THE INACTIVATION OF ACETYLCHOLINESTERASE BY TRIMETHYLOXONIUM ION, AN ACTIVE-SITE-DIRECTED METHYLATING AGENT

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Summary: Trimethyloxonium ion inactivates acetylcholinesterase from the electric eel and acetylcholinesterase on the surface of human red blood cells. Tetramethylammonium ion, which is a competitive inhibitor of acetylcholinesterase, protects against this inactivation. Trimethyloxonium ion does not inactivate the system that transports choline into the red blood cell. We conclude that trimethyloxonium ion is an affinity-labeling reagent for acetylcholinesterase and that red blood cell acetylcholinesterase is probably not a component of the choline transport system.

Trimethyloxonium ion ($(CH_3)_3\overset{+}{O}$) is a highly reactive methylating agent (1). It is very similar in structure to the trimethylammonium ($(CH_3)_3\overset{+}{N}$-) functional group. For this reason, we expected that trimethyloxonium ion might be an effective affinity-labeling reagent (2) for enzymes and transport systems that act on choline or choline derivatives. This paper reports the effect of this reagent upon the enzyme acetylcholinesterase (E.C. 3.1.1.7) (3) and upon the choline transport system of red blood cells (4).

MATERIALS

$(CH_3)_3OBF_4$ was purchased from Willow Brook Laboratories. Choline chloride, labeled with C^{14} in the methyl groups, specific activity 49 millicuries/millimole, was a New England Nuclear product. Acetylcholine chloride, choline chloride, and electric eel acetylcholinesterase, type V, were obtained from Sigma Chemical Company. The blood bank of the Mary Hitchcock Memorial Hospital provided human red blood cells in the form of units of whole blood that had been stored with citrate-phosphate-dextrose for 22 to 25 days.

EXPERIMENTAL PROCEDURES AND RESULTS

Hydrolysis of $(CH_3)_3O^+$: The hydrolysis of trimethyloxonium ion

yields methanol, dimethyl ether, and hydronium ion. The rates of this reaction were followed with a Radiometer pH stat apparatus, equipped with a thermostatted cell compartment that was flushed with nitrogen. The reaction was initiated by the addition of carefully weighed samples of $(CH_3)_3OBF_4$ to the temperature-equilibrated solvent. The rates were determined at 5.0° in water at pH 4.0 and in 0.1 mM potassium phosphate at pH 7.0, with a starting concentration of about 5 mM reactant. Under both conditions, there was an initial rapid uptake of NaOH due to the presence of about 20% hydrolysis products in the $(CH_3)_3OBF_4$, followed by a slower consumption of base that obeyed the first-order rate law. The total equivalents of base consumed were equal, within ±15%, to the moles of compound calculated from the weight. The half-time for hydrolysis was found to be 1.6 min at pH 4 and 1.8 min at pH 7. A control experiment showed that BF_4^-, added as $NaBF_4$, did not release acid or base under these conditions.

Inactivation of Eel Acetylcholinesterase: $(CH_3)_3OBF_4$ was added to a solution of 13 µgm/ml acetylcholinesterase, 0.5 mM potassium phosphate, pH 7, and in some cases, 10 mM $(CH_3)_4NCl$ that had been temperature-equilibrated at 5° and was under nitrogen in the reaction vessel of the pH stat apparatus. The reagent was introduced as either the solid or as a small aliquot of a concentrated aqueous solution at 0° that had been prepared a few seconds earlier. The pH of the reaction mixture was maintained at 7.0 by the addition of NaOH from the pH stat apparatus. After hydrolysis of the reagent was complete, the activity of acetylcholinesterase in a 50 or 100 µl aliquot was assayed by determining the initial rate of hydrolysis of acetylcholine with the pH stat apparatus. The assay mixture consisted of 5.0 ml of 1 mM acetylcholine-100 mM NaCl; the rate was measured at pH 7 and 25° under nitrogen with 4 mM NaOH as the titrant. Simultaneously with the modification procedure, another portion of the original

TABLE I. The Inactivation of Acetylcholinesterase by Trimethyloxonium Ion[a]

Enzyme	$(CH_3)_3\overset{+}{O}$, mM[b]	$(CH_3)_4\overset{+}{N}$, mM	V_i/V_o
eel	3.5	-	<0.05
	0.28	-	0.26
	0.25	-	0.27
	0.19[c]	-	0.34
	0.24	10	0.92
	0.23	10	1.0
red blood cell	10	-	0.20
	10	-	0.18
	10	10	0.53
	4.0	-	0.23
	4.0	4.0	0.59
	1.0	-	0.58
	1.0	10.0	0.80
	1.0	-	0.67
	1.0	10.0	0.97

[a]See the text for experimental details.

[b]Initial concentration

[c]10 mM NaCl present

solution of acetylcholinesterase was treated in the same manner except that the $(CH_3)_3OBF_4$ was replaced by the hydrolysis products of $(CH_3)_3OBF_4$ from a neutralized hydrolysate of the reagent. Subsequently, aliquots of this mixture were also assayed.[1] Table I presents the ratio of the activity of the enzyme after treatment with $(CH_3)_3OBF_4$ to that of the enzyme in the corresponding control experiment (V_i/V_o). Trimethyloxonium ion at an initial concentration of 3.5 mM completely inactivates the enzyme. Tetramethylammonium ion, which is a competitive inhibitor of eel acetylcholinesterase with a K_i of 1.2 mM (5), almost completely prevents the 70% inactivation caused by 0.25 mM reagent.

[1]Comparisons with the activity of the enzyme in the original solution have shown that the neutralized hydrolysate does not affect the activity.

Inactivation of red blood cell acetylcholinesterase: Acetylcholinesterase is located on the outer surface of the red blood cell membrane (6,7,8), and, for this reason, whole cells were treated with $(CH_3)_3OBF_4$. Before the modification reaction, the cells were separated from the plasma by centrifugation for 15 min at 1,400 x g, were washed three times with three volumes of 147 mM NaCl - 4 mM KCl - 1 mM $MgCl_2$ - 25 mM sodium phosphate - 0.1% glucose - 0.02% chloroamphenicol, pH 7.4, and were then suspended in this medium to 25% hematocrit. The cells were divided into three portions and cooled to 0°. $(CH_3)_3OBF_4$ was added to one portion; $(CH_3)_4NCl$ and then $(CH_3)_3OBF_4$ were added to the second portion; a hydrolysate of $(CH_3)_3OBF_4$ that was prepared by addition of the reagent to the medium for the cells was added to the third portion, in an amount equivalent to the amount of $(CH_3)_3OBF_4$ that was being used. After about an hour, 0.10 ml aliquots of the cell suspension were assayed for acetylcholinesterase. Each aliquot was added to 4.8 ml 155 mM NaCl in the reaction vessel of the pH stat apparatus; the pH was adjusted to 7.4 under nitrogen at 25°; and the reaction was initiated with 50 µl of 100 mM acetylcholine chloride. A correction was made for the fact that the small concentration of $(CH_3)_4\overset{+}{N}$ present into some of the assays decreases the rate by 5%. Table I summarizes the results in the form of the ratio of the activity in the cells treated with $(CH_3)_3OBF_4$ to that in the cells treated with hydrolysate.[2] As with the eel enzyme, $(CH_3)_3OBF_4$ inactivates and $(CH_3)_4NCl$ protects against inactivation. The fact that 4 mM and 10 mM reagent both cause approximately 80% inactivation suggests that 20% of the observed activity may be due to another esterase that is not inactivated.

In other experiments the rates of reaction of 10 mM $(CH_3)_3OBF_4$

[2]Comparisons with the activity of the enzyme in the original cell suspension have shown that the hydrolysate of $(CH_3)_3OBF_4$ has no effect upon the activity.

TABLE II: The Effect of Trimethyloxonium Ion on the Transport of Choline into the Red Blood Cell[a]

$(CH_3)_3O^+$, mM[b]	V_i/V_o
10	0.88[c]
10	0.82
10	1.0[d]
1	1.0

[a] See the text for experimental details.

[b] Initial concentration

[c] Done with choline-free cells, prepared by incubation of cells overnight in the medium at 5% hematocrit (4).

[d] These cells were readjusted to pH 7.4 before the assay of choline uptake. In the other cases with 10 mM reagent, the pH during the assay was 6.7 due to acid released in the hydrolysis of the $(CH_3)_3OBF_4$.

in the 25% suspension of cells and in the medium for the cells were followed at 5° and pH 7.4 by the pH stat method described above. The reactions followed first-order kinetics, and the half-time in the presence and absence of cells was 45 seconds. Thus, reaction with the cells themselves is not the predominant pathway for disappearance of the reagent. The $(CH_3)_3OBF_4$ caused no hemolysis of the cells.

The Choline Transport System: Red blood cells were washed and treated with either $(CH_3)_3OBF_4$ or the hydrolysate of $(CH_3)_3OBF_4$, according to the procedure described above. The cells were temperature-equilibrated in a shaker bath at 37°, and C^{14}-labeled choline chloride from a stock solution was added to give a concentration of 50 μM (4). At 1, 15, and 30 minutes after the addition of choline, 2 ml samples of the cell suspension were mixed with 10 ml of ice-

cold 2 mM $HgCl_2$ - 1.25 mM KI - 160 mM NaCl to stop the transport process (9,10). With each sample, the cells were separated by centrifugation, washed once with 10 ml of $HgCl_2$ reagent, and then lysed with 1.5 ml 10% trichloroacetic acid. The radioactivity in a portion of the supernatant from the lysate was determined with a scintillation counter. In one experiment, samples were also taken after 11 and 20 hours. After 11 hours the concentration of C^{14}-choline within the cells did not change and was approximately equal to that in the medium. During the first 30 minutes, the internal C^{14} choline reaches about 20% of the equilibrium value. The rates of choline uptake were taken to be the average rates for the first 30 minutes. Table II gives the ratios of the rate found after $(CH_3)_3OBF_4$ treatment to that found after treatment with the hydrolysate of $(CH_3)_3OBF_4$.[3] There is little, if any, inactivation of the transport system under conditions that inactivate 80% of the red blood acetylcholinesterase. This finding indicates that the acetylcholinesterase and the transport system for choline are distinct from each other (see 11, 8).

CONCLUSION

Trimethyloxonium ion, or derivatives of it, may prove to be useful affinity-labeling reagents for enzymes that act upon choline or choline derivatives, for the acetylcholine receptor protein (12), and possibly for some choline transport systems, such as the one in synaptosomes(13). The availability of a method for preparing C^{14}-labeled $(CH_3)_3OBF_4$ (14) will aid in the identification of the amino-acid residues that are methylated.

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[3] Other experiments showed that the hydrolysate has no effect on the rate.

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