INVESTIGATION OF THE *NAJA NAJA SIAMENSIS* TOXIN BINDING SITE OF THE CHOLINERGIC RECEPTOR PROTEIN FROM *TORPEDO* ELECTRIC TISSUE

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1. Introduction

The acetylcholine receptor protein has been purified from both *Electrophorus* and *Torpedo* in many different laboratories [1–4]. The molecule appears to consist of at least five subunits [5]. SDS polyacrylamide electrophoresis of the purified protein shows two bands indicating the existence of at least two different types of subunits in the protein molecule [1,4,5]. Furthermore the number of substrate molecules bound per oligomer is lower than the number of subunits [6,7]. From these data one could conclude that the receptor molecule is composed not only of chemically but also functionally different types of polypeptide chains. In this paper we present evidence which gives experimental support to this conclusion. By covalently binding radioactively labelled *Naja naja siamensis* toxin to the receptor molecule we have shown that only one of these subunits binds the toxin.

2. Material and methods

2.1. *Purification of the receptor protein*

Acetylcholine receptor was purified from the electric organ of *Torpedo Marmorata* by a modification of the method of Karlsson et. al. [8]. An affinity gel was prepared with the principal neurotoxin of *Naja Naja Siamensis* (purified according to [9] as substrate (10 mg/40 ml Sepharose 2B). 500 g of electric tissue is homogenized in 1 litre water submerged in an ice bath for 1 min with an Ultra-Turrax homogenizer. The solution also contained 10^{-4} M phenylmethylsulfonylfluoride. All subsequent steps are performed at 4°C. The resulting suspension is centrifuged at 500 *g* for 10 min. The supernatant is then centrifuged at 30 000 *g* for 20 min and the pellet is resuspended in 200 ml of Ringer's solution containing 1% Triton X-100. This detergent-solubilized crude receptor fraction is stirred overnight with 80 ml affinity gel and subsequently poured into a 2.0 × 60 cm column. The column is then washed with 200 ml 0.1 M Tris–HCl, 0.1 M NaCl, 1% Triton, pH 7.4 and then eluted with 100 ml of this same buffer containing, in addition, 0.5 M carbamoylcholine. The eluted receptor is then dialysed overnight against 3 litre of 0.01 M Tris–HCl, 0.01 M NaCl, 1% Triton X-100, pH 7.4 and over the next 24 hr the buffer is replaced three more times. The dialysed material is once again mixed with fresh affinity gel and the procedure through dialysis repeated.

The dialysed receptor protein is then concentrated by adsorption on a small (4 × 0.9) DE 52 (Whatman) ion exchange column that has previously been equilibrated with 0.01 M Tris–HCl, 0.01 M NaCl, 0.1% Triton X-100, pH 7.4. The column is washed with 50 ml of this buffer and the purified protein eluted with 0.1 M Tris–HCl, 0.5 M NaCl, 0.1% Triton X-100, pH 7.4. Protein is determined by the method of Lowry [10]. Activity of the receptor protein is assayed with ^{125}I-labelled *Naja Naja Siamensis* toxin by the method of Changeux [1]. Most preparations bind approximately 5 μmoles toxin/g protein.

2.2. *Cross-linking with suberimidate*

Cross-linking of receptor–toxin complex with suberimidate was performed as described by Hucho and Changeux [5] except that 100 μg of purified receptor were first incubated for 1 hr with 60 pmoles ^{125}I-toxin before addition of suberimidate.

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2.3. *Polyacrylamide electrophoresis*

Disc gel electrophoresis in the presence of 1% Triton X-100 on 5% polyacrylamide gels was performed according to the method of Ornstein and Davis [11].

SDS electrophoresis in 5% polyacrylamide gels was carried out as described by Hucho et al. [5] with the modification that an E–C vertical gel apparatus with a slab of polyacrylamide (17.5 × 13 cm) was used. After electrophoresis the slab was stained, destained [5] and dried on filter paper under vacuum at 80°C. The dried gel was then subjected to autoradiography for 48 hr on Kodak Regulix BB14 Radiographic Film and the film scanned at 550 nm by a densitometer attached to an Eppendorf Spectrometer.

3. Results

Disc electrophoresis of the purified receptor protein under non-denaturing conditions shows a single band (fig. 1) indicating homogeneity of the protein. SDS polyacrylamide electrophoresis under denaturing conditions shows in contrast several bands (fig. 2). The relative amounts of the higher molecular weight components depends on the age of the preparation i.e., they increase with time. The molecular weights of these components as calculated from the calibration curve in fig. 4 are 37 000, 49 000, 74 000, 93 000, 148 000. The bands at mol. wt. 37 000 and 49 000 correspond to the two subunits of the protein isolated from Electrophorus [1–5].

A ^{123}I-*Naja Naja* toxin–receptor complex was cross-linked with suberimidate [12,5]. This complex

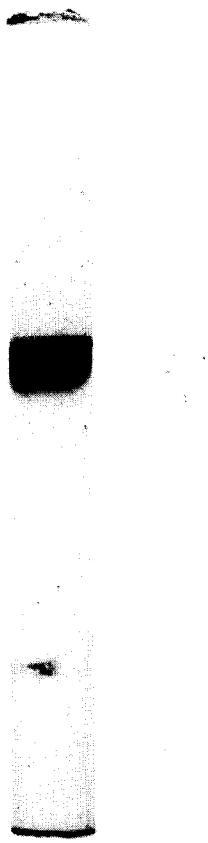


Fig. 1. Disc gel electrophoresis of purified receptor protein from *Torpedo* electric tissue. 5% polyacrylamide gel, 50 μg receptor protein. For experimental details see section 2.3.

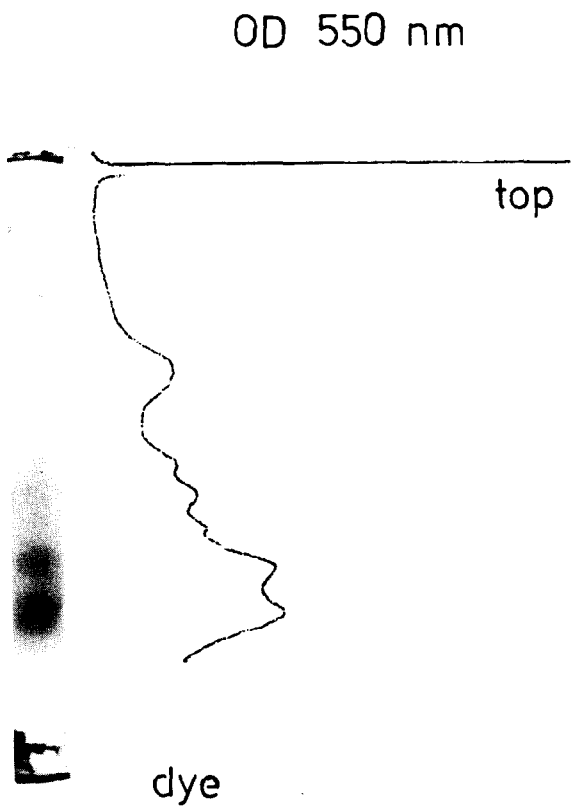
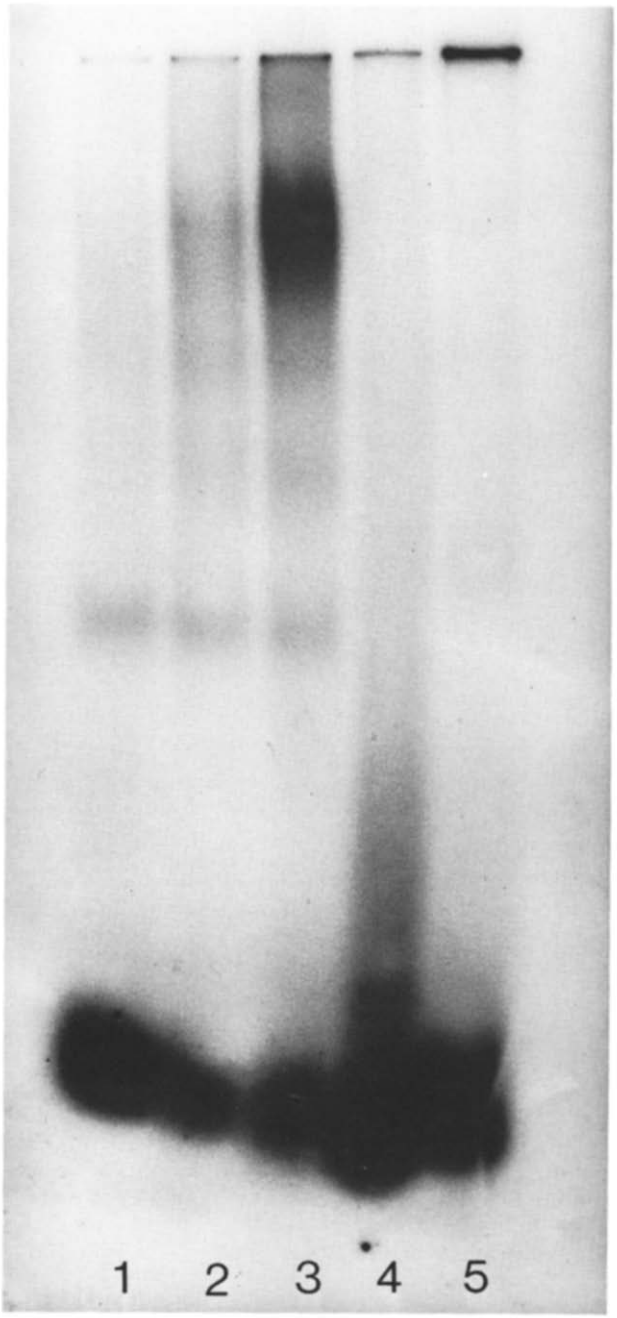


Fig. 2. SDS-polyacrylamide gel electrophoresis of purified receptor protein. For experimental details see section 2.3.

was then electrophoresed in SDS and subsequently the dried gel was autoradiographed. Fig. 3 shows five bands. The bands correspond to mol. wt. of 191 000, 167 000, 130 000, 82 000 and 53 000. Neither *Naja Naja* toxin alone nor a mixture of toxin with control proteins such as pyruvate kinase yield significant bands under these conditions (fig. 3, sample well 4 and 5).



unbound
^{125}I-toxin

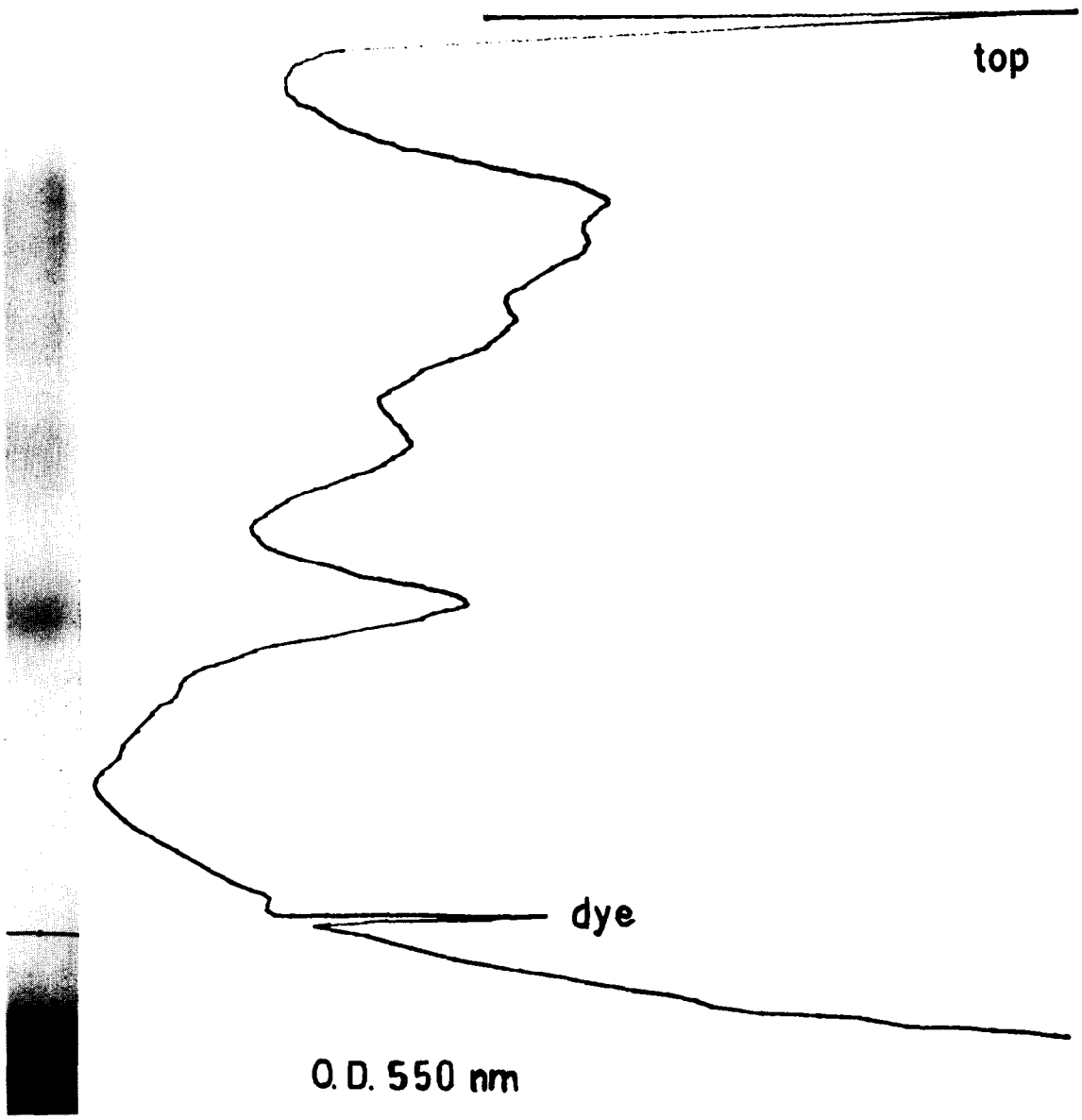


Fig. 3. Densitometer scan and autoradiograph of SDS-polyacrylamide gel electrophoresis of receptor–toxin complex cross-linked with suberimidate. The samples contained 1) 100 μg receptor protein, 60 pmoles ^{125}I-toxin, 0.1 mg/ml suberimidate. 2) 100 μg receptor protein, 60 pmoles ^{125}I-toxin, 0.5 mg/ml suberimidate. 3) 100 μg receptor protein, 60 pmoles ^{125}I-toxin, 1 mg/ml suberimidate. 4) controls: electrophoresis buffer, 60 pmoles ^{125}I-toxin, 1 mg/ml suberimidate. 5) 100 μg pyruvate kinase, 60 pmoles ^{125}I-toxin, 1 mg/ml suberimidate. All solutions contain 0.2 M triethanloamine, pH 8.5.

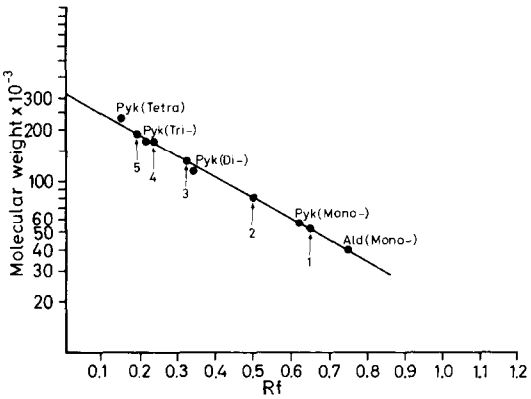


Fig. 4. Molecular weight determination of the receptor–toxin complex, Pyk: pyruvate kinase. Ald: Aldolase. 1, 2, 3, 4, and 5 refers to the bands of the autoradiograph of fig. 3.

4. Discussion

The results shown in fig. 3 indicate that only one of the two low molecular weight bands in the stained gel (fig. 2) exhibits radioactivity. We interpret this to mean that only one of the two subunits binds *Naja Naja* toxin. The control experiment with pyruvate kinase indicates that only proteins which specifically bind the toxin show significant radioactivity under our conditions of cross-linking.

Since cross-linking with suberimidate requires amino groups in appropriate proximity there is the possibility that the second subunit although binding the toxin did not react. However, for the following reasons we consider this unlikely: In earlier experiments the two subunits have been cross-linked to each other indicating that they both are reactive towards suberimidate [5]. Furthermore the subunits of all proteins investigated by this method could be cross-linked probably because of the general abundance of amino groups. According to Davis and Stark [12] the number of bands obtained by this method corresponds to the number of subunits in the molecule. This would mean that the *Torpedo* receptor protein like the receptor from Electrophorus [5] appears to be composed of at least five subunits. None of the radioactively, labelled bands (fig. 3) can be due to impurities since our protein is homogeneous (fig. 1). In contrast to Coomassie Blue stained gels the autoradiograph reveals only proteins that specifically bind toxin.

The molecular weights corresponding to the bands are difficult to evaluate because they include the toxin molecules and we have no way to determine how many toxin molecules are bound in each band. From the specific activity of our protein we would assume that only one or two toxin molecules are present. The molecular weight of the low molecular weight band corresponds to 53 000. Substracting 8000 dalton for the toxin from the molecular weight of this band we obtain 45 000.

In conclusion only one of the two subunits of the *Torpedo* receptor protein appears to bind *Naja Naja* toxin. It remains to be determined if the second band corresponds to a degradation product of this subunit that has lost its binding capacity or if it has a different function.

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References

[1] Meunier, J.-C., Sealock, R., Olsen, R. and Changeux, J.-P. (1974) Eur. J. Biochem. 45, 371.
[2] Klett, R., Fulpius, B., Cooper, D., Smith, M., Reich, E. and Possani, L. (1973) J. Biol. Chem. 248, 6841.
[3] Schmidt, J. and Raftery, M. A. (1973) Biochemistry 12, 852.
[4] Biesecker, G. (1973) Biochemistry 12, 4403.
[5] Hucho, F. and Changeux, J. P. (1973) FEBS Lett. 38, 11.
[6] Martinez-Carrion, M. and Raftery, M. A. (1973) Biochem. Biophys. Res. Commun. 55, 1156.
[7] McNamee, M. G., Weill, C. L. and Karlin, A. in Protein-Ligand Interactions (H. Sund and G. Blauer eds) Walter de Gruyter, Berlin, New York in press.
[8] Karlsson, E., Heilbronn, E. and Widlund, L. (1972) FEBS Lett. 28, 107.
[9] Karlsson, E., Arnberg, H. and Eaker, D. (1971) Eur. J. Biochem. 21, 1.
[10] Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265.
[11] Ornstein, L. and Davies, B. J. (1964) Ann. New York Acad. Sci. 121, 321.
[12] Davies, G. E. and Stark, G. R. (1970) Proc. Natl. Acad. Sci. U.S. 66, 651.