LACTOPEROXIDASE CATALYZED MEMBRANE SURFACE LABELING OF THE ACETYLCHOLINE RECEPTOR FROM *TORPEDO CALIFORNICA**

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SUMMARY Acetylcholine receptor from Torpedo californica has been iodinated by lactoperoxidase. All polypeptides of the purified receptor in emulphogene solution are labeled but the membrane bound receptor is preferentially labeled on the 40,000, 50,000 and 60,000 MW subunits. The 40,000, 50,000 and 60,000 MW subunits exhibit exposed regions on the membrane surface but the 65,000 MW subunit is predominantly shielded from labeling by membrane interactions.

INTRODUCTION

The acetylcholine receptor from Torpedo californica electroplaques has been characterized biochemically both as a purified molecule (1) and in highly enriched membrane preparations (2). Little information exists, however, on its disposition in the postsynaptic membrane. One approach to determining topographical features of membrane proteins is by chemical or enzymatic labeling (3). Lactoperoxidase catalyzes selective iodination of membrane protein regions exposed to the aqueous phase (4). To avoid non-selective chemical iodination, the reaction mixture must contain an excess of iodinatable substrate and low levels of both H_2O_2 and

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Abbreviations Used: AcChR, acetylcholine receptor; DEAE, diethylaminoethyl cellulose; SDS, sodium dodecyl sulfate; CBB, coomassie brilliant blue; MW, molecular weight; LPO, lactoperoxidase.

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I^- (5). Under these conditions, lactoperoxidase has been used to determine the vectorial arrangement of proteins in several membrane systems (6).

In this communication we present evidence based on LPO catalysed iodination that the topography of acetylcholine receptor polypeptide chains differs depending on whether the protein is in its native membrane environment or in detergent solution. In addition, not all of the polypeptides comprising the AcChR are readily available for labeling in the membrane associated state.

MATERIALS AND METHODS

AcChR enriched membrane fragments were prepared as previously described (7,8) from Torpedo californica electroplax from freshly killed animals or from electric organs stored at -90°C using calcium free phosphate buffer (10 mM sodium phosphate, 0.4 M NaCl, 1 mM EDTA, 0.02% NaN_3 pH 7.4). $Na^{125}I$ (carrier free) was obtained from New England Nuclear in 0.1 N NaOH and diluted 10 fold. Lactoperoxidase was obtained from Worthington Biochemical Corp. A specific activity of 17.8 units/mg was measured using the Worthington Enzyme Manual (1972, Worthington Biochemical Corp., Freehold, New Jersey) assay for peroxidase at pH 7.3 instead of 6.0.

For the labeling reaction, membrane fragments were washed three times to remove azide and suspended in Ca^{++} free Ringers (0.25 M NaCl, 5 mM KCl, 2 mM $MgCl_2$, 5 mM Tris-Cl pH 7.4). To reduce any $^{125}I_2$ present, 80 λ of 20 μM sodium sulfate was added to 0.16 ml of 10 mCi/ml $Na^{125}I$ in 10 mM NaOH. After 20 minutes incubation at room temperature, 16 λ of 0.1 N HCl was added. Aliquots of 12 λ of the $Na^{125}I$ mixture were combined with 5.5 λ of 20 μM H_2O_2 and added at 5 minute intervals to the reaction mixture. The reaction mixture contained 5 μM AcChR in membrane fragments (13 mg/ml total protein), 0.13 μM LPO, 0.25 M NaCl, 5 mM KCl, 2 mM $MgCl_2$, 55 mM Tris-Cl pH 7.4 in a total volume of 90 λ. A total of 5 to 9 additions of $Na^{125}I$ plus H_2O_2 were made, each delivering approximately 0.3 μM $Na^{125}I$ and 0.35 μM H_2O_2 to the reaction. A control sample was treated identically except for the omission of LPO. Following the reaction incubation, the membranes were washed twice with and suspended in Ca^{++} free Ringers. SDS-polyacrylamide gel electrophoresis of the samples was performed according to Laemmli (14) except that 12.5% acrylamide, 0.1% bis-acrylamide was used in the running gel. After staining with CBB, gels were scanned at 550 nm on a Gilford model 240 spectrometer with a linear gel transport. Gels were swollen in 10% acetic acid and sliced into 1 mm slices on a Hoefer gel slicer. Slices were counted for ^{125}I on a Beckman Biogamma II scintillation spectrometer.

Triton solubilized AcChR was purified as described previously (9). The solubilized receptor (3.5 mg) was bound to Whatman DEAE DE-52 anion exchanger (0.6g) and washed overnight with 250 ml of 20 mM Tris-Cl, 0.1% Emulphogene BC720 (G.A.F. Corp) pH 7.4 at 5°C. AcChR was eluted in 20 mM Tris-Cl, 0.1% Emulphogene, 1 M NaCl pH 7.4, dialyzed against 20 mM Tris-Cl, 0.1% Emulphogene pH 7.4 and concentrated to 3 mg/ml protein. The detergent-exchanged AcChR was iodinated by the same procedure used for membrane fragments with the inclusion of 0.025% emulphogene in the reaction mixture. Following the iodination, AcChR was dialyzed against 20 mM phosphate, 0.1% Triton X-100 pH 7.4 for 1½ days at 5°C and analyzed for ^{125}I counts on SDS gels.

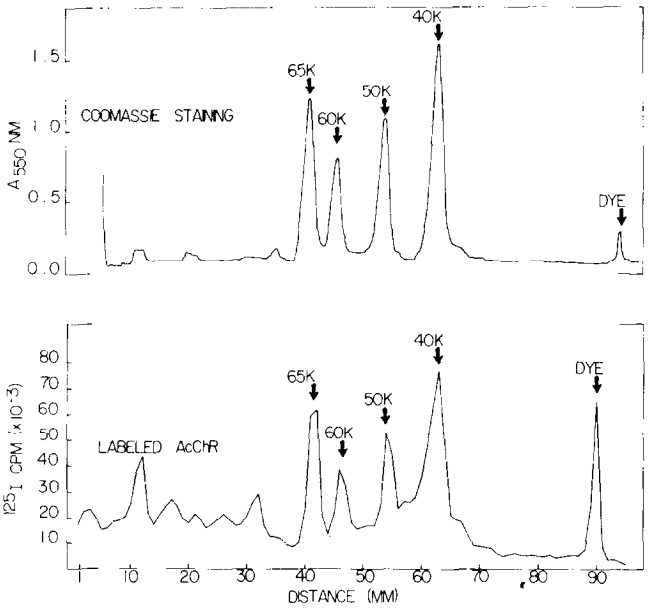

FIGURE 1: LPO catalyzed iodination of solubilized AcChR in emulphogene solution. The upper figure shows the CBB staining pattern of a gel containing 80 µg of AcChR. The lower figure shows the ^{125}I counts in 1 mm thick slices of an AcChR gel containing 25 µg of AcChR. The positions of the receptor subunits and the top of the bromophenol blue dye band are shown.

RESULTS AND DISCUSSION

Solubilized AcChR in Triton X-100 was not susceptible to iodination by lactoperoxidase (LPO). This failure apparently arose from inhibition of LPO by Triton. When Emulphogene was exchanged for Triton, all subunits of AcChR labeled efficiently as shown in Fig. 1. The labeling efficiency of each subunit was proportional to its CBB staining density. Thus, in solubilized AcChR, ^{125}I incorporation is proportional to the amount of protein present in each subunit, with no apparent subunit specificity.

LPO labeled the 40,000, 50,000 and 60,000 MW subunits of AcChR in membrane fragments (Fig. 2) but at 1000 fold lower efficiencies than for solubilized AcChR. In several trials, the 65,000 MW AcChR subunit was

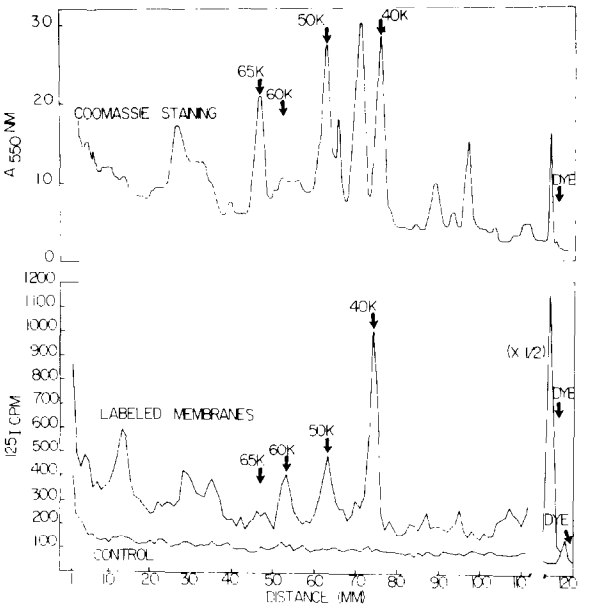

FIGURE 2: LPO catalyzed iodination of electroplaque membrane fragments. The upper figure shows the CBB staining pattern of a gel containing 400 µg total protein. The lower figure shows the ^{125}I counts in 1 mm thick slices of a labeled membrane gel containing 200 µg total protein and of a control gel containing 200 µg total protein which was treated identically except that LPO was omitted from the reaction mixture. The positions of the receptor subunits and the top of the bromophenol blue dye band are marked.

labeled little, or not at all, in membrane fragments. Membrane fragments isolated in Ca^{++} free phosphate buffer routinely showed little CBB staining density in the region of the 60,000 MW AcChR subunit even though a distinct ^{125}I labeling peak was present (Fig. 2). The 60,000 MW subunit appears to be present but shielded from CBB staining in these preparations. When the labeling reaction was performed near pH 6.5, the 65,000 MW AcChR subunit incorporated the label, apparently due to the enhanced $^{125}I_2$ production by LPO at this pH (10). Such chemical labeling of proteins provides no information on their vectorial arrangement in membranes because I_2 can penetrate the lipid phase. The LPO-free control membrane

(Fig. 2) showed no indication of AcChR labeling but exhibited minor peaks at the top and bottom of the gel from chemical iodination. No changes were noted in the CBB staining pattern of either the control or labeled membranes following the labeling reaction.

Several hypotheses were tested for the low labeling efficiency of AcChR in the membrane. The Ca^{++} free Ringers buffer used for the labeling reaction inhibited LPO catalyzed iodination of tyrosine by approximately 40%, but this inhibition is only a small fraction of the labeling decrease seen in membrane bound AcChR. When the labeling was attempted with 10 fold lower AcChR concentrations in 10 mM phosphate buffer, where LPO is fully active, large amounts of self iodinated LPO were generated which stayed with the membranes through several washes. Self-iodination was not a significant side reaction in Ca^{++} free Ringers since no ^{125}I-LPO was found in either the membrane pellet or the reaction supernatant. Thus, competitive self iodination by LPO did not limit AcChR labeling in the membrane preparations.

Membrane fragment preparations from electroplaques contain broken membrane pieces and may contain a mixture of right-side out and inside out vesicles (8). Any subunit of AcChR which is accessible from either side of the membrane should be iodinated by lactoperoxidase. The 40,000, 50,000 and 60,000 MW subunits of AcChR were iodinated in membrane fragments in nearly the same proportions as observed in solubilized receptor. All three subunits thus contain regions which extend from the membrane into the soluble phase. The 1000 fold lower labeling efficiency of AcChR in membrane fragments compared to solubilized AcChR may reflect shielding of AcChR labeling sites by the membranes or the presence of LPO inhibitors in membrane fragment preparations.

The 65,000 MW subunit presumably exhibits some exposed membrane surface regions because it contains water soluble glycolytic residues (1) and is susceptible to phosphorylation by protein kinases (11). These

putative exposed regions are not readily iodinated, however, in membrane fragment preparations. They may be shielded from labeling by surface glycolytic residues or may be deficient in LPO substrates, tyrosine and histidine residues (12). LPO labeling sites which are exposed on the solubilized AcChR are predominantly masked by the membrane interaction. These sites must be largely buried in the hydrophobic core of the membrane or otherwise shielded from labeling. Such shielding could arise from altered interactions of the 65,000 MW subunit with neighboring subunits due to conformational differences between solubilized and membrane-bound AcChR.

In summary, the membrane surface accessibility of three of the four AcChR subunits revealed by LPO labeling is not surprising since all the receptor subunits contain glycolytic residues which presumably extend into the soluble phase and all contain rather high contents of polar amino acids for a membrane protein (13). The ready accessibility of water soluble agonists, antagonists, and snake toxins to the receptor indicates that considerable exposure to the solvent phase may exist. The largely buried nature of the 65,000 MW subunit, suggested by this study, makes it a candidate for involvement in the trans-membrane ion conduction catalyzed by AcChR. Further investigation should reveal if the 30,000, 40,000 and 50,000 MW subunits actually traverse the membrane and the role of the buried segments of the 65,000 MW subunit.

REFERENCES

1. Vandlen, R.L. and Raftery, M.A. (1977) Biochemistry, submitted.
2. Raftery, M.A., Bode, J., Vandlen, R., Michaelson, D., Deutsch, J. and Moody, T. (1975) Proc. FEBS Mtg, Budapest, Hungarian Acad. Sci. 39-65.
3. Carraway, K.L., (1975) Biochem. Biophys. Acta 415, 379-410.
4. Morrison, M. (1974) Methods in Enzymology 32b, 103-109.
5. David, G.S. and Reisfeld, R.A. (1974) Biochem. 13, 1014-1021.
6. Morrison, M., Gates, R.E., and Huber, C.T. (1974) in Membrane Transformations in Neoplasia (Eds. Shultz, J. and Block, R.E.) New York Academic Press, pp. 35-50.
7. Duguid, J. and Raftery, M.A. (1973) Biochem. 12, 3593-3597.

8. Reed, K., Vandlen, R., Bode, J., Duguid, J. and Raftery, M.A. (1975) Arch. Biochem. Biophys. 167, 138-144.
9. Vandlen, R.L., Schmidt, F., and Raftery, M.A. (1976) J. Macromol. Sci. Chem. A10, 73-109.
10. Morrison, M. and Bayse, G. (1973) in Oxidases and Related Redox Systems (Eds. King, T.E., Mason, H.S., and Morrison, M.) Baltimore, University Park Press, pp. 375-388.
11. Gordon, A.S., Davis, C.G., Milfay, D. and Diamond, I. (1977) Nature 267, 539-540.
12. Morrison, M. and Schonbaum, G.R.,(1976) Ann. Rev. Biochem. 45, 861-888.
13. Raftery, M.A., Vandlen, R.L., Reed, K.L., and Lee, T. (1976) Cold Spring Harbor Symp. Quant. Biol. 15, 193-202.
14. Laemmli, U.K. (1970) Nature 227, 680-685.