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ON THE LOCATION OF ACTIVE SERINES OF MEMBRANE ACETYLCHOLINESTERASE STUDIED BY THE ESR METHOD

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Summary

1 An attempt was made to find out the causes of the discrepancy between the ESR spectra of membrane acetylcholinesterase (EC 3.1.1.7) obtained by Morrisett and co-workers and those obtained by the present authors.

2. In order to see whether the discrepancy was due to the different spin-labeling procedures, the same membrane acetylcholinesterase preparations were spin-labeled with the same compound, using the two different spin-labeling procedures. The enzyme activity was determined with pH-static titration and the ESR spectra recorded.

3. It was found that after spin-labeling according to Morrisett and co-workers, there were from 10—100 times more spin-label molecules bound to the enzyme preparations than there were active serines in them.

4. Using the method of Morrisett and co-workers, the majority of spin-label molecules was found to be bound to sites outside the active serines whereas the spin-labeling procedures of the present authors proved to be selective for active serines; the discrepancy in ESR spectra is explained.

Introduction

In an ESR study of membrane acetylcholinesterase (EC 3.1.1.7) from several tissues, Morrisett et al. [1] found spectra indicating fast molecular motion of spin labels and concluded that the active site of membrane acetylcholinesterase is on its surface, and is exposed to small molecules. On the other hand, in an ESR study of membrane acetylcholinesterase of the *Torpedo marmorata* electric organ, we obtained spectra indicating that the spin-label motion is strongly restricted by the surrounding groups [2], suggesting that the active

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Abbreviations: MeSL, 1-oxyl-2,2,6,6-tetramethyl4-piperidinylmethyl-phosphonofluoridate; BW 284C51, 1,5-bis(4-allyldimethylammoniumphenyl)pentan-3-on dibromide; 2-PAM, 1-methyl-2-hydroxy-iminomethyl-pyridinium iodide.

center is located in a pocket of enzyme surface, although a different serine spin label was used in our study.

The aim of the present work was to find out whether the above discrepancy is due to the different membrane acetylcholinesterase preparations, or different spin labels, or in different spin-labeling procedures. Morrisett et al. [1] used the spin label at a concentration more than 6000 times higher than used by Sentjurc et al. [2]; the former authors incubated the enzyme with the irreversible serine spin label for 10 h while the latter authors did so for 0.5 h. In order to see whether the discrepancy was due to the spin-labeling procedures, both the bovine-erythrocyte membrane acetylcholinesterase and *Torpedo marmorata* membrane acetylcholinesterase were spin-labeled with the compound used by Morrisett et al. [1] using each of the spin-labeling procedures. The criterion for the evaluation of the two procedures was the selectivity of the binding of spin-label molecules to the active serines of membrane acetylcholinesterase preparations.

Methods

All concentrations are final. The enzyme preparations were spin-labeled with MeSL (1-oxyl-2,2,6,6-tetramethyl-4-piperidinylmethyl-phosphonofluoridate). Control samples were prepared according to the procedures described below, but without MeSL.

1. Preparation and spin-labeling procedure of membrane acetylcholinesterase of Torpedo marmorata electric organ

1.1. The electric organ (8 g) was sheared in modified elasmobranch Ringer solution [3] in which the bicarbonate buffer was replaced by 2 mM Tris (elasmobranch medium), pH 7.7; after shearing, the pH was immediately adjusted with 0.1 M NaOH to 7.4 and the preparation centrifuged for 30 min at 40 000 × *g* and 0°C. (For other details, see [2]).

1.2. Using the spin-labeling procedure of Sentjurc et al. [2], 0.2 g of the sediment (procedure 1.1.) was suspended in 30 ml of elasmobranch medium, pH 7.4, and incubated for 30 min with 2.5 μM MeSL at room temperature, with continuous stirring. After adding 150 ml of elasmobranch medium, the suspension was washed by centrifugation under the same conditions as above, and the sediment washed three times with 180 ml of elasmobranch medium and, finally, with 50 ml of elasmobranch medium for 60 min.

1.3. Using the spin-labeling procedure of Morrisett et al. [1], 0.2 g of the sediment (procedure 1.1.) was suspended in 3 ml of 7.03 mM phosphate buffer, pH 7.4; after adding 0.1 ml of 1 M MeSL which had been dissolved in benzene, the mixture was stirred at 4°C for 10 h and, finally, the unreacted reagent was washed away.

2. Preparation and spin-labeling procedure of membrane acetylcholinesterase of bovine erythrocytes

2.1. Bovine-erythrocyte membranes were prepared by the method of Dodge et al. [4], using 20 mM phosphate buffer, pH 7.4.

2.2. 30 ml of erythrocyte membrane suspension prepared from about 250 ml

of blood, were spin-labeled using procedure 1.2., except that the last centrifugation was run at 150 000 × *g*.

2.3. The bovine-erythrocyte membranes were spin-labeled according to the procedure described by Morrisett et al. [1].

3. The selectivity of MeSL binding

The selectivity of MeSL binding was tested as follows: eserine was used as the protective substance. In this case, the enzyme preparation was preincubated with 1 or 10 μM eserine for 30 min before spin label was added; further, the first washing medium (procedures 1.2. and 2.2.) or the first two washing media (procedures 1.3. and 2.3.) also contained eserine, at the same concentration as during preincubation. Since Morrisett et al. [1] used 1 μM BW 284C51 (1,5-bis(4-allyldimethylammoniumphenyl)pentan-3-on dibromide) as the protective substance, its effect on the MeSL binding was tested under the same conditions as those for eserine.

The reactivation by 2-PAM (1-methyl-2-hydroxy-iminomethyl-pyridinium iodide) of spin-labeled acetylcholinesterase was used as an additional test of the selectivity of spin-label binding. After incubation of acetylcholinesterase with the spin label (1.2.), the enzyme preparation was incubated in 180 ml of 50 mM 2-PAM solution for 90 min at 37°C. The suspension was centrifuged as described in procedure 1.1., and the sediment washed two times by elasmobranch medium with 50 mM 2-PAM and, finally, three times with elasmobranch medium alone.

The binding selectivity was checked by active serine vs. MeSL stoichiometry. The number of active serines of the non-spin labeled preparation was calculated from the enzyme activity and the corresponding turnover numbers. In the present calculations the turnover numbers $4.8 \cdot 10^5$ and $2.3 \cdot 10^5$ molecules of ACh cleaved per mol of active site per min were used for membrane acetylcholinesterase of *Torpedo marmorata* electric organ [5] and bovine erythrocytes [6], respectively. The number of MeSL molecules bound to active serines was calculated from the reduction of the enzyme activity; the number of MeSL molecules bound to the enzyme preparation was estimated from the intensity of ESR spectra.

The selectivity of MeSL binding was compared using both procedures: the membrane acetylcholinesterase preparation was spin-labeled using either procedure 1.2. or procedure 2.2. After determination of the enzyme activity and recording the ESR spectra, the same preparation was spin-labeled for the second time, using either procedure 1.3. or 2.3.

4. Measurements

For the measurement of enzyme activity, an aliquot of suspension of the membrane acetylcholinesterase preparation, depending on the enzyme activity, was added to 45 ml of either elasmobranch or mammalian Ringer solution containing 1 μM *N,N'*-diisopropylphosphorodiamine (iso-OMPA), and titrated with 0.01 M NaOH, at pH 7.4 and 25°C and 1 mM ACh using a Radiometer titration set: autoburette ABU 13, pH-meter 26 and titrator 11.

For recording the ESR spectra, 0.1 g of the enzyme preparation was placed in a quartz tissue cell and measured on a Varian E-9 X band spectrometer,

modulation frequency 100 kHz, modulation amplitude 2 G and microwave power 50 mW; no saturation effect was observed at this power. The last supernatant was also measured.

Results and Discussion

Supernatants. If the spin-labeling procedures 1.2. and 2.2. were used, no measurable amounts of MeSL were found in the last supernatant; if, however, the spin-labeling procedures 1.3. and 2.3. were used, traces ($<10^{12}$ molecules) of MeSL were recorded.

Membrane acetylcholinesterase of Torpedo marmorata electric organ. The ESR spectra of membrane acetylcholinesterase preparations spin-labeled using either procedure 1.2. (Fig. 1) or 1.3. (Fig. 2) differ both in shape and intensity. The reasons for this discrepancy can be deduced from the data in Table I.

If the procedure of Sentjurc et al. [2] was used, 10 μM eserine protects more than 95% and 1 μM eserine about 90% of the enzyme activity; on the other hand 1 μM BW 284C51 protects only about 20% of it. Since eserine displays a high selectivity for the catalytic centers of *Torpedo marmorata* membrane acetylcholinesterase [2], we conclude that the MeSL molecules are bound to active serines of the enzyme. Furthermore, BW 284C51 is inadequate as a protective substance because most of the enzyme activity was inhibited after spin labeling.

The selective binding of MeSL to active sites of the enzymes according to the method of Sentjurc et al. [2] was also demonstrated in experiments with 50 mM 2-PAM as reactivator because the reactivation of enzyme activity (approx. 35%) is consistent with the reduction of the intensity of ESR spectra (approx. 40%). It should be noted that 0.5 mM 2-PAM did not reactivate the enzyme. About 15% reactivation was obtained by 1,3-bis(4-hydroxyiminomethyl-pyridinium (1))-propane dibromide (TMB_4). Since it is known that

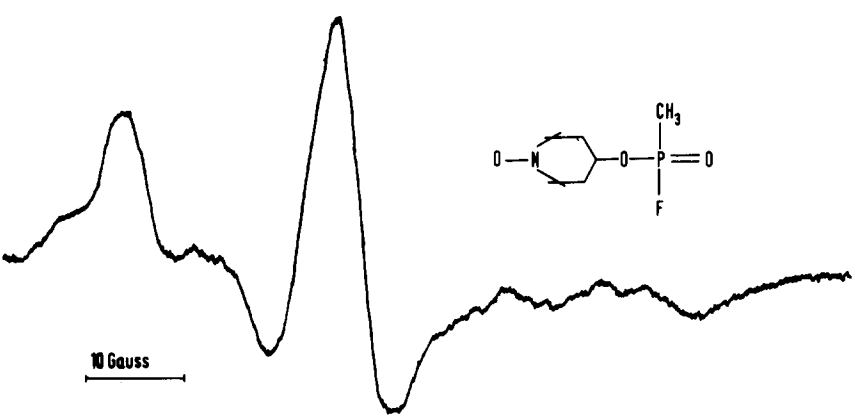

Fig. 1. ESR spectrum of 1-oxyl-2,2,6,6-tetramethyl-4-piperidinylmethyl-phosphonofluoridate bound to a membrane acetylcholinesterase preparation of *Torpedo marmorata* electric organ after the spin-labeling procedure of Sentjurc et al.: after 30 min of incubation of the enzyme preparation with 2.5 μM spin label, the preparation was washed 5 times by centrifugation; the number of bound spin-label molecules in the preparation was approx. $3 \cdot 10^{14}$; ν, 9280 MHz; H, 3240—3320 G; room temperature.

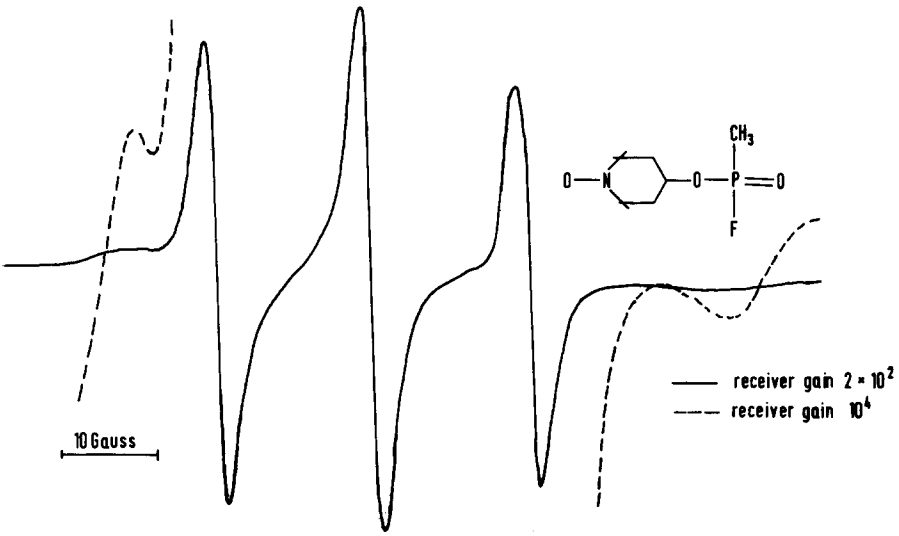

Fig. 2. ESR spectrum of 1-oxyl-2,2,6,6-tetramethyl-4-piperidinylmethyl-phoshonofluoridate bound to membrane acetylcholinesterase preparation of *Torpedo marmorata* electric organ after the spin-labeling procedure of Morrisett et al. [1]: after 10 h of incubation of enzyme preparation with 33 mM spin-label, the preparation was washed six times by centrifugation; the number of bound spin-label molecules in the preparation was approx. $3.5 \cdot 10^{15}$; ν, 9280 MHz; H, 3240—3320 G; room temperature.

acetylcholinesterase inhibited by some fluorophosphates can not be completely reactivated by nucleophilic site-directing reagents [7,8], the incomplete reactivation of the enzyme is not surprising.

If, however, the procedure of Morrisett et al. [1] was used, the protective effect of eserine even at 10 μM was only 40%. Apparently, the concentration of MeSL was too high and the incubation time too long to enable eserine to protect the active serine from irreversible binding of MeSL.

Furthermore, it can be seen from Table I that the stoichiometry of the number of active serines before spin-labeling to the number of bound MeSL molecules is about 1: 1 in the case of the spin-labeling procedure of Sentjurc et al. [2] only, while in the case of the spin-labeling procedure of Morrisett et al. [1], it is from about 1 : 15 to about 1 : 23. The finding that after the pro-

TABLE I

COMPARISON OF TWO SPIN-LABELING PROCEDURES

The number of spin label molecules bound to 0.1 g of the membrane acetylcholinesterase preparations of *Torpedo marmorata* electric organ, $\times 10^{14}$.

Spin-labeling in the presence of	Spin-labeling according to Sentjurc et al.		Spin-labeling according to Morrisett et al.	
	From enzyme activity	From ESR spectra	From enzyme activity	From ESR spectra
—	2.5	3.0	2.3	35
10 μM eserine	0.1	<0.5	1.3	30
1 μM eserine	0.3	<0.5	1.4	25
1 μM BW	2.1	3.0	2.1	40

cedure of Morrisett et al. [1] there are up to 20 times more bound MeSL molecules than there are catalytic sites in the preparation, shows that this spin-labeling procedure is not selective for active serines.

The conclusion is corroborated by the following experiments. Initially, the membrane acetylcholinesterase preparation was spin-labeled according to procedure of Sentjurc et al. [2]; the enzyme preparation was devoid of activity and the number of MeSL molecules bound to the membrane acetylcholinesterase preparation was nearly the same as the number of active serines (Table I); The same preparation was then additionally spin-labeled according to Morrisett et al. [1]. The ESR spectrum was equal to that in Fig. 2. Though all catalytic sites of the preparation were already irreversibly inhibited by MeSL molecules bound during the first spin-labeling, about ten times more MeSL molecules were found in the preparation after additional spin-labeling; this shows that after the procedure of Morrisett et al. [1] about 90% of the MeSL molecules are bound to sites other than active serines. A higher receiver gain reveals that the ESR spectrum of non-selectively bound MeSL is superimposed on the spectrum of MeSL bound to active serines (Fig. 2).

Non-selective binding of MeSL molecules to the enzyme preparation was also encountered in experiments in which the spin-labeling procedure was followed under conditions (1 mM MeSL, 5 min, 5°C) described by Morrisett and Broomfield [9] as selective for partially-purified acetylcholineasterase from the electric organ of *Electrophorus electricus*. In these experiments the incubation time was six times shorter and the concentration of MeSL 400 times higher than in the procedure of Sentjurc et al. [2]. Therefore, it may be concluded that in the procedure of Morrisett et al. [1] the primary reason for non-selective binding is too high a concentration of the spin label.

Membrane acetylcholinesterase of bovine erythrocytes. Experiments and results with bovine erythrocytes were analogous to those with *Torpedo marmorata* membrane acetylcholinesterase, and thus will be described only in brief. In order to get a reliable ESR signal, about 10^{14} MeSL molecules should be bound in a 0.1-g sample of membrane acetylcholinesterase. On the other hand, 10^{14} active serines are found only in about 15 ml of erythrocyte ghost suspension. After procedure 2.2., the enzyme activity was completely inhibited by MeSL and the volume of erythrocyte ghosts containing about 10^{14} MeSL molecules was still about 3 ml. Thus, no more than $3.5 \cdot 10^{12}$ active serines were obtained in the volume of the tissue cell as a consequence of which no ESR signal was recorded. However, when bovine-erythrocyte membrane acetylcholinesterase was spin-labeled according to procedure 2.3., ESR spectra indicating about $1 \cdot 10^{15}$ MeSL molecules were found (Fig. 3). The shape of the spectrum points to a high degree of rotational freedom of the nitroxyl radical about the bond of attachement, similar to the spectra described by Morrisett et al. [1]. Since benzene is used in procedure 2.3., it was used also in procedure 2.2., but did not influence the ESR spectra.

The selectivity of MeSL binding to bovine membrane acetylcholinesterase prepared according to procedure 2.3. was tested as for *Torpedo marmorata* membrane acetylcholinesterase: after spin-labeling procedure 2.2., the same preparation was additionally spin-labeled using procedure 2.3.. The shape of the spectrum was similar to that in Fig. 3. The number of MeSL molecules

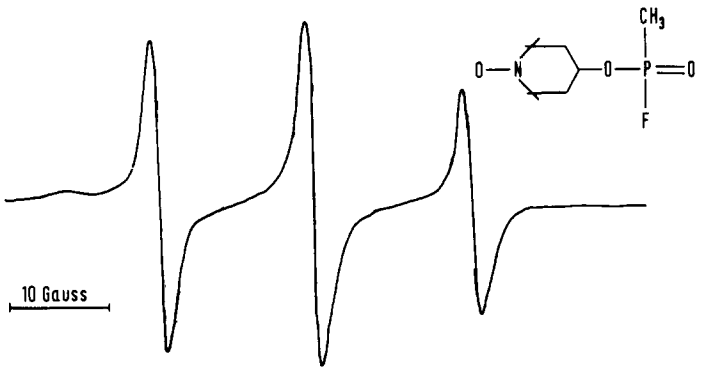

Fig. 3. ESR spectrum of 1-oxyl-2,2,6,6-tetramethyl-4-piperidinylmethyl-phosphonofluoridate bound to membrane acetylcholinesterase preparation of bovine-erythrocyte ghosts after the spin-labeling procedure of Morrisett et al. [1]: after 10 h of incubation of enzyme preparation with 33 mM spin label, the preparation was washed six times by centrifugation; the number of bound spin label molecules in the preparation was approx. $1 \cdot 10^{15}$; ν, 9280 MHz; H, 3240—3320 G; room temperature.

bound in this enzyme preparation was found to be about a hundred times higher than the number of active serines in it.

Conclusions

From both the intensity and the shape of the described spectra, we conclude that the active serines of membrane acetylcholinesterase of *Torpedo marmorata* electric organ are located in a pocket of the enzyme surface. Because of the limited sensitivity of the ESR spectrometer, it was impossible to detect MeSL bound to active serines of membrane acetylcholinesterase of bovine erythrocytes and therefore to draw conclusions about the microgeography around the active serine. Since after a spin-labeling procedure according to 2.3., there are up to a hundred times more MeSL molecules bound in the membrane acetylcholinesterase preparations than active serines in it, the suggestion of Morrisett et al. [1] that the active site of membrane acetylcholinesterase is well exposed on the enzyme surface, is apparently based on unselective spin-labeling.

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