Affinity chromatography and immunosorption with acetylcholine receptor attached to nylon tubes

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Nylon-linked proteins were used for affinity trapping and chromatography. As representative examples purified acetylcholine receptor, α -cobratoxin and bovine serum albumin were coupled to the activated matrix to serve as biospecific ligands. In particular, acetylcholine receptor was coupled without significant loss of biochemical properties. The resulting affinity tubes bind receptor-specific ligands including immunoglobulins and thus can be used for affinity-chromatographic purposes and immunoassays.

Purification of nicotinic acetylcholine receptor by affinity chromatography is now a well established procedure (Maelicke *et al.*, 1977a,b; Heidmann & Changeux, 1978). The converse, i.e. use of the purified receptor or its subunits as an affinity adsorbent for the purification of receptor-binding proteins (neurotoxins, antibodies), has been reported (Martinez *et al.*, 1977; Merlie *et al.*, 1979) but is seldom fully satisfactory. Large loss of binding capacity after linkage and/or non-covalent adsorption to the gel matrix and a general sensitivity of the receptor to variations in solvent conditions, among other complications, have hampered this application. Thus, although affinity adsorbents employing neurotoxin can often be used for several years without a decrease in yield or discriminating power, receptor affinity adsorbents tend to inactivate rapidly, often permitting only a few applications.

There certainly exists a need for an efficient and, if possible, simple procedure for trapping and purification of compounds interacting biospecificity with the receptor. (i) Quantitative studies with receptor-directed antibodies often require purification or separation from other serum components. (ii) A simple trapping device for such antibodies may serve as a tool to diagnose and monitor myasthenia. (iii) Other as yet unknown compounds interacting with the acetylcholine receptor may be discovered.

This situation has prompted us to search for new means of covalently attaching the purified receptor to a solid support suitable for affinity trapping, affinity chromatography and immunosorption. We particularly aimed at designing a device permitting

repetitive applications, the use of biological fluids such as whole blood, implantation in animals and direct immunoassays. All of these conditions are satisfied by tubular nylon supports. Here we report on the biochemical properties of such nylon affinity tubes with serum albumin, α -cobratoxin and purified acetylcholine receptor as representative examples of attached ligands.

Materials and methods

Preparations of acetylcholine receptor from *Torpedo marmorata* (Rüchel *et al.*, 1981), the α -neurotoxin (α -cobratoxin) from *Naja naja siamensis* and its mono-³H-labelled derivative (Maelicke *et al.*, 1977b) and α -bungarotoxin and its mono-¹²⁵I-labelled derivative (Miskin *et al.*, 1978) were all carried out as described in the cited references. Rabbit immunization (Miskin *et al.*, 1978), purification of antibodies directed against the acetylcholine receptor (Lindstrom *et al.*, 1979) and ¹²⁵I-labelling of these proteins (Harboe & Ingild, 1973) were carried out as described in the cited references. Bovine serum albumin and glutaraldehyde were supplied by Sigma Chemical Co., St. Louis, MO, U.S.A. 3-(3-Dimethylaminopropyl)-ethyl-carbodi-imide hydrochloride was obtained from Bio-Rad, München, Germany. Di-isopropyl fluorophosphate was from Serva, Heidelberg, Germany. Aprotinin (Trasylol) was a gift from Bayer AG, Leverkusen, Germany. Hexamethonium and all other reagents were supplied by E. Merck G.m.b.H., Darmstadt, Germany. Nylon tubing (1 mm internal

diameter) was obtained from Portex, Hythe, Kent, U.K.

Activation of the nylon tube (Sundaram & Hornby, 1970; Sundaram, 1977) consisted of initial partial hydrolysis with 3.5 M-HCl at 70°C by incubation for 7 min followed by a thorough wash with cold water for several hours. This hydrolysis cleaves the amide bonds releasing carboxy and NH_3^+ groups.

Cross-linking of α -cobratoxin to the partially hydrolysed nylon tubes with glutaraldehyde (Sundaram & Hornby, 1970; Rüchel *et al.*, 1981) was performed in two stages. Partially hydrolysed tubes were first perfused for 2 h with a 5% (w/v) glutaraldehyde solution in 0.05 M-sodium borate buffer, pH 9.5, and then washed for 15 min with cold buffer followed by perfusion for 24 h with a 10 mM solution of α -cobratoxin to which a small amount of ^{125}I -labelled toxin had been added. The tubes were then thoroughly washed with cold 1 M-NaCl and water and were stored filled with a solution consisting of 0.1 M-NaCl, 0.05 M-Tris/HCl, pH 7.4, 0.1 M-benzamidine, 0.002% (w/v) chlorhexidine, 0.02% (w/v) NaN_3 , at 4°C in a moist container. Concentration of coupled toxin was determined by measuring the amount of radioactivity retained using a Searle 1185 gamma counter.

Coupling of ligands with 3-(3-dimethylaminopropyl)-1-ethylcarbodi-imide hydrochloride was by the general procedure of Gordon *et al.* (1974). Routinely, the activated (partially hydrolysed) tubes were first perfused with cold water adjusted to pH 5 with 10 mM-HCl followed by perfusion for 2 h with 100 mM-3-(3-dimethylaminopropyl)-1-ethylcarbodi-imide hydrochloride at the same solvent conditions. The tube was then washed for 5 min with water or buffer adjusted to the desired coupling pH (pH 5 for bovine serum albumin and neurotoxins, pH 6 for acetylcholine receptor), followed by perfusion for 7 h (bovine serum albumin and toxins) or 24 h (acetylcholine receptor) with a cold buffer containing the protein to be coupled. Coupling yields are markedly lower when acetate buffer is used instead of pH-adjusted water. In a representative experiment with receptor, between 10% and 18% were coupled in water, whereas only 0.3% were coupled in 0.1 M-acetate buffer of the same pH. Thus coupling was routinely performed with protein solutions in water adjusted with HCl to the desired coupling pH. In most instances pH control or adjustment during coupling was not required. Finally, the tube was washed with 0.3 M-NaCl and water and stored filled with buffer at 4°C. Since rather low concentrations of receptor were employed in the coupling reactions with the activated tubes, complete saturation of activated groups at the nylon surface was assured by perfusing the tubes for a further 7 h with a buffer solution containing 10 μM -bovine serum albumin. All ac-

tivation and coupling procedures were performed in the cold room. To improve the reaction conditions nylon tubes were coiled around a rod and solutions were pumped at 0.2 ml/min. For most experiments tubes of 0.1 cm internal diameter and of 30 cm length were used.

The concentration of attached toxin was determined by including in the coupling solution a portion of ^{125}I -labelled toxin and then following the rest of the procedure as described in the cross-linking reaction using glutaraldehyde (see above). The amount of bovine serum albumin coupled was determined by the difference of the u.v.-absorption measurements of the coupling solution. The amount of receptor coupled was estimated by assaying the receptor solutions (Maelicke *et al.*, 1977b) before and after perfusion of the activated tubes. Perfusion with ^{125}I -labelled toxin and counting the radioactivity of the thoroughly washed tubes was employed to measure the concentration of nylon-linked receptor able to bind its ligands.

Results

Application of immobilized proteins for trapping or chromatography of specific ligands depends crucially on the preservation of biospecific properties during the coupling reaction. To optimize these conditions for the solubilized acetylcholine receptor we have first tested the toxin-binding properties of receptor cross-linked by various bifunctional reagents. Of the reagents tested, glutaraldehyde and the water-soluble 3-(3-dimethylaminopropyl)-1-ethylcarbodi-imide hydrochloride caused the smallest changes. Only the affinity but not the total number of available toxin-binding sites were decreased after several hours of treatment of the solubilized receptor with low concentration of glutaraldehyde (Rüchel *et al.*, 1981). At concentrations of up to 30 mM, 3-(3-dimethylaminopropyl)-1-ethylcarbodi-imide hydrochloride was better suited than glutaraldehyde in that it did not have any detectable effect on the toxin-binding properties of the receptor, though considerable cross-linking was observed (Rüchel *et al.*, 1981). Hence, this procedure was selected for all coupling reactions involving the purified receptor.

To standardize all coupling procedures they were also performed with bovine serum albumin and α -cobratoxin. The former was considered representative for typical water-soluble proteins and maximal coupling efficiency. The latter with its small size and rigid three-dimensional structure (Walkinshaw *et al.*, 1980) served as an example of a protein with low coupling efficiency. The coupling procedures for bovine serum albumin, α -cobratoxin and acetylcholine receptor described in the Materials and methods section yielded the concentrations of coupled ligand listed in Table 1. Consistently,

Table 1. *Efficiency of protein coupling to activated nylon tubes*

Coupling efficiency is expressed in pmol of attached ligand per cm length of nylon tube. The tubes had an internal diameter of 0.1 cm. Coupling efficiency for bovine serum albumin was arbitrarily set as 100%. Dividing the efficiency values by 0.3183 ($2\pi r$) yields pmol of attached ligand per cm² of nylon surface. Coupling of α -cobratoxin was usually performed with a 100-fold coupling of acetylcholine receptor, with only a small excess of ligand over the concentration of available sites (as measured by coupling with bovine serum albumin).

Coupling reagent	Ligand	Concentration of coupled ligands	
		(pmol/cm)	(%)
3-(3-dimethylaminopropyl)-1-ethylcarbodi-imide	Bovine serum albumin	420	100
	α -Cobratoxin	41	10
	Acetylcholine receptor	137	33
Glutaraldehyde	Bovine serum albumin	121	100
	α -Cobratoxin	15	12
	Toxin + bovine serum albumin	0.6	0.5

* To decrease the density of attached toxin groups, coupling with glutaraldehyde was also performed in the presence of bovine serum albumin (in a ratio of 1:25). These 'low-density' tubes had a relatively much higher trapping capacity for receptor and most of the bound receptor could be detached from toxin by perfusion with 0.1 M-hexamethonium.

coupling was least efficient with α -cobratoxin and most efficient with bovine serum albumin, with the densities of coupled bovine serum albumin molecules at the nylon surface approaching that of a closely packed monomolecular layer. Probably because of its larger size (mol.wt. 300000; Doster *et al.*, 1980) coupling efficiency for acetylcholine receptor was only one-third that determined for bovine serum albumin.

The binding capacities of nylon-linked toxin and receptor were determined by two procedures. In the first, the concentration of bound ligand and in the second, the concentration of displaceable bound ligand was determined. For coupled receptor, toxin was used as biospecific ligand and vice versa. The effect of accelerated dissociation of receptor-toxin complexes in the presence of high concentrations of low-molecular-weight ligands (Maelicke *et al.*, 1977b; Maelicke & McConnell, 1978; Kang & Maelicke, 1980) was employed to determine the concentration of displaceable ligand. The results are listed in Table 2. As previously found for α -cobratoxin linked to CNBr-activated agarose (Rüchel *et al.*, 1981; Maelicke *et al.*, 1977b) only a fraction (26%) of the attached receptor retained the capacity to bind α -toxin. Attachment of the receptor to the tubes was very stable as detected by toxin-binding tests after various periods of perfusion with buffers containing low and high salt concentrations. A small decrease in toxin-binding capacity with time (3–4% after 10 days but no effect within the first 48 h) may be attributed to proteolytic nicking of covalently attached receptor molecules rather than to dissociation of linkages with the nylon surface.

Elution of toxin bound to receptor-linked nylon tube was analysed as follows. After perfusion with a

toxin solution containing a portion of ³H-labelled α -cobratoxin the tube was washed with buffer containing 0.5 M-NaCl until the protein content of the effluent was below the detection limit. The tube was then perfused with buffer containing 5 mM-hexamethonium, the amount of toxin displaced being determined as the radioactivity of the effluent (Fig. 1). Toxin was displaced completely (Table 2) and rapidly as may be expected from conditions of accelerated dissociation of toxin-receptor complexes (Maelicke *et al.*, 1977b; Kang & Maelicke, 1980). Furthermore, this treatment did not affect the concentration of active receptor molecules linked to the nylon surface as was determined by a second binding experiment with the toxin. In repetitive experiments of this kind, the tubes lost less than 15% activity over a period of 4 weeks as long as they were stored filled with a buffer containing proteinase inhibitors (0.1 M-benzamide, 1 μ M-Trasyolol, 1 mM-di-isopropyl fluorophosphate).

Having shown that (i) receptor can be linked efficiently to activated nylon surfaces, (ii) these affinity surfaces bind biospecific ligands with high affinity and in a reversible fashion and (iii) nylon-linked receptor retains its typical properties even after repetitive cycles of ligand adsorption, desorption, washing and storing, we then used the affinity tubes to investigate why apparently not all of the ligand trapped by these tubes is detached in the consecutive washing and dissociation experiments. Similar studies with agarose-linked neurotoxins (Klett *et al.*, 1973; Rüchel *et al.*, 1981) suggested that there was a correlation between the density of immobilized affinity ligand and the capacity of reversibly adsorbing biospecific ligands. To test this hypothesis, α -cobratoxin was coupled at various densities to the nylon surface and the relative

Table 2. Efficiency of protein-coupled nylon tubes in trapping and reversible binding of biospecific ligands

Capacities of:			
Ligand linked to nylon tube			
Type	Trapping	Reversible binding	
	Concentration (pmol/cm)	Density (pmol/cm ²)	(% of concentration of linked ligand)
Acetylcholine receptor	137	436	36
α -Cobratoxin	0.6	2	Bound ligand
			α -Neurotoxin
			32
α -Cobratoxin	15	48	(% of trapped ligand)
			89
			83
α -Cobratoxin	41	131	Coupling reagent
			3-(3-dimethylaminopropyl)-1-ethylcarbodi-imide hydrochloride
			0.42
α -Cobratoxin	41	131	Acetylcholine receptor
			3.8
			0.35
α -Cobratoxin	41	131	Acetylcholine receptor
			7
			0.3

Fig. 1. Displacement of α -cobratotoxin from nylon-tube-linked acetylcholine receptor

Acetylcholine receptor was linked by the 3-(3-dimethylaminopropyl)-1-ethylcarbodi-imide hydrochloride procedure to 3 cm of partially hydrolysed nylon tubing. From the remaining amount of receptor in the coupling solution the concentration of nylon-linked receptor was calculated to be 108 pmol/cm length of nylon tube; 1 cm of the receptor-linked tube was incubated for 24 h at 4°C with 5 ml of 0.1 M-NaCl/0.05% Tween 80/0.05 M-Tris/HCl (pH 7.4), containing 320 pmol of [³H]-pyridoxamine-labelled α -cobratotoxin (sp. radioactivity 4 Ci/mmol). From the remaining amount of toxin in the incubation solution, the concentration of receptor-bound toxin was calculated to be 22.3 pmol or 21% of the molar concentration of nylon-linked receptor. Before further experiments the tube was then washed with buffer containing 0.5 M-NaCl. To check the spontaneous rate of receptor-toxin complex dissociation, the tube was perfused at 22°C with a sterile solution of 0.1 M-NaCl/0.05% Tween 80/0.05 M-Tris/HCl (pH 7.4) at a rate of 10 ml/h. The effluent was collected in 1 ml portions and counted for radioactivity with Quickzint 212 in a scintillation counter. Under these conditions toxin was released from the column at a rate of 0.10 pmol/h (46 ± 9 c.p.m./ml). The tube was then perfused in the same conditions with a buffer of the same composition except that it contained 5 mM-hexamethonium chloride. Results for toxin in the effluent in the absence of hexamethonium (x) and in the presence of hexamethonium (●) are shown. Half-lives of dissociation were $\tau = 110$ h in the absence of hexamethonium and $\tau = 1.7$ h in the presence of hexamethonium. The inset shows the logarithm of the difference between the concentration of totally displaceable toxin (c.p.m._∞) and toxin displaced after any given time t (c.p.m.) plotted against t . The linear functional relationship indicates that displacement of α -cobratotoxin from nylon-tube-linked acetylcholine receptor conforms to a first-order rate law.

efficiencies of trapping and reversible binding of ligands were analysed (Table 2). Both trapping capacity and capacity of reversible binding decreased with increasing density of toxin covalently attached to the nylon surface. The effect was

particularly pronounced for the capacity of reversible binding. Consequently, as with toxin-agarose columns, the toxin-linked nylon tubes initially trap irreversibly a considerable amount of receptor but with repetitive applications become increasingly efficient in reversible binding of acetylcholine receptor. This particular property of immobilized toxin is probably due to the low rate of spontaneous dissociation of receptor-toxin complexes (Fig. 1; Maelicke *et al.*, 1977b; Kang & Maelicke, 1980). With half-times of dissociation of the order of 100 h, any spontaneous dissociation of receptor bound to nylon-linked toxin is negligible within the time range of typical chromatography runs.

The binding capacities of the affinity tubes (capacities of trapping and reversible binding) depended only on the density of affinity ligands (binding sites) and not on the total concentration of nylon-linked protein. This is shown by the coupling experiments with toxin in the presence of a 25-fold excess of bovine serum albumin (Table 2). The 'low-density' tubes obtained in this way were most efficient in both trapping and reversible binding of receptor.

The above experiments provide a rationale for the chromatographic properties of receptor on both nylon-linked toxin and agarose-linked toxin. At high coupling densities of toxin, receptor can interact with two (or more) toxins at the affinity tube surface. These coupled equilibria considerably decrease the probability of receptor dissociation, thus causing an increased amount of receptor to remain attached even in the presence of large concentrations of competing small ligand. At lower densities of toxin such multi-site attachment of receptor is less likely and the capacity for reversible binding of receptor therefore increases. Continuing this line of arguments, irreversible trapping should then only occur with ligands having more than one binding site (receptor), whereas the reverse (toxin chromatography on receptor affinity tubes) should not show such complications. This was indeed observed (Fig. 1, Table 2).

Taken together, the experiments with receptor and toxin linked to nylon surfaces indicate that these affinity matrices behave in a predictable fashion and thus can be used for new applications. As an example we investigated the use of receptor-linked nylon tubes for trapping and chromatography of anti-(receptor immunoglobulins). From a 3 cm receptor affinity tube we first took 0.5 cm to determine with ^{125}I -labelled α -bungarotoxin the density of toxin-binding sites. The remaining tube (2.5 cm) was then perfused for 1 h with an antiserum from immunized rabbits and the remaining density of toxin-binding sites was determined. As expected the anti-(receptor immunoglobulins) of the serum competed with toxin for receptor binding in a

concentration-dependent fashion. A series of experiments of this kind showed that the procedure was applicable to the determination of antibody titres in various immunopreparations and sera. Owing to the high density of nylon-linked receptor the sensitivity of the assay matched or exceeded those of radioimmunoassays commonly employed with the receptor (Moshly-Rosen *et al.*, 1979; Martinez *et al.*, 1977; Gomez *et al.*, 1979; James *et al.*, 1980; Tzartos & Lindstrom, 1980). Similar results were obtained with nylon-linked toxin and anti-(toxin immunoglobulins) from serum of immunized rabbits.

Both anti-(toxin immunoglobulins) and anti-(receptor immunoglobulins) can be dissociated from the affinity tubes by short treatment with 0.1 M-acetic acid/0.1 M- NH_3 (Martinez *et al.*, 1977) or with 0.1 M-glycine/HCl, pH 2.8 (Merlie *et al.*, 1979). When eluted directly into 0.3 M-sodium phosphate, pH 7.5, the immunoglobulins largely retain their immunogenic properties, as was shown by titre determination using radioimmunoassay (Martinez *et al.*, 1977; James *et al.*, 1980).

When the pH of the perfusing solution was adjusted to around 7 immediately after dissociation of the immunoglobulins, nylon-linked toxin regained its ability to bind both antibodies and receptor in consecutive experiments. Nylon-linked receptor was considerably more sensitive to the pH changes required to dissociate bound antibodies. Recovery of toxin-binding properties after low-pH treatment typically varied between 60% and 90% and was very sensitive to both time of exposure to low pH and experimental procedure. Higher than usual concentration of non-ionic detergent, high ionic strength and the presence of low-molecular-weight ligands of the receptor all favoured recovery of biospecific properties after re-adjustment of pH. Although not yet completely satisfactory and reproducible, repetitive use of receptor-linked nylon tubes therefore seems feasible.

Conclusions

Bovine serum albumin, α -cobratoxin and purified acetylcholine receptor protein were covalently linked to the inner surface of nylon tubes to form a novel affinity matrix with the following advantageous properties. (i) High densities of coupled affinity ligand can be obtained. (ii) The biospecific properties of the affinity ligands are preserved during coupling. (iii) The affinity tubes can be re-used. (iv) The hydrophobic nylon surface appears to be of particular advantage when solubilized membrane proteins are used as affinity ligands. With these properties, receptor-linked nylon tubes can be used for titre determination and chromatography of antibody-containing fluids, including blood, ascites fluid and cell culture media.

Of the possible applications of this matrix, determination of the concentration of circulating antibodies and other proteins in body fluids appears most striking. This is because nylon tubes with high densities of coupled affinity ligand can serve as a concentrating device, thereby amplifying the sensitivity of such assays. Various therapeutic applications can also be envisaged. Other important applications are the purification of antisera and cell culture media and the trapping of cells with specific surface properties.

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