ON THE IRREVERSIBLE BINDING OF *p*-(TRIMETHYLAMMONIUM) BENZENEDIAZONIUM FLUOROBORATE (TDF) TO ACETYLCHOLINESTERASE FROM ELECTROGENIC TISSUE

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1. Introduction

Para-(trimethylammonium) benzenediazonium fluoroborate (TDF) is a reagent which has been successfully used for the affinity labeling of sites specific for acetylcholine (ACh) and its congeners: the active site of anti-phenyltrimethylammonium antibodies [1], the active center of acetylcholinesterase (AChE) from bovine erythrocyte [2], the ACh receptor from the excitable membrane of the eel electroplax [3]. The results presented in this letter concern the effect of TDF on AChE from the electrogenic tissue of *Electrophorus electricus*. It is shown that the covalent bonding of TDF to AChE results first in a dramatic decrease of the catalytic hydrolysis of both polar and neutral substrates. However, after a few hours of incubation with TDF, and while the enzyme is still inactive with a polar substrate, a selective recovery of the capacity to hydrolyse a neutral substrate is observed. The results are interpreted in terms of the binding of TDF to several categories of topographically distinct sites.

2. Materials and methods

Enzyme: a purified preparation of AChE from electric eel, type V, from Sigma Chemicals Co., of specific activity: 80 mmoles ATCh/mg protein/hr was used for routine work and was stored as a solution in 5×10^{-2} M phosphate buffer pH 7.0 with 1.5 mg of proteins per ml. The essential results were repeated on pure AChE, of specific activity: 250 mmoles ATCh/mg protein/hr, a gift of Dr. W.Leuzinger from Columbia University.

Assay: acetylthiocholine (ATCh) hydrolysis was followed at 412 mμ by the method of Ellman et al. [4] in a medium containing: 5×10^{-4} M ATCh, 5×10^{-4} M DTNB, 5×10^{-2} M sodium phosphate pH 7.0, 7.5 mμg/ml proteins in a final volume of 1.0 ml. Indophenyl acetate (IPA) hydrolysis was measured at 625 mμ in a medium containing 7.5×10^{-4} M IPA, 5×10^{-2} M sodium phosphate pH 7.0, and 7.5 μg/ml protein in a final volume of 1.0 ml [5].

Chemicals: acetylthiocholine chloride (ATCh), sodium 5-5′ dithiobis 2-nitrobenzoate (DTNB) were from Sigma Chemicals Co., decamethonium bromide (Deca) from K and K Lab.; indophenyl acetate (IPA) from Eastman Organic Chem., para-(trimethyl ammonium) benzene diazonium fluoroborate (TDF) was a gift of Dr. H.Mautner from Yale University. Stock solutions of IPA were made in 95% ethyl alcohol, of TDF in 10^{-2} M HCl.

3. Results and discussion

In fig. 1 are compared the effects of TDF on the catalytic activity of AChE, using either a polar substrate: ATCh, or a neutral one: IPA. In this experiment, the AChE solution is made 10^{-4} M with TDF at zero time. Subsequently, samples are diluted at the indicated time and immediately assayed with both substrates. ATCh being the substrate, a complete inactivation occurs, the kinetics of which does not follow a simple exponential decay. When IPA hydrolysis is measured, during the first 10 minutes, a rapid decrease in activity is observed, as well, but this inactivation phase is followed by a slow reactivation of the enzyme. The slow phase reaches completion,

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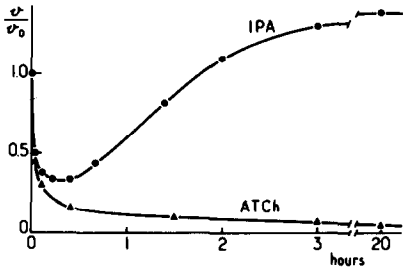

Fig. 1. Time course of the reaction between TDF and AChE followed with a charged substrate: acetylthiocholine (ATCh), and a neutral one: indophenyl-acetate (IPA). At zero time, AChE (1.6 mg/ml) is made 10^{-4} M with TDF. At the indicated time samples are diluted: 200,000 folds for the assay with ACTh and 200 folds for the assay with IPA. Conditions of assay: see section 2 of the text.

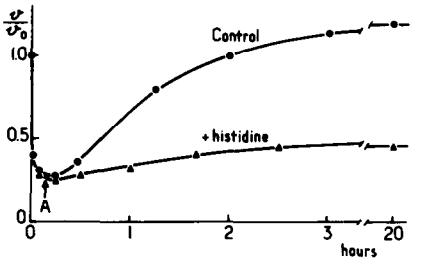

Fig. 2. Blockade of the slow (reactivation) phase by 0.25 M histidine. Histidine is added at the end of the inhibition phase (arrow A). Same conditions as for fig. 1. Assay on IPA.

under the present conditions, after approximately three hours.

The early inhibition phase, observed with ATCh and IPA is simply explained on the basis of TDF binding at, or close to, the active center, in such a manner that the accessibility of both substrates to the esteratic site is sterically hindered. This interpretation is supported by the observation that reversible competitive inhibitors considerably protect the enzyme against irreversible inhibition by TDF. Furthermore, the dissociation constant of such inhibitors is found to be the same when measured either via their antagonism towards TDF or by the classical kinetic analysis using ATCh as a substrate [6].

Several hypothesis can be proposed to explain the slow recovery of the catalytic activity towards IPA hydrolysis:

1. a spontaneous dissociation of an AChE-TDF complex;
2. a slow structural re-organization of AChE, following the covalent binding of a first TDF molecule to the active site, e.g., the intra-molecular migration of bound TDF;
3. the covalent binding of additional TDF molecules to peripheral anionic sites distinct from the anionic site of the active center.

Hypothesis 1 does not account for the observation that the re-activated enzyme no longer hydrolyses polar substrates (i.e., ATCh). A specific prediction of hypothesis 2 is that the re-activation phase should occur in the absence of TDF. In order to test this second hypothesis unreacted TDF was, at the end of the inhibition phase (i.e., after about 10 minutes incubation), either quenched by adding an excess of histidine, or rapidly eliminated by filtering the reaction mixture on a Sephadex G-25 column. In both cases, the re-activation rate was instantaneously decreased (for the effect of histidine, see fig. 2). Inhibition of the slow phase was also obtained by adding under the same conditions, an excess of Deca. In other words, the re-activation phase can be explained on the basis of a secundary slow reaction between TDF and additional sites distinct from the active site. The simplest hypothesis to account for the re-activation process would be that binding of TDF to these "additional" sites promotes a structural reorganisation of the active site through indirect and thus "allosteric" interactions mediated through a conformational transition of the enzyme molecule.

After an extensive reaction with TDF, an irreversibly modified enzyme (M-AChE) is obtained, some properties of which differ markedly from those of the native enzyme (AChE). The M-AChE hydrolyzes IPA about 40% faster than AChE, although its ability to hydrolyse ATCh is almost completely lost. The M-AChE shows the same sedimentation coefficient (about 11S) and the same apparent affinity for IPA ($K_M = 8.3 \times 10^{-4}$ M) as the native enzyme. But, its sensitivity to the bis-quaternary ammonium inhibitor Deca – $Me_3\overset{+}{N}(CH_2)_{10}\overset{+}{N}Me_3$ – is markedly altered. As illustrated on fig. 3, Deca inhibits the splitting of IPA by the native protein, but not by the modified enzyme;

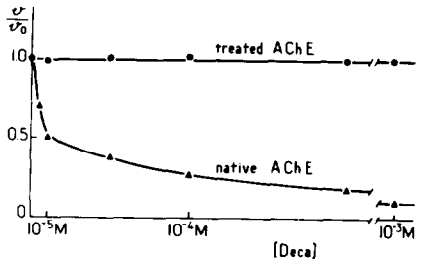

Fig. 3. Compared sensitivity to decamethonium (Deca) of native AChE and AChE extensively treated by TDF. Same conditions as for fig. 1. Assay on IPA. Treated AChE has been exposed to 10^{-4} M TDF for at least three hours.

The sum of these results is in agreement with the suggestion earlier made by one of us [7] that there exist, on AChE molecules, specific sites for quaternary nitrogens, which are topographically distinct from the anionic site of the active center. The evidence for such "regulatory" sites is of interest in connection with the observation that "in vivo", TDF blocks irreversibly the depolarization of the electroplax membrane by ACh, in other words, that TDF is an affinity labeling reagent of the ACh-receptor [3].

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