# LIGHT AND HEAVY FORMS OF THE ACETYLCHOLINE RECEPTOR FROM *TORPEDO MARMORATA* ELECTRIC ORGAN

## Morphological identification using reconstituted vesicles

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### 1. Introduction

The acetylcholine receptor (AChR) protein from *Torpedo* electric organ may exist under two molecular forms referred to as 9 S 'light' (L) or 13 S 'heavy' (H), with respective $M_r$-values of 250 000 and 500 000. These two forms have the same number of $\alpha$-toxin sites per mass of protein and give identical patterns of four ($\alpha\beta\gamma\delta$) polypeptide chains after polyacrylamide gel electrophoresis under reducing and denaturing conditions (see references in [1,2]). The H form can be converted into the L one in the presence of disulfide bond-reducing agents such as dithiothreitol or $\beta$-mercaptoethanol [3–5]. The data are consistent with the interpretation that the H form results from the association of two molecules of the L form via an intermolecular disulfide bridge between the $\delta$, 66 000 $M_r$, chains.

Ultrastructural studies of the purified AChR carried out in particular by electron microscopy after negative staining [6–8] and by neutron scattering [9] have led to the conclusion that the molecule is a prolate cylinder with dimensions ~11 × 8 nm. It is most frequently visualised end-on as a 'rosette' also characteristic of the molecule in its membrane-bound state [6,8,10–14]. Up till now, EM studies of the isolated L and H forms by negative staining have not revealed obvious differences.

Here, we have taken advantage of the reconstitution of the AChR into large single-walled lipid vesicles to reinvestigate this point. We observed a clear and quantitative difference between preparations containing the H and L forms of the AChR. The H form is identified with coplanar pairs or 'doublets' of the typical receptor rosettes present in preparations of the L form.

*Abbreviations:* ACh, acetylcholine; AChR, acetylcholine receptor; SDS, sodium dodecyl sulphate; EDTA, ethylenediaminetetraacetic acid; EGTA, ethyleneglycol-bis-($\beta$-aminoethyl ether) *N,N'*-tetraacetic acid; $\alpha$-Bgt, $\alpha$-bungarotoxin; DTT, dithiotreitol; H and L, the heavy (500 000 $M_r$) and light (250 000 $M_r$) forms of solubilized AChR, respectively

### 2. Materials and methods

#### 2.1. *Purification and reconstitution procedures*

AChR-rich membranes were purified from *Torpedo marmorata* electric organ as in [3], solubilized, and the AChR reincorporated into large vesicles of asolectin as in [15]. Briefly, the membranes are first solubilized in Na-cholate asolectin solution, centrifuged, dialysed for 1–3 h at room temperature using a Spectra Por/2 membrane (Bio Rad) and rapidly filtered on a column of Sephadex G-25; the reconstitution procedure was performed throughout in Na-buffer I (100 mM NaCl, 10 mM phosphate buffer, 0.02% $NaN_3$, pH 7.4).

Alternatively, purification and reconstitution were carried out under conditions designed to limit proteolysis and preserve a high proportion of H form: protease inhibitors (3 mM EDTA, 1 mM EGTA, 5 units/ml aprotinin) [16] were present throughout the purification procedure, with, in addition, 5 mM *N*-ethylmaleimide during the two steps of homogenization [4].

For the reconstitution procedure, the solubilization medium was supplemented with 5 mM *N*-ethylmaleimide and 5 mM EDTA and Na-buffer I with 5 mM EDTA. These reconstituted preparations contained the typical $\alpha\beta\gamma\delta$ chains of the AChR (see [1,2]). All the preparations used in this study responded to ACh by an increase in $^{22}Na^{+}$ permeability (see [15]).

2.2 *Quantification of the L and H forms*

Nearly complete reduction of the AChR to the L form was achieved by incubating the reconstituted vesicles overnight at 4°C with 5 mM dithiothreitol [4]: before $\alpha$-toxin labelling or EM observation, the excess dithiotreitol and free sulphydryls were blocked with 10 mM *N*-ethylmaleimide. This treatment did not interfere with $\alpha$-toxin binding. In some cases, partial reduction to the L form was achieved by 1–2 h incubation with 0.5% $\beta$-mercaptoethanol.

For $^{125}$I-labelled $\alpha$-bungarotoxin ($^{125}$I-$\alpha$-Bgt) labelling, samples of the reduced and unreduced reconstituted preparations were incubated 7–12 h at 4°C with $10^{-8}$ M $^{125}$I-$\alpha$-Bgt (a 2–4-fold excess over the receptor sites). A 100-fold excess of cold $\alpha$-Bgt was then added and the samples solubilized with 3–5% sodium cholate. Partial labelling (with a 2–4-fold excess of receptor sites) without addition of cold $\alpha$-Bgt was used on some occasions and gave within 1–2% the same H/L ratios as total labelling. The solubilized samples (100–200 $\mu$l) were layered on 11 ml 5–20% sucrose gradients containing 1% sodium cholate and centrifuged for 12–15 h at 39 000 rev./min at 4°C in a Beckman SW41 rotor. About 30 fractions were collected from the bottom of the tubes and counted for $^{125}$I in a Multigamma LKB counter. The radioactivity remaining associated with the centrifugation tubes after collection was also measured and is given as fraction 0 in fig.2. The data are expressed

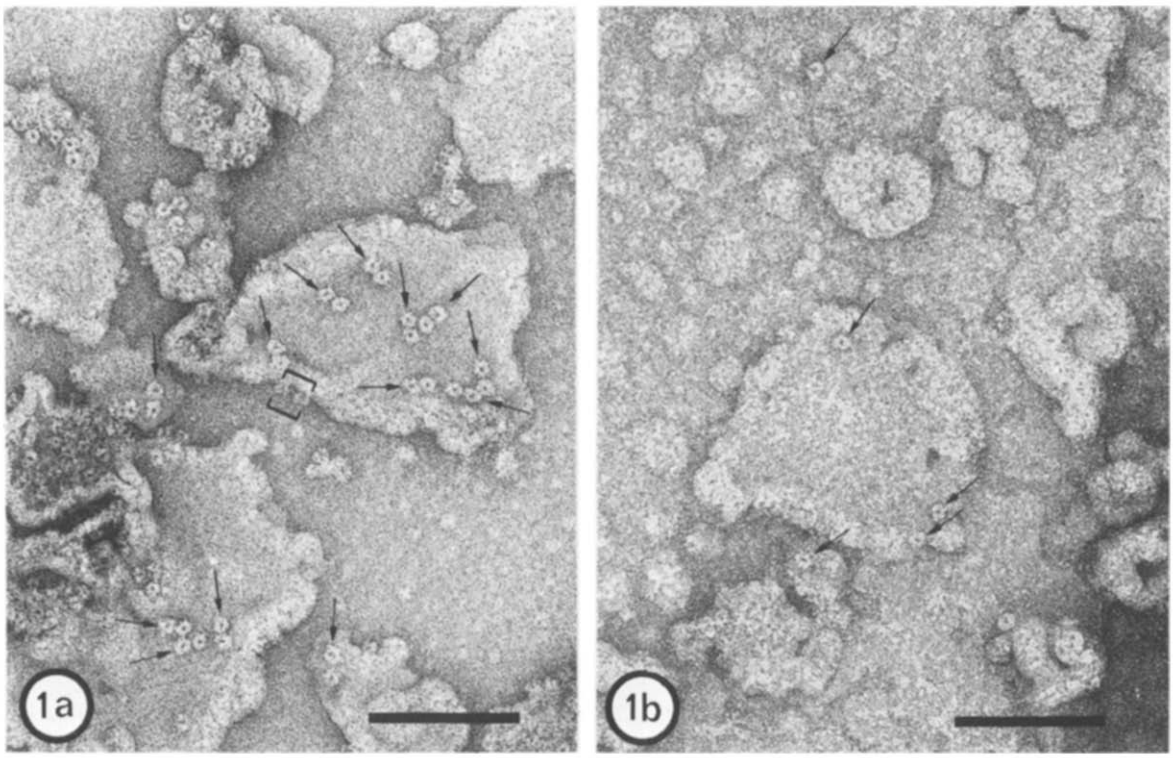


Fig.1. The heavy (H) and light (L) forms of the AChR observed by electron microscopy on negatively stained preparation of reconstituted vesicles. a) H-form: The majority of the rosettes appear as paired structures or doublets (arrows). At this relatively high protein-to-lipid ratio, the rosettes tend to further associate into small aggregates or chains. Note the side view of one doublet (brackets) × 200 000. Bar = 100 nm. b) L-form (at a lower protein-to-lipid ratio; same preparation as fig.2b): Most of the rosettes now appear isolated (arrows) × 200 000. Bar = 100 nm.

as the ratio (area of the peak of H-form)/(sum of the areas of the peaks of H and L forms). Heavier forms (see [5]) never represented >10–15% of the total bound $^{125}$I-α-Bgt.

### 2.3. *Electron microscopy*

For negative staining, the samples were spread onto a thin carbon film supported by a perforated thin plastic–carbon film on a copper grid [17]. Uranyl formate (1% aqueous solution) was prepared according to [18]. Micrographs were taken in a Philips model 400 electron microscope operating at 80 kV and fitted with 50 μm objective aperture. For quantitative analysis, instrument magnifications of 20 000 were used.

Automated calculations of center-to-center distance between AChR rosettes (and group pattern determinations) were made using the Phymetron image analyser coupled to a Data General Nova 3D computer at the Unité Calcul of the Institut Pasteur. The central electron-dense area of each rosette was manually selected on 300 000-times enlarged prints. Quantification of isolated rosettes and doublets were made on 100 000-times enlarged prints using an optical aid. Several hundred rosettes were counted for each experiment. When clusters of rosettes larger than doublets represented more than a few % of the population of rosettes present in the sample, the preparation was not used for quantification (e.g., fig.1a). Such associations were mainly non-covalent as judged from sedimentation analysis of solubilized samples.

### 2.4. *Chemicals*

$^{125}$I-Labelled α-bungarotoxin was purchased from New England Nuclear (Boston, MA), sodium cholate from Merck, asolectin (batch no. 98036) from Associated Concentrates (Woodside, NY), aprotinin from Sigma.

## 3. Results and discussion

In fig.1 are presented electron micrographs of two negatively-stained preparations of AChR reconstituted into asolectin vesicles (see section 2). In fig.1a, the AChR was mainly in its heavy (H) form and in fig.1b in its light (L) form. The preparation containing the L form displayed randomly-distributed AChR rosettes while close associations of two rosettes into 'AChR *doublets*' were frequently encountered in preparations rich in H form.

In order to establish the existence of a correlation between the occurence of doublets and that of the H form, reconstituted vesicles were prepared under conditions yielding different H/L ratios (fig.2; see section 2). Electron microscopic observations were facilitated by using large (~100 nm) vesicles (see [15]) and low surface densities of rosettes (fig.3a). Computer-aided analysis of the distribution of rosettes in the plane of the lipid bilayer demonstrated the higher than stochastic frequency of center-to-center distance of 9 ± 2 nm (fig.3b); this latter value corresponds to the mean

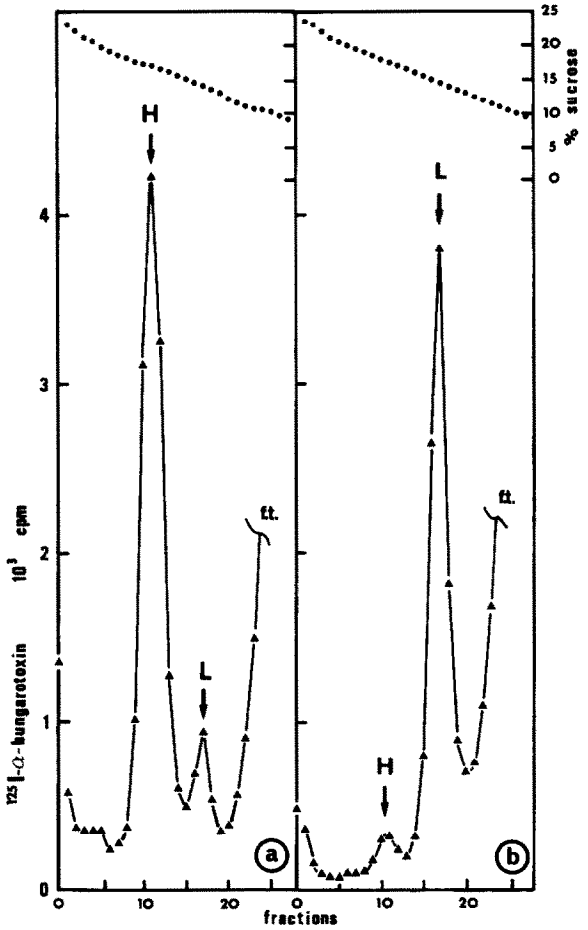


Fig.2. Analysis by sucrose gradient sedimentation velocity of the ratio of heavy to light forms of a preparation of reconstituted vesicles before and after reduction by dithiothreitol. Purification of the AChR-rich membranes, solubilisation of the AChR and reconstitution were conducted in the presence of *N*-ethylmaleimide and antiproteolytic agents; labelling with $^{125}$I-α-Bgt and sedimentation analysis were performed (a) without and (b) after reduction with dithiothreitol (see section 2); f.t., unbound $^{125}$I-α-Bgt.

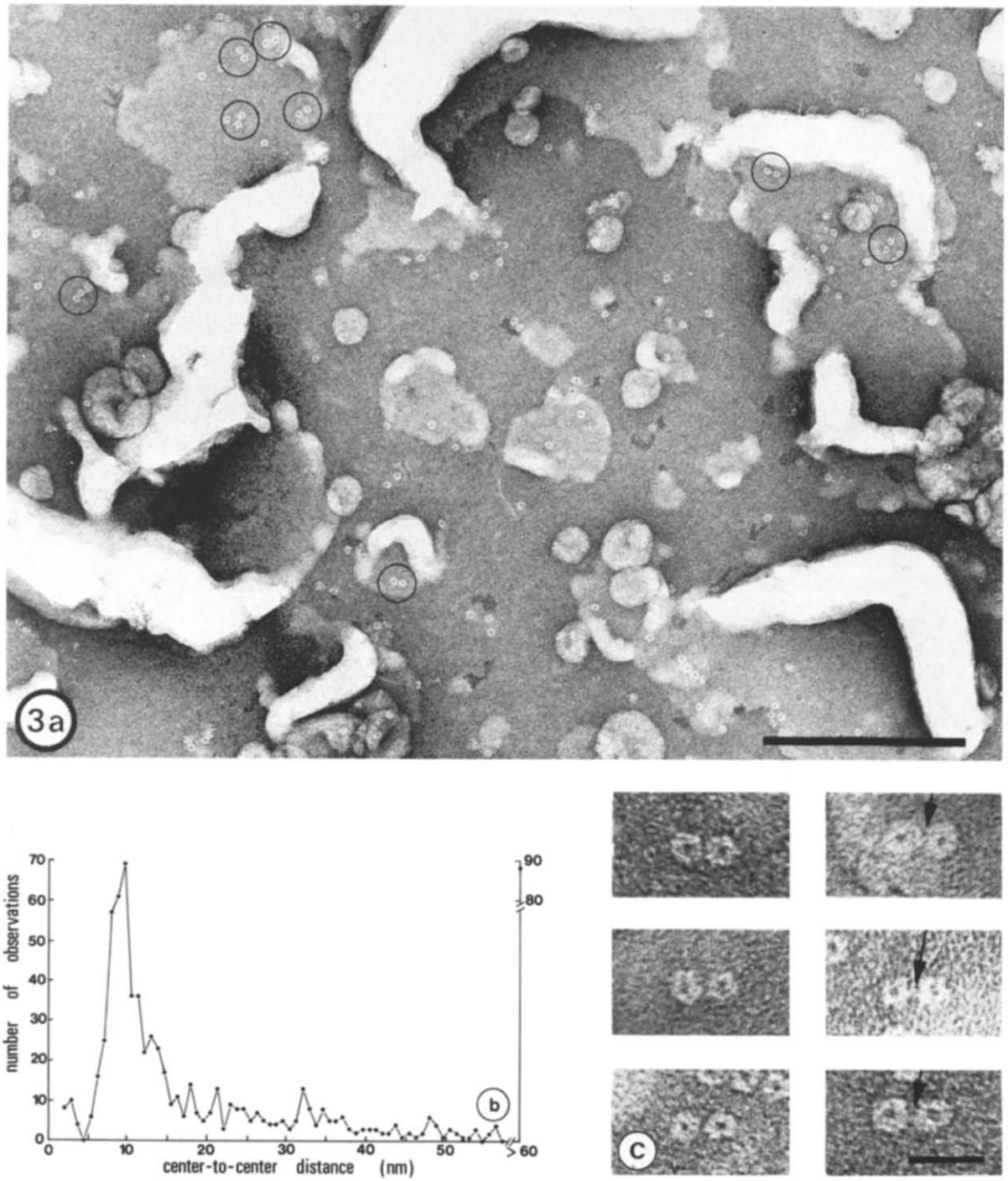


Fig.3. Quantitative analysis of the fraction of AChR 'doublets' in a reconstituted preparation. (a) Low magnification electron micrograph showing several large lipid vesicles displaying scattered rosettes; the identification of the AChR doublets is unambiguous (circled areas). Some rosettes are also present outside the vesicles. × 160 000. Bar = 200 nm. (b) Distribution of the centre-to-centre distance of individual ACh-receptor rosettes to their nearest neighbour (preparation shown in (a); 740 measurements). A sharp peak at 9 ± 2 nm corresponds to the mean centre-to-centre distance of the rosettes within doublets. (c) Gallery of selected negatively stained doublets. In some doublets (arrows) the two rosettes appear bridged by a short stem. × 600 000. Bar = 20 nm.

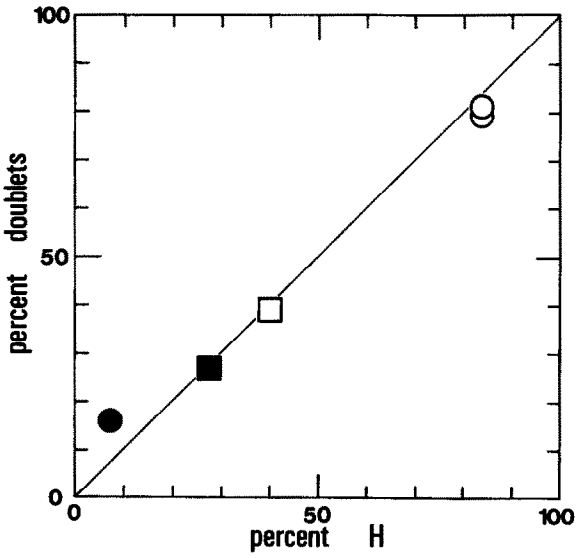


Fig.4. Correlation between the fraction of AChR doublets and that of the heavy form of the AChR in reconstituted vesicles. Five different preparations were analysed for their amount of doublets on electron micrographs (% of rosettes in doublets relative to the sum of isolated plus paired rosettes) and in H form (% of α-toxin binding sites under the H form relative to H + L. The AChR was purified, solubilized and reconstituted into asolectin vesicles either in the absence of protective agents (□) and then partially reduced with β-mercaptoethanol (■), or in the presence of *N*-ethylmaleimide and antiproteolytic agents (○; two preparations) and then extensively reduced with dithiotreitol (●). See section 2.

centre-to-centre distances between rosettes in doublets (fig.3c). When the ratio of paired to isolated rosettes was compared to that of H to L form as determined by sucrose gradient sedimentation velocity analysis, a one-to-one relationship was found, indicating that doublets and isolated rosettes represent, respectively, the morphological counterparts of the heavy and light forms of the cholinergic receptor (fig.4).

The functional significance of the H and L forms of the AChR protein is not yet clear. No obvious differences in ligand binding properties, allosteric interactions, conformational transitions or ion-transport properties have been detected between H and L [1,2].

On the other hand, in replicas of the postsynaptic membrane obtained by freeze-etching or freeze-fracturing, the rosettes exposed at the external surface of the membrane (ES) often form extensive double rows interdigitated with particle-free areas [8,11,12]. The distance between the centres of rosettes in adjacent rows is ~9–10 nm, a value similar to that found in the doublets visualized by negative staining (fig.3b). These morphological data derived from independent techniques suggest that the rows result from the alignment of AChR H form doublets.

Whether the presence of double rows reflects interactions between receptor molecules or with other stabilizing membrane components remains to be established. In particular, recent data indicate that a 43 000 $M_r$ polypeptide, which seems to be associated as an extrinsic component with the cytoplasmic surface of the postsynaptic membrane, affects the thermal stability of the receptor protein [19] and its rotational [20,21] and lateral [14] mobility. In addition, it has been suggested that both the basal lamina ([22,23]; see references in [24]) and the phosphorylation of the receptor protein itself (see references in [25]) may contribute to the genesis and maturation of the postsynaptic domain.

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