ELECTROPHORUS ACETYLCHOLINESTERASE: A GLYCOPROTEIN; MOLECULAR WEIGHT OF ITS SUBUNITS

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1. Introduction

The different forms of acetylcholinesterase (Ach E, EC 3.1.1.7) from electric eel (*Electrophorus electricus*) have already been described, and characterized by their sedimentation coefficients in a sucrose gradient [1, 2]. The physico–chemical characteristics of the three 'native' forms, A, C, D, with sedimentation coefficients 9.2 S, 14.2 S, and 18.4 S, respectively, are only compatible with asymmetric structures. This hypothesis has now been confirmed by electron microscopy [3]. Another active, globular form G(11.8 S) is obtained by tryptic, or other proteolytic treatment of A, C, and D; it appears as a tetramer in the micrographs.

We have used the same preparations, that were studied by electron micrography, to investigate their subunit composition by SDS acrylamide gel electrophoresis. These results, coupled with those from electron microscopy, lead to specific proposals about the molecular structure of the various forms.

2. Materials and methods

The acetylcholinesterase was extracted from electric organs by homogenisation in a buffer of high ionic strength (1 M NaCl, 0.05 M $MgCl_2$, 0.01 M Tris, pH 7.0), and purified as recently described [3]. The contaminants of the forms A, C, D, were estimated from the activity/protein profiles of sucrose gradients as less than 20%.

Routine electrophoreses were performed in 5 or 10% polyacrylamide gels containing 0.1% SDS, with a buffer of 0.1 M phosphate, pH 7.2, 0.1% SDS. Molecular weight calibration was effected with urease, bovine serum albumin, catalase, ovalbumin, aldolase and pepsin. Samples were incubated at 60°C for 5 min in 2% SDS or 2% 2-mercaptoethanol, and layered directly on to the gel using 6 M urea or 20% sucrose; the tracking dye was Bromophenol blue. Gels were stained with Coomassie brilliant blue or Amido black for proteins, periodate Schiff stain for carbohydrates and Oil Red for lipids. Dosage of sialic acid, both of the enzymes and extracted gel slices, was by a modified Warren procedure [4].

3. Results

Samples of all molecular forms, incubated with 2-mercaptoethanol, and run on 10% SDS gel show two major subunits (fig. 1). These have estimated molecular weights of 91 000 ± 5 000 and 62 000 ± 4 000 for all forms (the molecular weight calibration of the gels is shown in fig. 2). Bands of higher molecular weight are also present; in particular we notice an important band with an estimated molecular weight greater than 180 000, for all forms except G; no great precision can be given for the molecular weight in this region of the gel. The molecular weight determinations are quite reproducible, as shown in the histograms in fig. 3. All the main protein components also show a positive stain for carbohydrates. The relative intensity of

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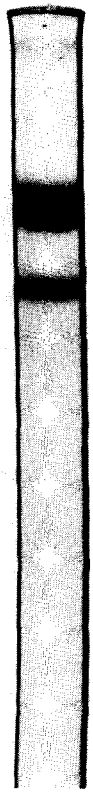

Fig. 1. The subunits of 'native' acetylcholinesterase. 10% polyacrylamide, 1% SDS gel electrophoresis of acetylcholinesterase (form D).

the two main bands is variable; the heavier band (h:91 000 ± 5 000) stains more intensely than the lighter one (1:62 000 ± 4 000). Similar staining ratios are observed for both Coomassie blue and Amido black protein stains.

The results in 5% acrylamide SDS gels are also given as histograms in fig. 3. The molecular weight of the smallest band [1] is estimated as 62 000 ± 3 000 for all forms, in agreement with the results on 10% gels. However, the heavier band (h) has an estimated molecular weight of 84 000 ± 5 000 for the forms A and D, but a value of 74 000 ± 4 000 for the forms C and G. Again, all the main components give a positive stain for the carbohydrates. The gels also show components running at twice the molecular weight of the subunits; the intensity of these bands is variable, but it can be diminished by incubation of samples with iodoacetamide after subunit dissociation. This evidence suggests that the bands are dimers. However, the band of molecular weight 170 000 ± 20 000 observed for A, C, and D is unaffected by treatment with iodoacetamide.

From 5% SDS gels of the form G, without 2-mercaptoethanol, we estimate the mass of the additional dimer bands as: 120 000 ± 15 000, 150 000 ± 15 000, and 175 000 ± 15 000; they are of comparable intensity. These bands probably represent dimers of the heavy and light subunits. The presence of the three types of dimers: h^2, hl, and l^2 after dissociation of the tetramer, would suggest a structure with an equal number of heavy and light subunits.

The calibration curves of 5% and 10% SDS acrylamide gels are shown in fig. 2. For the 10% gels we have a linear relationship between molecular weight and mobility. The calibration of the 5% gels is less satisfactory and two slopes can be drawn according to the prominence given to the dimeric protein markers. The slope which considers only the mobilities of the monomer marker proteins gives molecular weights increased by some 3 000–6 000 in the important 75 000–100 000 molecular weight range, compared to the calibration which gives equal weighting to the monomer and dimer protein markers. The absence fo suitable monomer markers in this 75 000–100 000 range is unfortunate. The observed variation in mobility with gel porosity may be attributed to the carbohydrate content of this subunit, which stains strongly for carbohydrates, since the migration of glycoproteins has been shown as anomalous [5].

For species C, D, and G, samples of 250 μg, 180 μg, and 300 μg, respectively, were incubated with SDS and 2-mercaptoethanol and run on 5% acrylamide, 0.1% SDS gels (analytical gels were run simultaneously). The gels were sectioned, extracted, and assayed for sialic acid [4, 6] (fig. 4); the position of the sialic acid corresponded well with the principal protein staining bands. The form A also stains positively for carbohydrates on acrylamide gels. The scarcity of the active form A did not permit a sialic acid determination, but we may deduce that this form also contains sialic acid.

The negatively charged sialic acid residues must contribute to the low isoelectric point of acetylcholinesterase, pH 5.35 for an autolysed preparation [8]

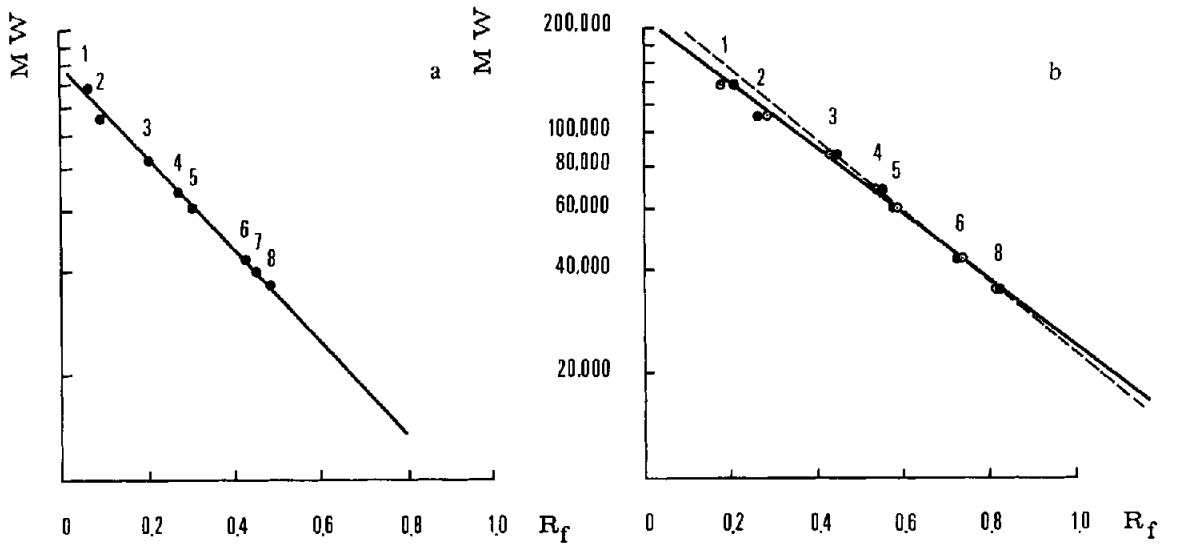

Fig. 2. Calibration of SDS polyacrylamide gels: a) 10% gels; b) 5% gels, full circles, open circles replicate calibrations. The full line considers all points, the broken line considers only the monomer protein markers. R_f is determined as the ratio between the distance of migration of the protein and the tracer dye (Bromophenol blue). The marker proteins are: 1) bovine serum albumin dimers; 2) catalase dimers; 3) urease; 4) bovine serum albumin; 5) catalase; 6) ovalbumin; 7) muscle aldolase; 8) pepsin.

and pH 5.0 for the enzymes extracted with saline solutions [9].

4. Discussion

The subunits, structure and molecular weights of acetylcholinesterase from electric eel have previously been studied by centrifugation in guanidine [10] and by SDS gel electrophoresis [11]. These two methods have given very different results; the former suggests that the enzyme is a tetramer with two different subunits of molecular weight 64 000. From gel electrophoresis experiments, Dudai and Silman [11] reported two subunits of molecular weight 88 000–100 000 and 64 000 for the trypsin treated enzymes, as well as for the total of tissue extract acetylcholinesterase.

We also find two subunits of molecular weight less than 100 000 which appear as common components of all acetylcholinesterase forms, whether native or obtained by proteolytic digestion.

The molecular weight of the lighter subunit is 62 000 ± 4 000, as estimated from electrophoresis in 10% or 5% SDS polyacrylamide gels. We estimated the molecular weight of the heavier subunit as 91 000 ± 5 000 from 10% gels, for all the molecular forms of acetylcholinesterase studied. However, the apparent molecular weight is lower in 5% SDS polyacrylamide gels, and different for A and D on the one hand, and C and G on the other hand: these differences, although they are significant, may be related to the preparation used if they result from a partial degradative process. It is tempting to assume that the lower apparent molecular weights observed in the higher porosity gels is due to the carbohydrate content of the subunit. However, it must be noted that this effect is not observed for the lighter subunit, and that an opposite effect of porosity has been demonstrated for a number of glycoproteins [12].

We have seen that dissociation of the tetramer G apparently produces all kinds of possible dimer bands (h^2, hl, and l^2) and we have mentioned that this would lead us to conclude that the tetramer is built out of two subunits of each kind; it would therefore appear that both kinds of subunits (heavy and light) occur in equal molecular numbers in acetylcholinesterase (since all native forms can be converted into G by tryptic digestion). However, it is striking that with three staining procedures (Coomassie blue and Amido black for proteins, Schiff reagent for

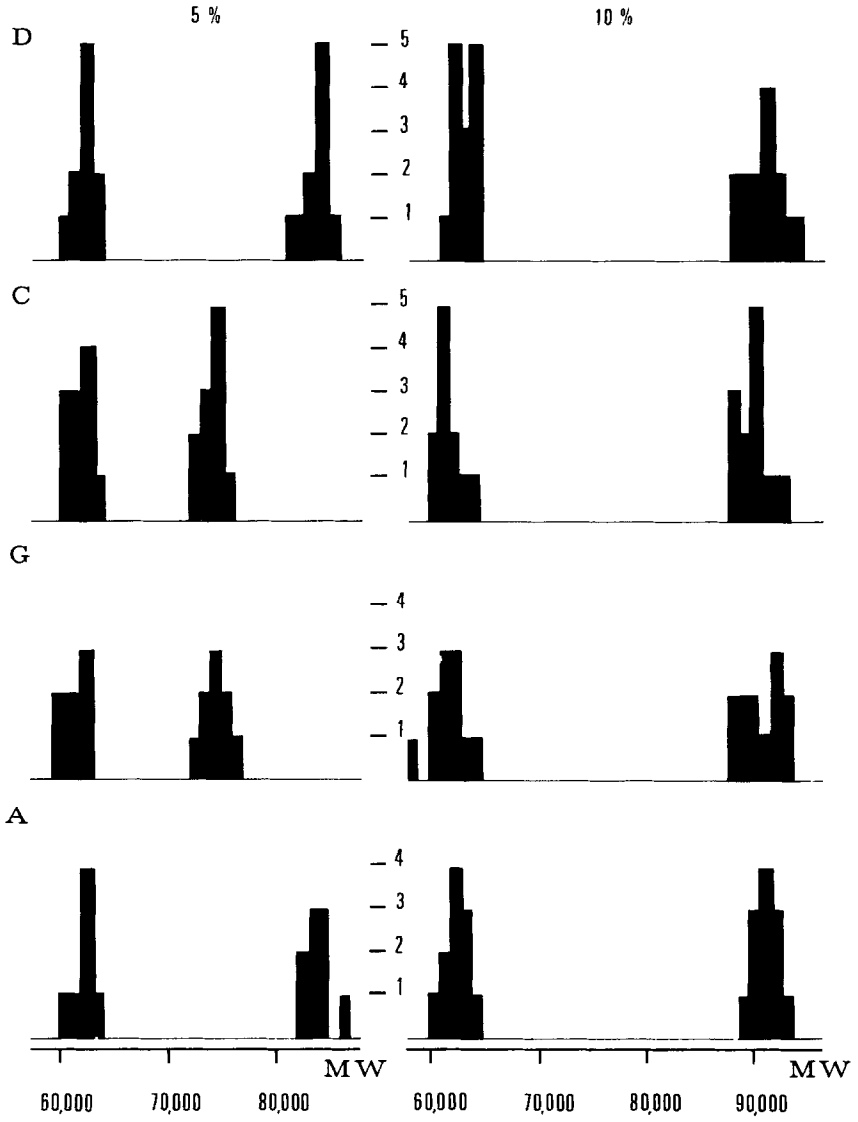

Fig. 3. Apparent molecular weights of the acetylcholinesterase subunits. Histograms showing the molecular weights of the subunits below 100 000. Results for 5% polyacrylamide gels are on the left, results for 10% polyacrylamide gels on the right. Reading down the diagram we have the result for the molecular forms D, C, G, and A.

saccharides), the heavier subunit seems from two to four times more abundant than the lighter one. It is unlikely that this large difference in intensity can be attributed simply to a greater diffusion of this more mobile subunit (although it seems relatively more important in the 10% gels, where it is less diffuse, than in the 5% gels). Although the general occurrence of both subunits argues for their existence prior to extraction and purification of acetylcholinesterase, the lighter of these two subunits may result from a secondary splitting of the protein chains, found *in vivo*. This same phenomenon was also noted by Dudai and Silman [11], who showed that the lighter subunit bound less radioactive DFP than the heavier one.

All gels contain several high molecular weight bands. The diminution of intensity of two of these

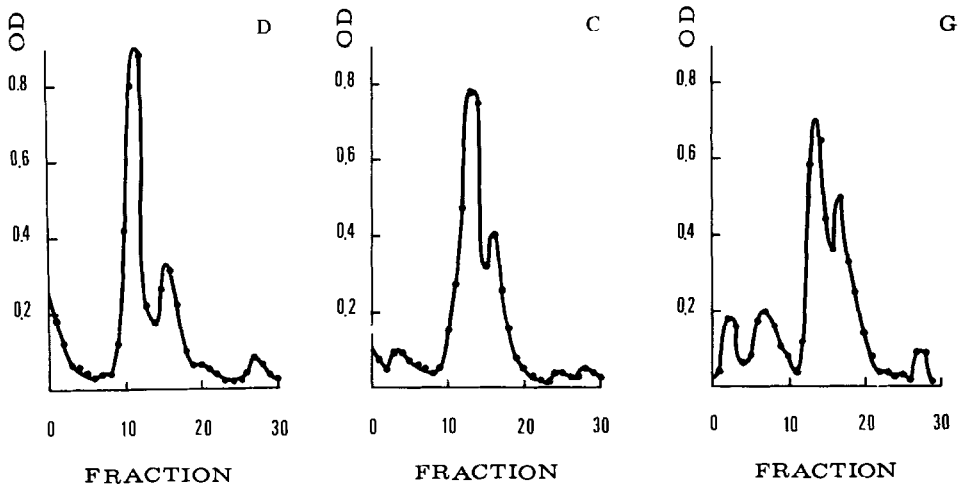

Fig. 4. Sialic acid in the acetylcholinesterase subunits. 5% polyacrylamide, 0.1% gels were sliced after electrophoresis (2 cm thick slices). The content of sialic acid in the slices is given in the graphs for a) D, b) C, c) G. The peaks correspond to the protein bands.

bands, at double the molecular weight of the above mentioned subunits, when treated with iodoacetamide, suggest that they are dimers, and that the subunits are therefore linked by disulphide bridges, which would be important in maintaining the structure of the enzymatic 'head'.

We have also observed that the native forms A, C, and D have a persistent high molecular weight band, greater than 170 000, which seems very large for a single subunit. Nevertheless it is possible that this subunit may be associated with the asymmetric character of the native forms [3,7]. The structure of these forms has recently been illustrated by electron micrographs [3]; the globular form G is shown as a tetramer and the forms A, C, and D are shown as a cluster of subunits (structure 'en grappe') stemmed by a rigid 'tail'. A simple model was discussed for these molecular structures. If we represent the tail as 'q' and accept for convenience the hypothesis that the heavy subunit 'h' and the light subunit 'l' are of equal distribution, the structures described would be:

h_2l_2	h_2l_2q	h_4l_4q	h_6l_6q
G	A	C	D

It must be emphasized that, although no gel, with or without SDS, stains for lipids, all the main components show a positive stain for both proteins and carbohydrates. This may be particularly significant since many surface membrane proteins have been proved to be glycoproteins: the glycoprotein nature of acetylcholinesterase would correlate well with its anticipated position at the surface of the synaptic membrane. Since neuraminidase does not modify the activity of the enzyme, the charged sialic acid is not critically involved in maintaining the ionic environment of the active sites, although it is involved in antigenic recognition sites [13] and low ionic strength aggregation [14].

The anomalous behaviour of some glycoproteins on SDS gels does not allow us to present a definitive model for the molecular forms of acetylcholinesterase and their interconversion. The determination of the molecular weights of the various species is in progress in this laboratory.

Acknowledgements

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