MOLECULAR WEIGHT AND QUATERNARY STRUCTURE OF THE CHOLINERGIC RECEPTOR PROTEIN EXTRACTED BY DETERGENTS FROM *ELECTROPHORUS ELECTRICUS* ELECTRIC TISSUE

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1 . Introduction

The cholinergic receptor protein can now be obtained from *Electrophorus electricus* electric organ in a highly purified state and in reasonable quantities [1–3]. It therefore becomes accessible to thorough biochemical investigation. The hydrodynamic properties of the receptor protein in detergent solution are quite unusual [4–6]: while gel filtration experiments indicate a Stokes radius of 70 Å close to that of β-galactosidase (mol.wt. 550 000), sedimentation in sucrose gradients in the presence of Triton X-100 shows an apparent sedimentation constant of 9.5 S, far lower than the 16 S found for β-galactosidase. Such a particular behaviour, which has been assigned to a significant binding of detergent to the solubilized protein [7] renders the determination of mol.wt. by hydrodynamic methods particularly difficult. This is why we turned to an entirely different technique: polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS) after complete or partial crosslinking of the receptor molecule by suberimidate [8]. In this paper we present estimates of the mol.wt. of the receptor protein given by this method and further show that this protein results from the assembly of several (possibly five) subunits belonging to two different molecular weight classes.

2. Materials and methods

2.1. *Preparation of the purified receptor protein*

The cholinergic receptor protein was purified from the electric organ of *Electrophorus electricus* as described wlsewhere [1, 10]. Its specific activity was 5400 nmoles of *Naja nigricollis* ^{3}H-α-toxin-[9] binding site per gram protein, as determined by the Millipore assay for receptor activity and the method of Lowry et al. [1] for assay of protein concentration.

2.2. *Cross-linking with suberimidate*

Synthesis of suberimidate, and cross-linking of the receptor protein as well as that of the calibration proteins was performed as described by Davies and Stark [8]. Tris buffer which interferes with the reaction was removed by overnight dialysis against 1000 vol of 0.2 M triethanolamine, pH 8.5, 1% Triton X-100. Subsequently, the protein solution was concentrated to about 0.8 mg/ml with dry Sephadex G100. For complete cross-linking suberimidate was added to a final concentration of 2 mg/ml. For partial cross-linking, the suberimidate concentration was varied between 0.1 and 1.0 mg/ml. The best subunit pattern of the receptor was obtained at 0.2 mg/ml suberimidate. Incubation time was always 3 hr at room temperature.

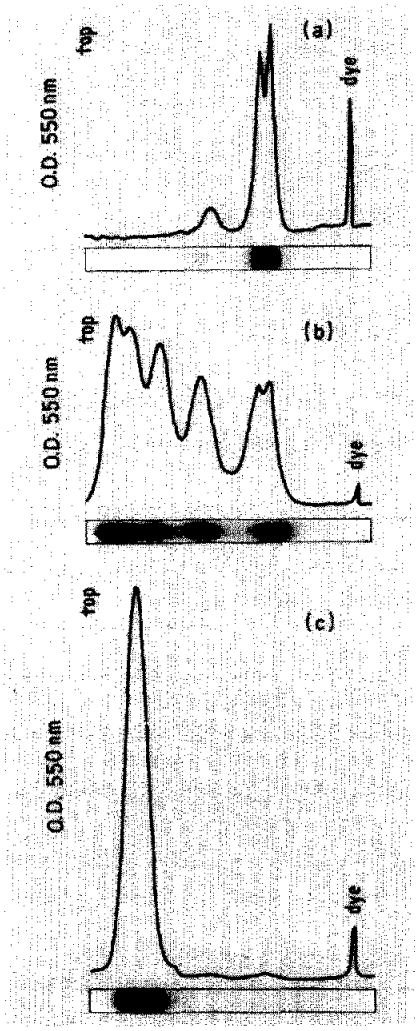

Fig. 1.Ha) SDS-polyacrylamide gel electrophoresis of purified cholinergic receptor protein. For experimental details see section 2.3; b) SDS-polyacrylamide gel electrophoresis of receptor protein partially cross-linked with suberimidate (0.2 mg/ml, for experimental details see sections 2.2 and 2.3); c) SDS-polyacrylamide gel electrophoresis of receptor protein completely cross-linked with suberimidate (2 mg/ml, for experimental details see section 2.2 and 2.3).

2.3. *Polyacrylamide gel electrophoresis in sodium dodecyl sulfate*

Electrophoresis was performed by a method similar to that described by Davies and Stark [8]. SDS and β-mercaptoethanol were added to the protein samples at final concentrations of 1%. After heating for 1 min in a boiling-water bath, 50–100 μl of the mixture,

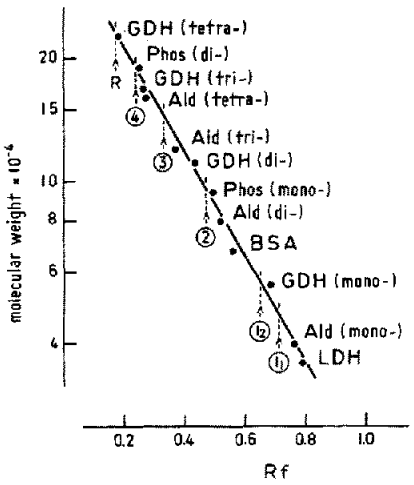

Fig. 2. Molecular weight determination of the receptor protein. Plot of the electrophoretic mobility of various calibration proteins versus molecular weight. For experimental details see sections 2.2 and 2.3. 1_1 and 1_2, 2, 3 and 4 refers respectively to the smallest subunits, dimer, trimer and tetramer. GDH: glutamate dehydrogenase (beef liver), Phos: phosphorylase b (rabbit muscle), Ald: Aldolase (rabbit muscle), BSA: bovine serum albumin, LDH: lactic dehydrogenase (rabbit muscle).

containing about 50 μg of protein, were applied to a 5% polyacrylamide gel containing 0.135% methylene bisacrylamide. Electrophoresis buffer was 0.1 M borate, 0.1 M acetate, 0.1% SDS, pH 8.5. Electrophoresis was done at 7mA per tube for about 1.5 hr.

The gels were stained by incubation for 6 hr with 1% Coomassie blue in 25% isopropanol, 10% acetic acid, 65% water. Destaining was accomplished by overnight shaking of the gels in 30% methanol, 10% acetic acid, 60% water at 37°C in the presence of a small amount of ion-exchange resin. The gels were scanned at 550 nM with Beckman Acta III recording spectrophotometer.

3. Results

The purified receptor gives a single and symmetrical band of protein by gel electrophoresis in the presence of non-denaturing detergents Sodium cholate or Emulphogen and both at pH 7.5 and 9.0. Under these conditions the ability to bind *N. nigricollis* ^{3}H-α-toxin follows almost exactly the protein profile [10].

Disc gel electrophoresis in the presence of SDS of the receptor protein solubilized by Triton X-100 gives two bands which stain for proteins (fig. 1.a). These bands migrate more slowly than the aldolase monomer but more quickly than the glutamate dehydrogenase monomer. The mol.wt. determined by interpolation (fig. 2) are 45 000 ± 3 000 and 54 000 ± 4 000. Scan of the gel obtained with the receptor prepared in Triton-X-100 indicates that the 45 000 mol.wt. species tends to be more abundant than the 54 000 one, possibly by a ratio of 3 to 2. A minor band is visible in the range of 90 000 and seems more important when the final step in the preparation of the receptor protein involves a sucrose gradient centrifugation in the presence of cholate rather than in the presence of Triton X-100. In this last case, the ratio of the 54 000 to 45 000 species appears modified.

Extensive cross-linking with 2 mg/ml suberimidate of the native protein prepared in Triton X-100 gives a single band (fig. 1.c). Its mobility is close to that of glutamate dehydrogenase tetramer (224 000 mol.wt.) and significantly lower than that of glutamate dehydrogenase pentamer (280 000 mol.wt) also obtained after crosslinking with suberimidate. From the calibration curve of fig. 2 we estimate the mol.wt. of the cross-linked receptor protein as 230 000 ± 15 000. The same result is obtained with gels containing 3.5% and 7.5% acrylamide.

Partial cross-linking of the receptor protein with 0.2 mg/ml suberimidate leads, as expected from a protein containing multiple subunits [8], to a gel pattern with several bands after electrophoresis in SDS. Repeatedly we found 6 bands (figs. 1.b,3). One of them, the slowest one, corresponds to the undissociated molecule. The two fastest represent the fully dissociated subunits (45 000 and 54 000 mol.wt.). The mol.wt. of the remaining three bands are, by interpolation, 190 000 ± 14 000, 145 000 ± 10 000, 95 000 ± 7 000 which roughly correspond to 4, 3 and 2 times the average mol.wt. of the smallest subunits (figs. 2, 3).

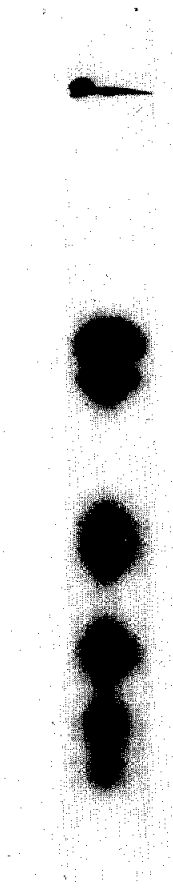

Fig. 3. SDS-polyacrylamide gel electrophoresis of the receptor protein after partial cross-linking by suberimidate. This gel clearly shows 6 distinct bands.

4. Discussion

The method of Davies and Stark [8] gives for the mol.wt. of the protein assembly obtained by detergent extraction, 230 000, a value significantly smaller than the apparent mol.wt. inferred from hydrodynamic measurements [7]: Approx. 320 000. The presence of a carbohydrate residue attached to the receptor protein as evidenced by the ability to bind plant lectins [10] does not seem to explain this difference; the same mol.wt. values are obtained by electrophoresis at different concentrations of polyacrylamide, and, in any case, a significant contribution of a carbohydrate moiety would lead to an overestimation of the mol.wt. As already discussed [7], another interpretation of the observed difference is that in solution of *non-denaturing* detergent an extensive binding of the detergent modifies the hydrodynamic properties of the protein moiety and leads to an overestimation of the mol.wt. On the other hand it is known with

some hydrophobic membrane proteins, cytochrome b_5 in particular [11], that SDS-gel electrophoresis gives consistent underestimation of mol.wt. of about 20% when water soluble proteins are used as standards. If the same correcting factor holds for the cholinergic receptor protein, then the exact mol.wt. should be close to 275 000.

The smallest subunits seen in the presence of SDS have mol.wt. (45 000 and 54 000) in the range of those already found by various authors with crude extracts [4, 5, 12] or purified preparations of receptor protein from *Electrophorus* [2] or *Torpedo* [13]. The nature of these two bands is not clear. It is possible that the low mol.wt. component represents a degradation product of the high mol.wt. one caused by proteolysis or loss of a carbohydrate moiety. Another alternative is that the two polypeptide chains are different and possess different functions, e.g. one would carry the cholinergic receptor site, the other would be more directly involved in ion translocation [14]. In this respect, one might mention that the values already reported for the mol.wt. of the chain labelled by ^{3}H-α-toxin [4, 5] or affinity reagents [12] lie between 40 000 and 45 000. In addition, even in the purest preparations, the molecular weight per ^{3}H-α-toxin or [^{3}H]decamethonium-binding site always seems much larger (between 100 000 and 150 000 1–3, 10, 13) than that of the dissociated subunits (45 000 or 54 000).

The 4 + 2 bands observed on SDS-gels after partial cross-linking of the native protein can be interpreted on the basis of a multiple subunits structure of the receptor protein obtained by detergent extraction. Five subunits would be associated together, but we do not know the exact stoichiometry of the 45 000 to 54 000 mol.wt. chains in this assembly; the rather broad bands observed after partial crosslinking prevent such an analysis. On the other hand, the observed broadening might result from a microheterogeneity caused by diverse associations of the two kinds of subunits.

If we consider seriously the observation that with fully dissociated molecules the smallest chain seems more abundant than the larger one, then a plausible but *still entirely hypothetical* structure is: 3 × 45 000 mol.wt. chains carrying the receptor sites + 2 × 54 000 mol. wt. chains involved in ion translocation. A different distribution of the two classes of subunits within the thickness of the membrane: those carrying the receptor site being exposed to the external surface, the others spanning the membrane, might explain such an uncommon and poorly symmetrical quaternary structure.

Finally, it is worth emphasizing that we still do not know what part of the molecular edifice involved in the control of ion translocation by cholinergic agonist is extracted by the detergent. Some critical structures might have been separated from the subunit carrying the receptor site, alternatively extraneous components might associate strongly with this subunit under our conditions of membrane solubilization.

Acknowledgements

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