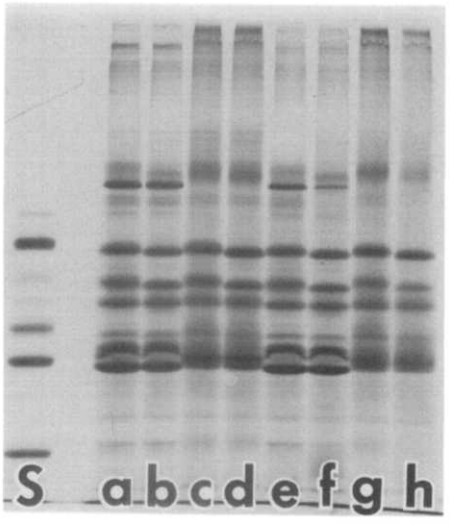


Fig. 7. Effects of neuraminidase and β-glucosidase on AChR membranes. AChR membranes (30 μg protein, 120 pmol α-toxin sites) were incubated with neuraminidase as described under Methods. After each incubation membranes were centrifuged, washed twice and subsequently oxidised with 10 mM $NaIO_4$ as in Fig. 3. Appropriate controls without enzyme were undertaken (see text). (a) Control. (b) Neuraminidase-treated membranes. (c and d) The same as a and b, but subsequently treated with 10 mM $NaIO_4$. (e) Control of the neuraminidase + β-glucosidase treatment. (f) Neuraminidase + β-glucosidase treated membranes. (g and h) The same as e and f but followed by 10 mM $NaIO_4$ treatment. S, standard proteins as in Fig. 3.

ness in thiols of this peptide (Sobel, Heidmann, Hofler and Changeux, 1978; Hamilton, McLaughlin and Karlin, 1979), and (iii) the solubility properties of this peptide (Sobel *et al.*, Saitoh and Changeux, 1980) all point to (a) the abundance of free-SH groups capable of self-aggregating the peptide and (b) the possible existence of two classes of -SH groups in this peptide, defined by their reactivity towards NEM and $NaIO_4$. One class is almost totally protected by NEM and, in the absence of such protection, leads to extensive crosslinking by $NaIO_4$ treatment. Such class of thiols could tentatively be referred to as the readily accessible type, topographically assigned to the surface of the peptide, and made responsible for inter-molecular crosslinking. It is known that $NaIO_4$, when used in almost stoichiometric amounts with -SH groups can readily oxidise multiple pairs of *vicinal* thiols in some proteins (Rippa, Bellini, Signorini and Dallochio, 1981). The second class of thiols, revealed after NEM protection, is quantitatively less significant and might correspond to -SH groups not accessible to the alkylating reagent but amenable to intra-molecular oxidation by $NaIO_4$, and thus presumably less reactive with $NaIO_4$. Finally, the ineffectiveness of pH 11 treatment to solubilize the *v*-peptide after $NaIO_4$ oxidation is paralleled by the lack of effects of alkaline extraction after blockage of free -SH groups by NEM alkylation (Barrantes, 1982a).

At the oligomeric level of organization of the AChR, a related effect of $NaIO_4$ is the shift towards the dimeric species observed in gradient centrifugation experiments (Table 1), specially when the $NaIO_4$ reaction is carried out on detergent-solubilized AChR. Either the accessibility or conformation of vicinal, oxidizable groups is modified upon solubilization, or the reactivity of such groups is enhanced in detergents. This is particularly evident on the electrophoretic properties of the α-chain: concentrations of $NaIO_4$ producing the splitting and displacement of the α-band when oxidation is performed on the membrane-bound AChR (Fig. 3) result in its complete

Table 1. Modification of acetylcholine receptor monomer–dimer ratio by $NaIO_4$ oxidation and glycosidase treatment

| Treatment | Monomer | Dimer |
|---|---|---|
| Control $NaIO_4$ oxidation | 62.3 ± 3.2 | 37.7 ± 3.2 |
| 10 mM $NaIO_4$, membrane-bound AChR | 55.6 ± 1.3 | 44.4 ± 1.3 |
| 10 mM $NaIO_4$, detergent-solubilised membranes | 50.4 ± 2.2 | 49.6 ± 2.2 |
| Control neuraminidase digestion | 64.6 ± 2.0 | 35.4 ± 2.0 |
| Neuraminidase digestion | 71.1 ± 3.4 | 28.9 ± 3.4 |
| Control neuraminidase + β-glucosidase | 75.6 ± 3.6 | 24.4 ± 3.6 |
| Neuraminidase + β-glucosidase digestion | 73.3 ± 3.8 | 26.7 ± 3.8 |

* Obtained by integration of the areas under each peak. Original data taken from two sucrose gradient centrifugation experiments as given in *Experimental Procedures*, including in each case the standard deviation of the mean.

Table 2. Effects of $NaIO_4$ and glycosidase treatments on the thermal stability of the acetylcholine receptor

| Treatment | Controls | Samples heated at 56°C |
|---|---|---|
| None | 100 | 70.0 (70.0) |
| 10 mM $NaIO_4$, 0°C, 10 min | 89.2 | 42.6 (47.8) |
| Citrate–phosphate buffer pH 5.6, 37°C, 15 min | 108.4 | 86.3 (79.6) |
| The same, plus neuraminidase | 103.0 | 84.2 (81.7) |
| Sodium acetate buffer pH 5.2, 37°C, 15 min | 107.4 | 27.9 (26.0) |
| The same, including neuraminidase + β-glucosidase digestion for 15 min | 69.8 | 9.5 (13.6) |

Receptor-rich membranes were exposed to the indicated treatment and subsequently heated at 56°C for 5 min. The samples were immediately cooled to 4°C and the binding of α-toxin carried out by standard procedures. Results are expressed as percentages of the control, untreated membranes. The binding of the heated samples with respect to the non-heated samples is also given (in parentheses).

absence from the gels when the reaction is undertaken in solution (Fig. 4B). These effects of $NaIO_4$ parallel recent observations on the 5,5′-dithio-*bis*-(nitrobenzoic acid) re-oxidation and oligomer formation of reduced receptor in sodium cholate (Barrantes, 1982a, b). Hamilton *et al.* (1979) had concluded from previous, similar studies (but using Triton X-100) that no reassociation of solubilized AChR occurs.

$NaIO_4$ is not a group-specific reagent, and various amino acid side chains can be the target of the $NaIO_4$

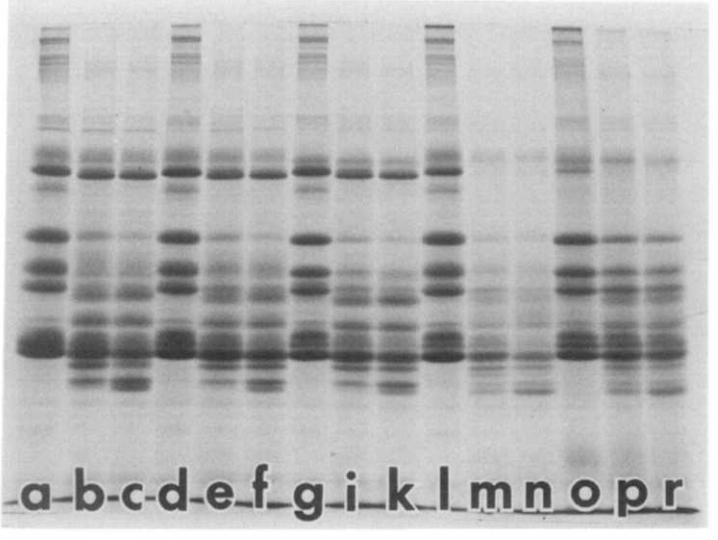


Fig. 8. Mild tryptic proteolysis of normal and glycosidase-treated AChR membranes. AChR membranes (35 μg protein, 113 α-toxin sites) were subjected successively to the following treatments: a–c, pH 7.4; d–f, pH 5.6, 15 min (control of the neuraminidase treatment); q–k, neuraminidase at pH 5.6 for 15 min; l–n, the same as d–f, followed by pH 5.2 for 15 min (Control of neuraminidase + glucosidase treatments); o–r, 15 min neuraminidase treatment at pH 5.6, and 15 min β-glucosidase digestion at pH 5.2. After the above treatments, samples were washed twice in an Airfuge and digested with 40 nM trypsin in a final volume of 50 μl for 5 min (b, e, i, m, p) or 35 min (c, f, k, n, r) at 25°C, diluted with cold buffer, pelleted in the Airfuge and washed twice. Tracks a, d, g, l, o are controls without trypsinization.

Table 3. Effects of $NaIO_4$, neuraminidase and β-glucosidase treatments on the binding of [$^3$H]α-bungarotoxin

| Treatment | Ratio $NaIO_4$:AChR | Max. equilibrium binding* |
|---|---|---|
| $NaIO_4$, 40 μM | 500 | 112% |
| , 2 mM | 2.500 | 97% |
| , 10 mM | 12.500 | 90% |
| Neuraminidase | | 95% |
| Neuraminidase + β-glucosidase | | 65% |

* Results are expressed as percentage of control binding on membranes not subjected to $NaIO_4$ or glycosidase treatments. Protein concentrations were determined before and after enzymic or chemical treatments.

reaction, such as tryptophan (Atassi, 1967; Azari and Phillips, 1970), tyrosine (Geoghegan, Dallas and Feeney, 1980), terminal serines (Dixon and Moret, 1964) or methionine (Knowles, 1965). Some of the present effects of $NaIO_4$ can most economically be accounted for by alterations of the membrane redox state, preferentially via thiol groups. The richness in free thiol groups in the *v*-peptide discussed above, and the low reagent concentrations needed to elicit changes in the *v*-peptide (Fig. 3) argue in favour of such groups being the main target of $NaIO_4$ attack. Periodate-labile residues apparently occur in unusually exposed locations, or on residues with exceptionally high reactivity. The protection exerted by NEM (Fig. 3B) reinforces this interpretation. A different site of action of $NaIO_4$ seems to be operative on the α-chain. Firstly, this chain does not appear to possess free (or exposed) sulphydryl groups in the membrane (Hamilton *et al.*, 1979). Secondly, the electrophoretic changes in the α-chain are independent of thiol group alkylation. Thirdly, the $NaIO_4$-mediated quenching of the intrinsic fluorescence (Fig. 5) half-saturates at the same concentration at which the splitting of the α-chain occurs (Fig. 3). Thus, given the experimental conditions used, tryptophan residues are likely to be involved in the quenching phenomenon.

*The carbohydrate moiety of the AChR subunits and its involvement in the structural and functional stability of the AChR*

$NaIO_4$ in general methods of protein chemistry has also found applicability in studies of the role and structure of carbohydrate groups in glycoproteins (Spiro, 1972). Aside from the various protein groups which have since been found to react with this ion (see above), the possible involvement of vicinal hydroxyl groups in the carbohydrate moiety of the AChR polypeptides should be discussed. As demonstrated by several groups (see Introduction) the AChR is a glycoprotein, the four polypeptide subunits possessing carbohydrate residues (Raftery *et al.*, 1976; Vandlen *et al.*, 1979; Lindstrom *et al.*, 1979). Here it is shown that the four subunits react with concanavalin A whereas the *v*-peptide does not (Fig. 6), in agreement with the findings of Meunier *et al.* (1974) on the lectin binding of the whole AChR. Wennogle and Changeux (1980) have also shown that the β and δ-subunits react with phytohemagglutinin A whereas the α-subunit does not.

Glycosidic treatment of the AChR membranes did not modify the observed effects of $NaIO_4$ (Fig. 7). Glycosidic cleavage *per se* selectively modified the electrophoretic mobility of the γ and δ subunits of the AChR, without affecting non-receptor polypeptides (Fig. 7). According to Lindstrom *et al.* (1979) the former are the two AChR chains having the highest sialic acid content. The results of Saitoh *et al.* (1979) on mobility alterations of the δ-subunit after digestion with a mixture of exo- and endo-glycosidases could also be explained on a similar basis. The slower

Table 4. Effect of neuraminidase + β-glucosidase digestion on the kinetics parameters of acetylcholine receptor–[$^3$H]α-cobrotoxin complex formation*

| | $k_a(M^{-1}s^{-1})$ | $k_d(s^{-1})$ | $t_{1/2}$(min) | $K_D$(nM) |
|---|---|---|---|---|
| Control membranes† | $3.08 \times 10^4$ | $8.35 \times 10^{-5}$ | 138 | 2.7 |
| β-Glucosidase + neuraminidase treated membranes | $1.05 \times 10^4$ | $5.50 \times 10^{-5}$ | 210 | 5.2 |

* Rate measurements were done using 3 nM toxin sites and 8.2 nM toxin and the Millipore filtration assay as described in Barrantes (1978). Data were plotted according to the integrated second-order rate equation.

† Control membranes refer to membranes incubated with pH 5.6 and 5.2 for periods equivalent to those used in the enzymic digestions as described under Methods.

migration of glycopeptides in comparison to that of the corresponding non-glycosylated polypeptides is probably due to the overall reduced $NaDodSO_4$ binding of the former (Glossman and Neville, 1971; Segrest, Jackson, Andrews and Marchesi, 1971). The increase in electrophoretic mobility observed upon removal of sialic acid residues has provided an explanation for the retarding effects of the glyco-moiety: The frictional drag offered by the sugar residues is manifested to a greater extent than the driving force of their negative charge (Strayer Leach, Collawn and Fish, 1980). The most dramatic electrophoretic changes in the mobility of the AChR chains is observed when comparing the mature glycosylated subunits with their corresponding non-glycosylated precursors (Anderson and Blobel, 1981), especially in the $\gamma$ and $\delta$ subunits. The fact that endoglycosidase D had no noticeable effect on the electrophoretic patterns while endoglycosidase H has been found to alter the mobility of the $\alpha$-chain (Merlie, Hofler and Sebbane, 1981) suggests that this subunit is glycosylated with at least one 'high mannose' or 'simple' *N*-linked oligosaccharide side chain (Merlie *et al.*, 1981).

In agreement with Saitoh *et al.* (1979) modifications of the membrane-bound AChR or its environment affect the thermal stability of the receptor. In the present work it is shown that in a narrow pH range (5.2–5.6) the thermal stability decreases (Table 2) and a concomitant increase in the proportion of AChR oligomers higher than dimers occurs, paralleled by a reduction in solubility of the AChR. In fact, although the antigenicity of the AChR is not altered by $NaIO_4$/glycosidase treatments, exposure to pH 4.5 causes the loss of antigenicity (Wonnacott *et al.*, 1980b). Putting all these pieces of information together, it appears that various properties of the AChR can be drastically affected within a critical pH range, around the isolectric point of either monomeric or dimeric AChR (Raftery, Schmitt and Clark, 1972; Ruechel, Walters and Maelicke, 1981). It is also in the acidic pH region where the greatest lability of the AChR to trypsin digestion was observed (Fig. 8). The paradoxical increase in the resistance to proteolysis upon $\beta$-glucosidase treatment (Fig. 8) contrasts with the usually enhanced lability of glycoproteins to trypsinization upon carbohydrate elimination. Klymowsky *et al.* (1980) recently reported that membrane-bound AChR was more sensitive to proteolytic degradation after alkaline extraction, and Lindstrom, Gullick, Conti-Tronconi and Ellisman (1980b) have shown the complete tryptic proteolysis of the four AChR chains in solubilised protein. Further, the membrane appears not to exert any hindrance to pepsin degradation. They also reported the cleavage of dimers into monomers upon trypsinization; here we show that neuraminidase and glycosidase digestions can also shift the equilibrium towards the monomer.

Finally, no modifications are observed upon $NaIO_4$ treatment on the kinetic and equilibrium binding properties of $\alpha$-toxins to the membrane-bound AChR (Table 3). Wonnacott *et al.* (1980a) reported a 20% reduction of equilibrium binding after reacting 2.5 mM $NaIO_4$ with the detergent-solubilised AChR, and Weinberg and Hall (1979) needed 4h reaction at the highest concentration we have used (10 mM reagent) to observe a 40–50% reduction in binding. Neuraminidase is also without effects on toxin binding but $\beta$-glucosidase treatment accomplishes a 35% decrease in binding (Table 3), i.e. the same obtained after concanavalin A treatment of the AChR (Boulter and Patrick, 1979).

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