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6-Coumarin Diazonium Salt: A Specific Affinity Label of the *Torpedo* Acetylcholinesterase Peripheral Site

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SUMMARY

A 6-coumarin diazonium salt was synthesized and tested on *Torpedo* acetylcholinesterase as a site-directed irreversible probe for quaternary ammonium binding. The rate of the inactivation was examined as a function of time, inhibitor concentration, and pH, which allowed the determination of the dissociation and the rate constants of this efficient affinity labeling process. Protection experiments using tetramethylammonium, edrophonium, and propidium demonstrated that the labeling reaction occurred exclusively at the peripheral quaternary ammonium binding site of the enzyme. This result was confirmed by the modification of propidium binding at the peripheral site after inactivation reaction, as directly determined by fluorescence. Mutations of the likely labeled amino acid residues, Tyr^{70} and Tyr^{121}, by histidine and phenylalanine indicated a predominant involvement of Tyr^{70} over Tyr^{121} in the coupling reaction.

The active site of *Torpedo* AChE is localized at the bottom of a deep and narrow gorge and contains a catalytic triad, $Ser^{200}/His^{440}/Glu^{327}$ (1). Among the 14 aromatic residues lining the surface of this gorge, it was shown that Trp^{84} and Phe^{330} are directly involved in the binding of the quaternary ammonium of acetylcholine (2, 3). In addition, AChE possesses a regulatory peripheral anionic site located near the top of the aromatic gorge. Ligands interacting at this peripheral site, such as *d*-tubocurarine and gallamine, modulate the hydrolytic activity of the enzyme. The binding of a substrate molecule at this site may also be responsible for excess substrate inhibition (4, 5). Photolabeling experiments as well as determination of the crystal structure of a decamethonium complex showed that Trp^{279} is involved in the complexation of quaternary ammonium within this peripheral site (3, 6). In addition, the mutation of Trp^{279} to alanine on human (7) and mouse (8) AChE reduced the binding of propidium, a selective peripheral ligand, without affecting the affinity of edrophonium, an active site specific ligand. Another mutation of Trp^{279} to arginine on mouse AChE strongly reduced the affinity of fasciculin, a 6750-kDa peptide that interacts with the AChE peripheral site of AChE (9). However, additional modeling and mutagenesis experiments on *Torpedo* (10, 11) and human (7) AChE suggested the involvement of other residues to the binding of quaternary ammonium at the peripheral site, especially Tyr^{70} and Tyr^{121}, located near the lip of the aromatic gorge. In this context, the present work describes a site-directed irreversible labeling of the peripheral site of *Torpedo* AChE, using a reactive diazonium coumarin derivative. The sensitivity of a series of mutants indicates that both tyrosines are involved at different levels in the inactivation process.

Materials and Methods

Synthesis

6-Nitrocoumarin 2. Coumarin **1** (5 g, 34 mmol) was nitrated by nitric acid (41 mmol) in concentrated sulfuric acid at −20° for 1 hr before the solution was poured on ice. The collected precipitate was dissolved in ethylacetate, washed with water, and dried over $MgSO_4$. The solvent was removed under vacuum, and the residue was recrystallized from ethylacetate/hexane (yield 90%).

***N*-Tertiobutyloxycarbonyl-6-aminocoumarin 3.** A solution of compound **2** (1 g, 5.2 mmol) in ethylacetate was added by drops to a suspension of iron (4 g) in 50% acetic acid. After 1.5 hr at 70°, the solution was neutralized and filtrated. The filtrate was extracted with ethylacetate, the solvent was removed under vacuum, and the residue was dissolved in tetrahydrofuran. Di-tertiobutyldicarbonate (2 eq) in tetrahydrofuran was added, and the solution was heated at 70° for 17 hr. After evaporation of the solvent, the solid residue was taken up in ethylacetate and washed with water before chromatography on silica gel (ethylacetate/hexane [3/7]; **3**: yield 35%).

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ABBREVIATIONS: AChE, acetylcholinesterase.

6-Coumarin diazonium salt 4. Five milligrams of 3 were treated with 500 μl of trifluoroacetic acid. After being stirred for 15 min at room temperature, the solution was cooled at −5°, and an aqueous sodium nitrite solution (1.2 Eq) was added over 15 min. 6-Coumarin diazonium was purified by high performance liquid chromatography on a C_{18} Hyperbond column (H_2O/0.06% trifluoroacetic acid) and stored at −30° in the dark; UV of 4 in H_2O, λmax = 257 nm, ε = 9600 M/cm.

AChE Preparation

AChE was obtained from electric organs of *Torpedo marmorata* (Station de Biologie Marine, Arcachon, France), solubilized by a mild proteolytic procedure, and purified by affinity chromatography (12). Activity was assayed spectrophotometrically on acetylthiocholine iodide (13).

Labeling Experiments

Due to the photosensitivity of 4, all of the experiments were performed in the absence of activating light (λ >450 nm).

Purified AChE was dialyzed overnight at 4° in 50 mM Tris·HCl buffer, pH 8.0, and then diluted at 20° in this buffer at a final concentration of 50 μg protein/ml. An aliquot of 5 μl was diluted (2000×) for initial enzyme activity determination. Then, 4 was added to the assay (final volume 1 ml) at various concentrations (6.6–98 μM). The inactivation of the enzyme was monitored on aliquots for 5 min, and the results were analyzed according to the kinetics scheme described previously (14).

For the protection experiments, the concentration of 4 was kept constant (50 μM) while the protective agents, either tetramethylammonium bromide (0.3–1 M), propidium (30 nM to 1.5 μM), or edrophonium (5 μM to 1 mM), were added at initial time. The affinity constants of the protective agents were determined as indicated previously (14).

Titration of residual peripheral sites after reaction of AChE with 4

AChE, diluted in 50 mM Tris·HCl, pH 8.0, was incubated in the absence or presence of 0.2 mM of 4. Inactivation was monitored using Ellman's assay. After 20 min, the solutions were dialyzed at 4° in the dark against Tris·HCl 50 mM buffer, pH 8.0. The fluorescence of propidium (0.2–4.7 μM) was then measured in this buffer (final volume 600 μl) at 20° in the presence of 130 μg dialyzed AChE.

Site-Directed Mutagenesis and Analysis of Mutants

Wild-type or mutated *Torpedo* AChE was obtained by transfecting COS cells with the corresponding DNA in the pEF vector as described by Harel *et al.* (8) and Duval *et al.* (15). The nature of the mutation has been checked by sequencing the gene after mutagenesis. Cells were extracted with 10 mM Tris·HCl buffer, pH 7.0, containing 10 mM $MgCl_2$, 0.1% Triton X-100, and 0.1 mg/ml bacitracin.

The concentration of active sites in the cell extracts was determined by titration with the organophosphate *O*-ethyl-*S*^{2}-diisopropylaminoethyl methylphosphothionate (16). Kinetics constants K_m and k_{cat} were determined using Ellman's assay with acetylthiocholine iodide (0.01–5 mM) as substrate. Interactions with inhibitors were monitored by determining residual activity after preincubation of AChE or its mutants for 1 hr with various concentrations of edrophonium or propidium.

Inactivation of Wild-Type or Mutated AChE

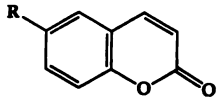
Cell extracts were diluted in 50 mM Tris·HCl, 40 mM $MgCl_2$, 0.1% Triton X-100, and 0.1 mg/ml bovine serum albumin, pH 8.0, and incubated at 25° in the dark with 0 (control) or 280 μM of the inhibitor 4 in a final volume of 100 μl. After 30 min, the decomposition of 4 was total (half-life in these conditions, 5 min), and the enzymatic activity was determined by the addition in the incubation medium of Ellman's buffer and 0.5 mM acetylthiocholine (final volume, 1 ml).

Results and Discussion

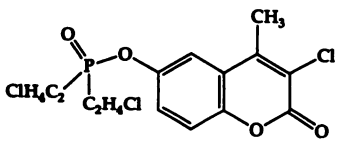
Aryldiazonium derivatives were described as reactive analogues of quaternary ammonium ions and have been used as such, either as affinity (14) or photoaffinity (12) probes for the cholinergic proteins, depending on their chemical reactivity in the absence of light. In this study, we synthesized a 6-coumarin diazonium derivative 4 from a corresponding 6-nitro coumarin 1 (Fig. 1), and we measured the stability of the diazonium salt in the dark. The observed half-life of 15 min at pH 8.0 and 20° classified 4 as a fairly reactive chemical species, without any light-activating process. According to the structure and to the chemical reactivity of 4, we examined the possibility of using this probe as an affinity label for a quaternary ammonium binding site of AChE. By incubation with probe 4 in the dark, we observed a time- and concentration-dependent inactivation of *Torpedo* AChE (Fig. 2A), which could be analyzed according to the following kinetic scheme:

$$E + I \underset{K_d}{\rightleftarrows} EI \xrightarrow{k} E \text{ inactivated}$$

and the deduced equation $t_{1/2} = 0.693/k + 0.693\ K_d/k_I$. We determined for 4 a K_d value of 49 μM (Fig. 2B), which is reminiscent of the affinities of related chemicals for the peripheral binding site of AChE, i.e., coumarin (26 μM) and haloxon (22 μM) (Fig. 1) (4). In parallel, an inactivation rate constant of $k = 0.51\ \text{min}^{-1}$ could be determined. The protection of AChE against inactivation by 4 was studied with various ligands. Tetramethylammonium bromide (1 M) efficiently protected the enzyme, showing a possible interaction of 4 with a quaternary ammonium binding site. The use of edrophonium as protecting ligand allowed to specify which binding site (active or peripheral) is involved in the labeling reaction. A concentration of 1 μM edrophonium, while occupying the active site (K_d = 0.25 μM), did not provide any protection of the enzyme against the inactivation by 4 (Table 1). Only higher concentrations of edrophonium were effective in this protection. From the variation of the half-time of inactivation as a function of edrophonium concentration (5 μM to 1 mM, Fig. 3A), we determined an affinity constant for edrophonium of 0.32 mM, a value that is closer to the described affinity of edrophonium for the peripheral site (0.4 mM) rather than for the active site (0.25 μM)(17). These results indicated that diazonium 4 selectively labeled the peripheral site of AChE. This was confirmed by using pro-



1 R = H
2 R = NO_2
3 R = $NH\text{-}CO_2\text{-}C(CH_3)_3$
4 R = N_2^+



Haloxon

Fig. 1. Structures of coumarin derivatives.

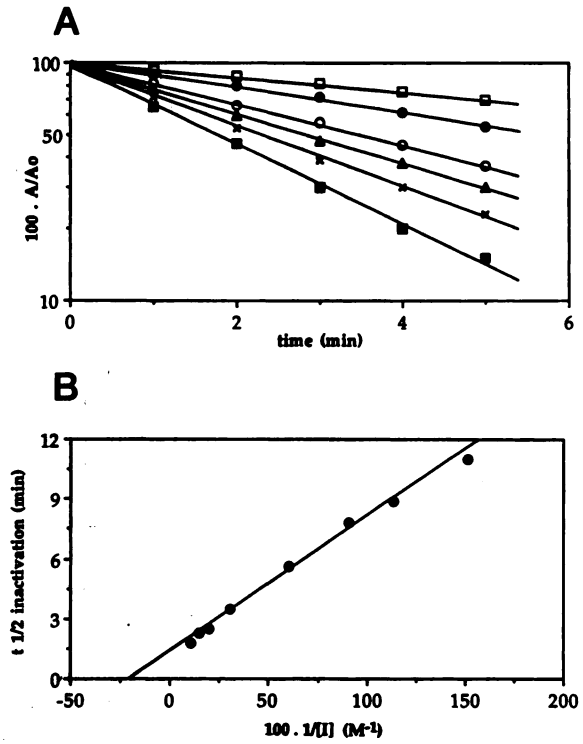


Fig. 2. Affinity labeling of AChE by the coumarin diazonium derivative **4**. Inactivation was performed in 50 mM Tris-HCl buffer, pH 8.0, at 20°. Initial activity (*Ao*) of an aliquot was determined using Ellman's assay. Then, the probe **4** was added at a final concentration of 6.6–98 μM, and the activity (*A*) was monitored at various times. A, Ratio *A/Ao* plotted as a function of time for the following concentrations of **4**: □ 6.6 μM, ● 16.5 μM, ○ 33 μM, ▲ 51 μM, × 66 μM, and ■ 98 μM (8.8 and 11 μM not shown). B, Time of half-inactivation (*t 1/2*) as a function of the inverse of inhibitor concentration. This plot allows the determination of K_d and *k*.

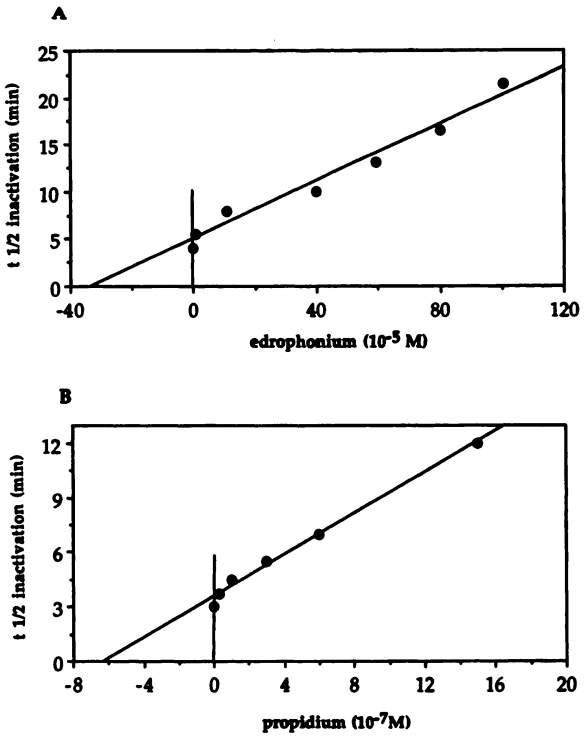


Fig. 3. Edrophonium (A) and propidium (B) protection of AChE inactivation by coumarin diazonium derivate **4**. Inactivation was performed with a constant concentration (50 μM) of **4**, in 50 mM Tris-HCl buffer, pH 8.0, at 20° and the addition at initial time different concentrations of edrophonium (5 μM to 1 mM) and propidium (30 nM to 1.5 μM). The residual activity was monitored at various times. Times of half-inactivation (*t 1/2*) were plotted as f(edrophonium) (A) or f(propidium) (B). These plots allow the determination of K_d for each protector.

TABLE 1

Protection of acetylcholinesterase by edrophonium against inactivation by probe 4

AChE was incubated in 50 mM Tris · HCl, pH 8.0, at 20° in the presence of 50 μM **4** and various concentrations of edrophonium. Activities were monitored on aliquots using Ellman's assay. The residual activities in table are given for 5-min incubation.

Edrophonium	Residual activity
M	%
0	42
10^{-6}	41
$5\ 10^{-6}$	61
10^{-3}	87

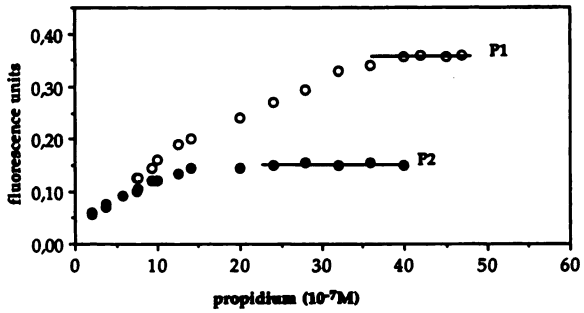


Fig. 4. Titration of residual peripheral sites in AChE labelled by **4**. AChE was incubated for 20 min in the absence (○) or presence (●) of 0.2 mM **4**. After dialysis, the fluorescence of propidium was measured in the presence of 130 μg AChE.

pidium as a peripheral site-selective protective agent. Using increasing concentrations of this ligand (30 nM to 1.5 μM), we observed a progressive protection of the enzyme against **4**. We determined an affinity for propidium of 0.63 μM (Fig. 3B), in agreement with its known affinity for the peripheral site (17).

The labeling of AChE by **4** at the peripheral site was further confirmed by the direct titration of the propidium-binding sites (18). Fluorescence measurements showed (Fig. 4) that the inactivation of the enzyme by **4** induces a 60% loss of propidium-binding capacity, in good agreement with the 80% inactivation of hydrolytic activity. To evaluate the possibility of artifactual fluorescence results due to impurities in our study of the propidium binding to AChE, we checked the homogeneity of the AChE preparation by spectrofluorimetric titration of both active and peripheral sites with *N*-methylacridinium and propidium, respectively (not shown). The K_d of propidium for the peripheral site was 2.4×10^{-7} M, and we obtained a 1:1 ratio between peripheral and active site, indicating the absence of interference by propidium-binding contaminants. We cannot exclude, however, the possibility that the fluorescence of bound propidium might be different in the case of the native and alkylated enzymes.

At this point, probe **4** appeared as an efficient and selective affinity label of the AChE peripheral site. Additional information was obtained by the pH dependence study of this labeling reaction. Table 2 shows the variation of the kinetic constants observed at pH 6, 7.2, and 8, respectively. Pronounced increases in both affinities and alkylation rates are

TABLE 2

Effect of pH on the affinity labeling of AChE

Inactivations were performed at 20° in 50 mM Tris · HCl buffer, pH 8.0, or 50 mM KPO_4 buffer, pH 6.0 or 7.2, with various concentrations of probe 4. For each pH, concentration dependence was analyzed as shown in Fig. 2 to determine the two constants: k (rate constant) and K_d (dissociation constant).

pH	K_d	k
	M	min^{-1}
6.0	$1.5\ 10^{-4}$	0.12
7.2	$2.3\ 10^{-4}$	0.15
8.0	$4.9\ 10^{-5}$	0.51

observed at higher pH values only. However, in the absence of a complete titration curve due to the instability of 4 at higher pH values, it is impossible to draw unambiguous conclusions on the chemical nature of the target residue involved in the alkylation reaction. Nevertheless, the shape of the inactivation rate as a function of pH indicates that the modified residue should have an apparent pK_a of >7.5. This value is compatible with the labeling of a cysteine or a tyrosine residue (respective pK_a values in solution, 8.3 and 10.1). Chemical modifications have previously shown the importance of tyrosine residues for the activity of the enzyme (19, 20). We examined the presence of such residues near Trp^{279}, known to belong to the peripheral site in the three dimensional structure of *Torpedo californica* AChE (1, 3, 6). Two tyrosines, Tyr^{70} and Tyr^{121}, are positioned in the aromatic gorge just below Trp^{279}, whereas Cys^{231}, the only free cysteine of the enzyme, is not located in this area. Two other nucleophilic residues, Glu^{278} and Ser^{291}, which might be able to react with an electrophilic species like 4, are located near Trp^{279}, but their respective pK_a values would not show the observed pH dependence of the inactivation. Thus, Tyr^{70} and Tyr^{121} appear as the best candidates to be alkylated by probe 4.

The possible involvement of Tyr^{70} and Tyr^{121} in the labeling process was studied by site-directed mutagenesis. These residues were replaced by phenylalanine or histidine, and the effects of these mutations were analyzed by a series of kinetic measurements and binding capacities as well as by the irreversible enzyme inactivation induced by 4 (Table 3). Phenylalanine and histidine were chosen not only as aromatic substitutes for tyrosine but also for their differentiated nucleophilic properties; although the phenylalanine residues should be chemically inert toward diazonium 4, the histidine mutants are expected to show increased nucleophilicity.

Analysis of the catalytic constants K_m and k_{cat} for acetylthiocholine shows that the catalytic efficiency (k_{cat}/K_m) is strongly reduced (27-fold) for the $Tyr^{121}His$ mutant, whereas the other mutants are less affected (Table 3). Interestingly, the $Tyr^{70}His$ mutant shows a slight increase in the k_{cat} that is, to our knowledge, a unique feature for a mutation at this residue or to a corresponding position in related enzymes.

The IC_{50} values for edrophonium and propidium were determined for the different mutants to assess their active site and peripheral site binding abilities, respectively (Table 3). The binding of the active site probe edrophonium was more affected in the case of the histidine mutants, with a stronger effect for the 121 position (1.7-fold compared with the wild-type). The fact that residue 121 is located in the catalytic gorge closer to the active site might be a possible explanation for this predominant influence of the position 121. On the other site, a negative interaction of the ligand with the histidine ring, which is partially charged at neutral pH, can explain the difference between $Tyr^{121}His$ and $Tyr^{121}Phe$.

Concerning the binding of propidium, we observed, as expected, a reversed order of influence between the positions 70 and 121 compared with the binding of edrophonium. This might be explained by the fact that residue 70 is closer to the Trp^{279} that is considered to occupy a central position in the peripheral site. As for the binding of edrophonium, the histidine mutants exert a very pronounced effect i.e., the $Tyr^{121}His$ mutant shows a 9-fold decrease in propidium binding that increases to a 50-fold decrease for the $Tyr^{70}His$ mutant. Again, this can be explained by a prejudiciable ionic interaction between diazonium 4 and the histidine residue. In the case of the phenylalanine mutants, the effect is smaller (4.6-fold for $Tyr^{70}Phe$ and 2.8-fold for $Tyr^{121}Phe$) but also indicates a predominant effect for propidium binding of position 70 over 121.

Finally, we studied, for the different mutants, the effect on the enzyme inactivation by the coumarin diazonium probe 4 at an initial concentration of 280 μM (Table 3). Considering at first the mutations at position 70, there is a dramatic difference between mutants $Tyr^{70}His$ and $Tyr^{70}Phe$. Although $Tyr^{70}His$ remains almost as sensitive as the wild-type to the inactivation, the $Tyr^{70}Phe$ mutant is markedly less sensitive. It must be noted that the affinity of $Tyr^{70}His$ for propidium is markedly more reduced that the one of $Tyr^{70}Phe$, compared with the wild-type, and we may assume that this parallels their affinity for probe 4. Thus, the rates of alkylation with this reagent follow the order of nucleophilicity His > Tyr ≫ Phe. Histidines and tyrosines are known to form stable azo adducts with aryldiazonium ions (21). In a different set of experiments using lower probe concentration (45 μM), only

TABLE 3

Substrate and inhibitors interactions with AChE and its mutants

AChE type	K_m[a]	Relative k_{cat}[b]	Relative k_{cat}/K_m[c]	IC_{50} edrophonium	IC_{50} propidium	Residual activity[d]
	mM			μM	μM	%
Wild-type	0.08	1.00	1.00	3.8	2.8	28
$Tyr^{70}Phe$	0.06	0.86	1.17	4.2	13	73
$Tyr^{70}His$	0.20	1.19	0.49	4.3	140	37
$Tyr^{121}Phe$	0.10	0.71	0.59	4.6	7.9	59
$Tyr^{121}His$	0.67	0.29	0.04	6.6	25	64
$Tyr^{70}Phe/Tyr^{121}Phe$	0.15	0.83	0.46	5.5	44	82

[a] Substrate is acetylthiocholine.
[b] Relative k_{cat} is defined as k_{cat} mutant/k_{cat} wild-type.
[c] Relative catalytic efficiency is defined as [(k_{cat}/K_m) mutant]/[(k_{cat}/K_m) wild-type].
[d] Residual hydrolysis of acetylthiocholine after 30-min incubation in the presence of an initial concentration of 280 μM of 4.

the Tyr^{70}His mutant was partially inactivated, whereas all other mutant enzyme as well as the wild-type enzyme remained totally unaffected (not shown). These experiments emphasize the efficiency of the labeling reaction by probe 4 for the Tyr^{70}His mutant. However, the 27% inactivation observed with the nonreactive Tyr^{70}Phe mutant (Table 3) indicates the contribution of other nucleophilic species within the peripheral site.

The possible involvement of Tyr^{121} in the labeling process was similarly studied. Both Tyr^{121}His and Tyr^{121}Phe mutants show a similar decrease in the inactivation reaction compared to the wild-type (Table 3). Again, if we consider the propidium binding as a possible reference for the binding of probe 4, the Tyr^{121}His mutant appears more reactive than the wild-type. The direct comparison between Tyr^{70}His and Tyr^{121}His indicates a predominant contribution of residue 70 in the alkylation reaction. Similarly, a comparison of the inactivation reaction of the Tyr^{121}Phe and Tyr^{70}Phe mutants indicates that the contribution of Tyr^{70} appears to be predominant over that of Tyr^{121} in the wild-type enzyme. In addition, the fact that the double mutant Tyr^{70}Phe/Tyr^{121}Phe is still inactivated (18%) shows that apart from Tyr^{70} and Tyr^{121}, other nucleophilic residues may react with probe 4.

Three-dimensional models suggest that residues Tyr^{70} and Tyr^{121} are both close to the quaternary ammonium group of peripheral site ligands (7, 10, 11). However, these modeling experiments have only been partially confirmed by mutational studies, i.e., Tyr$^{124(121)}$Ala[1] of human AChE does not affect the affinity of propidium, whereas the affinity of Tyr$^{72(70)}$Ala mutant is reduced 4-fold (7).

Taken together, these results have demonstrated that coumarin diazonium salt 4 is a specific affinity label for the AChE peripheral site and support the idea that Tyr^{70} is predominantly involved over Tyr^{121} in the binding of peripheral site ligands and in the irreversible alkylation reaction induced by probe 4. Coumarin diazonium 4, therefore, constitutes an interesting new tool for the investigation of the functional interaction existing between the active and peripheral sites of AChE.

[1] The number in italics indicates the number of the homologous residue in *Torpedo* AChE.

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