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AN ESR STUDY OF THE INFLUENCE OF SOME PHYSICO-CHEMICAL FACTORS ON THE CONFORMATION OF A POSTSYNAPTIC ACETYLCHOLINESTERASE

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Summary

1. In a previous ESR study of a membrane acetylcholinesterase (EC 3.1.1.7) we found, contrary to observations by other authors, spectra indicating that the active serine might be located in a pocket of the enzyme surface. In order to inquire into this possibility, ESR spectra were studied under the influence of different physico-chemical factors known to cause an unfolding of proteins.

2. The active serine of the postsynaptic membrane acetylcholinesterase of *Torpedo marmorata* electric organ was spin labeled using 1-oxyl-2,2,6,6-tetramethyl-4-piperidinyletoxyphosphonofluoridate.

3. The effect of the chosen physico-chemical factors was an increase in the rotational freedom of spin labels; this result corroborates the suggestion that the active center of our acetylcholinesterase preparation is located in a pocket.

Introduction

The spin label technique has been found useful in the study of the shape of the active surface in enzymes as well as of their conformational changes [1—3]. Spin labels usually used in ESR studies of biomacromolecules are nitroxide spin labels which exhibit a strong anisotropic hyperfine interaction between the unpaired electron and the N^{14} nucleus. Therefore, the lineshape of the ESR spectrum is sensitive to the changes of molecular motional freedom over a wide range of rates represented by the rotational correlation times from 10^{-11} to 10^{-7} s. Any conformational change in the macromolecule in the vicinity of the

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Abbreviations: EtOSL, 1-oxyl-2,2,6,6-tetramethyl-4-piperidinylethoxyphosphonofluoridate; MeSL, 1-oxyl-2,2,6,6-tetramethyl-4-piperidinylmethylphosphonofluoridate.

spin label can be reflected in the motional freedom of the label and consequently on the ESR spectra.

In ESR studies of horse serum butyrylcholinesterase and of either soluble or membrane acetylcholinesterases (EC 3.1.1.7) (human or bovine erythrocytes, rat brain nerve ending particles and electric eel) the spectra indicate fast molecular motion of spin labels bound to serine residues in the active site [4—7]; some authors conclude that the active site is well exposed on the surface of the enzymes [4,5]. On the other hand, in our ESR study of membrane acetylcholinesterase with EtOSL selectively bound to the active serine, spectra were obtained indicating that spin label motion is strongly restricted by the surrounding groups and that, consequently, the active center is located in a pocket of the enzyme surface [8]. In order to test this possibility, ESR spectra of a membrane acetylcholinesterase were studied under the influence of different physico chemical factors most of which are known to change the conformation of proteins up to their unfolding, and thus more and more exposing the spin label molecules. In the present paper the effects of urea, pH, ammonium sulphate, chloroform and temperature on ESR spectra were studied.

Materials and Methods

Membrane acetylcholinesterase preparation and the spin labeling procedure

The *Torpedo marmorata* electric organ (8 g) was sheared in modified elasmobranch Ringer solution [9] in which the bicarbonate buffer was replaced by 2 mM Tris (elasmobranch medium), pH 7.7; immediately after shearing, the pH was adjusted with 0.1 M NaOH to 7.4 and the preparation centrifuged for 30 min at 40 000 $\times g$ and 0°C; 0.2 g of the sediment was suspended in 30 ml of elasmobranch medium, pH 7.4, and incubated for 30 min with 2.5 μM EtOSL or MeSL at room temperature and continuous stirring. After adding 150 ml of elasmobranch medium, the suspension was washed by centrifugation under the same conditions as above, the sediment washed three times with 180 ml of elasmobranch medium and, finally, with 50 ml of elasmobranch medium for 60 min. For details see ref. 8.

Selectivity of spin label binding

The selectivity of the binding of spin label molecules to active serines was borne out with eserine as the protective substance and by the correspondence between the number of the bound spin label molecules and the number of active serines in the enzyme preparation. The enzyme preparation was preincubated with 0.1 to 10 μM eserine for 30 min before the spin label was added; further, the first washing medium also contained eserine, in the same concentration as during preincubation. The number of the active serines of the non-spin labeled preparation was calculated from the enzyme activity and the catalytic center activity of *Torpedo marmorata* electric organ [10]. On the other hand, the number of spin label molecules bound to active serines was calculated from the reduction of the enzyme activity; the number of spin label molecules bound to the enzyme preparation was estimated from the intensity of the ESR spectra, which was compared with the intensity of the Varian weak pitch standard (0.00033% of pitch in KCl) in a dual sample cavity [11].

Measurements

The enzyme activity was measured as follows: an aliquot of the membrane acetylcholinesterase preparation, depending on the enzyme activity, was added to 45 ml of elasmobranch medium and titrated with 0.01 M NaOH, at pH 7.4 and 25°C and 1 mM acetylcholine using a Radiometer titration set: autoburette ABU 13, pH-meter 26 and titrator 11.

For recording the ESR spectra, 0.1 ml of sediment was inserted in a quartz tissue cell and measured on a Varian E-9 X band spectrometer, modulation frequency 100 kHz, modulation amplitude 2 G, microwave power 50 mW. No power saturation effect was observed up to 80 mW, either for the broader or the narrower spectral components characteristic of denaturated samples. It seems that owing to the low factor of merit (Q) the value of the actual microwave field in the tissue sample was below the level at which saturation can be achieved. The high power was chosen because in the preparation there are only about 10^{14} spin label molecules and the lines are broad (Fig. 1). Therefore only at this power level was a satisfactory signal to noise ratio achieved.

The total protein content was determined according to Lowry et al. [12] and the total lipid content according to Folch et al. [13].

Effects of physico-chemical factors

In order to study the effects of physico-chemical factors, the spin labeled membrane acetylcholinesterase preparation was exposed to urea, low pH, am-

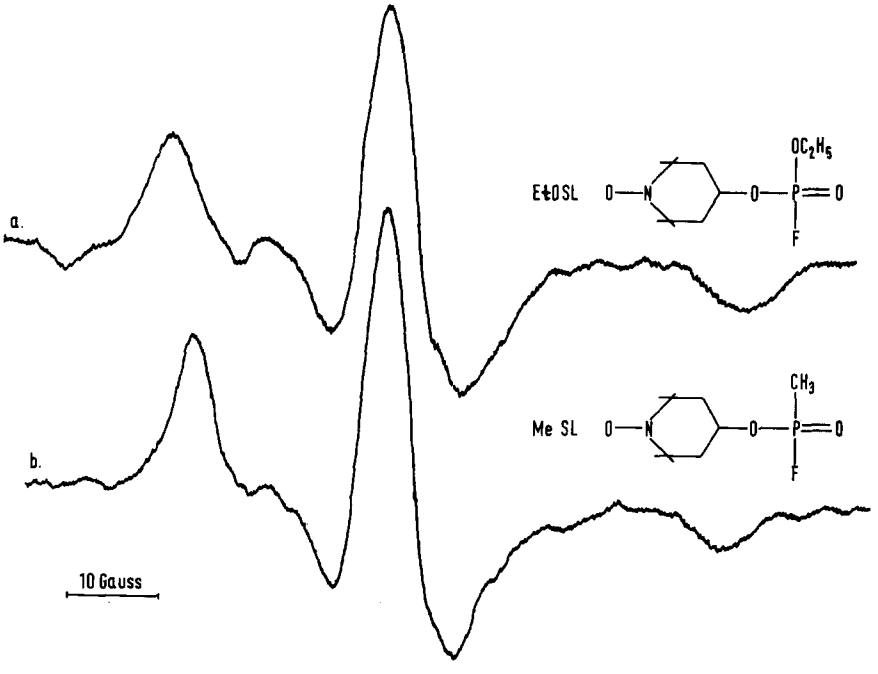

Fig. 1. ESR spectra of the spin labels bound to 0.1 ml of the membrane acetylcholinesterase of *Torpedo marmorata* electric organ: the enzyme preparation was incubated for 30 min with 2.5 μM spin label, and the unreacted reagent washed away; free active serine concentration in the sample was approx. 1 μM and that of spin labeled active serines approx. 4 μM; ν = 9280 mHz; H = 3240—3320 G; at room temperature.

monium sulphate and high temperature and the spectra recorded either in their presence or absence. The non-spin labeled membrane acetylcholinesterase preparation was also exposed to the chosen physico-chemical factors and the enzyme activity pH-metrically measured at pH 7.4 either in their presence or absence. For other experimental conditions see the corresponding figures and tables.

In some experiments, 30 ml of spin labeled or non-spin labeled membrane acetylcholinesterase suspension was shaken with 30 ml of chloroform and the water phase used for the subsequent preparation of membrane acetylcholinesterase.

Results and Discussion

After the preparation was incubated with 2.5 μM spin label and subsequently washed, the last supernatant was devoid of the spin label.

If 1 μM eserine was used as the protective substance, the number of spin label molecules bound to membrane acetylcholinesterase was reduced to 5—10% (100% = number of spin label molecules bound to membrane acetylcholinesterase in the absence of eserine). Further, the stoichiometry of the number of active serines inhibited by the spin label to the number of bound spin label molecules was found to be about 1 : 1. Both the effect of eserine and the stoichiometry lead to the conclusion that practically no spin label molecules are bound to locations other than active serines.

Protein and lipid composition of main variously treated membrane acetylcholinesterase preparations is shown in Table I.

The ESR spectrum of EtOSL bound to membrane acetylcholinesterase is shown in Fig. 1; it can be seen that the motion of the EtOSL molecules is restricted. The distance between the two outermost extrema: A_{max} = 63 ± 0.2 G (x ± S.E., n = 20) is close to the rigid limit. The order of magnitude of the estimated correlation time is 10^{-7} sec.

The effect of urea on the ESR spectrum (Fig. 2) and on the enzyme activity (Table II) was followed in the concentration range from 0.33 to 8 M.

From Fig. 2 it can be concluded that after treatment with a 2 M urea solution the spectra are superpositions of at least two populations of EtOSL mole-

TABLE I

PROTEIN AND LIPID COMPOSITION

Protein and lipid composition of 0.1 g samples of the variously treated membrane acetylcholinesterase preparations*

Treatment	Dry weight (mg)	Proteins (mg)	Lipids (mg)
Control	13	5	7
8 M urea**	34	3	28
Chloroform	17	11	5
pH = 3	25	9	12

* Values represent the average of two determinations.
** After treatment the urea was washed out.

TABLE II

INFLUENCE OF UREA

The influence of urea on the enzyme activity of membrane bound acetylcholinesterase of *Torpedo marmorata* electric tissue*

Concentration of urea (M)	Percent activity (1 mM ACh)	
	In the presence of urea	Urea*** washed out
0.38	100**	100**
1	98	100
2	69	93
3	33	68
4	9	5
5	0	0

* Values represent average of two or three determinations.
** Control
*** Preincubation time with urea was 30 min.

cules with different motional freedoms. The presence of two types of spectra means that exchange between different populations occurs at a rate below 10^8 s^{-1}. A fraction of EtOSL molecules qualified by a higher motional freedom indicates that in a fraction of membrane acetylcholinesterase most steric hindrances are removed. This fraction increases with the increasing urea concen-

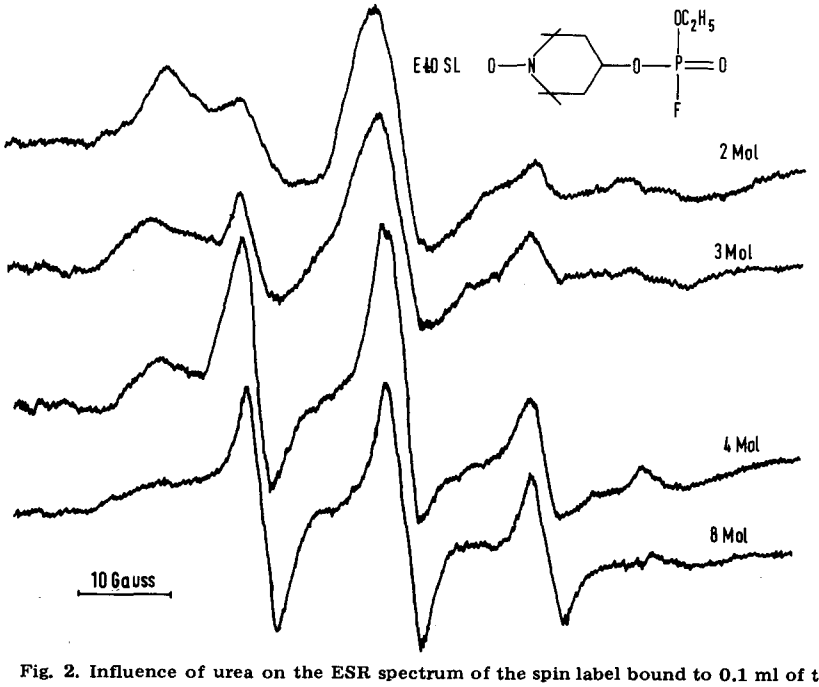

Fig. 2. Influence of urea on the ESR spectrum of the spin label bound to 0.1 ml of the membrane acetylcholinesterase of *Torpedo marmorata* electric organ; the enzyme preparation was incubated for 30 min with 2.5 μM 1-oxyl-2,2,6,6-tetramethyl-4-piperidinyletoxyphosphonofluoridate, and the unreacted reagent washed away using elasmobranch medium with urea; free active serine concentration in the sample was approx. 1 μM and that of spin labeled active serines approx. 4 μM; ν = 9280 mHz; H = 3240—3320 G; at room temperature.

tration in accordance with the decreasing enzyme activity. At 8 M urea, all EtOSL molecules exhibit a fast rotational motion with τ_c in the range of 10^{-8}—10^{-9} s. At this urea concentration no enzyme activity was observed. Identical results were obtained if after incubation with urea the preparation was washed by repeated centrifugation before recording the ESR spectra, and thus the change in the spectrum can not be ascribed to a possible solubilization of membrane acetylcholinesterase under the influence of urea.

The effect of pH was followed from pH 7.4 to 3 (Fig. 3). The effect of preincubation at pH in the range from 7.4 to 3 on the enzyme activity, measured at 7.4, is shown in Table III. At pH 4 the lineshape of the spectrum is practically still the same as at pH 7.4, although the hydrolytic activity decreases by more than 50%. At pH 3 a marked change is observed in the ESR spectrum. It seems that at this pH only a fraction of spin label molecules remains immobilized, whereas the other one displays fast rotational motion as it does in the case of 2 M urea. After incubation of membrane acetylcholinesterase at pH 3, no hydrolytic activity is found.

In the presence of 2.2 M $(NH_4)_2SO_4$ no change in the shape of the spectrum of EtOSL molecules bound to the membrane acetylcholinesterase preparation was found. After 30 min of incubation of membrane acetylcholinesterase with $(NH_4)_2SO_4$ at the same concentration, and after washing out the salt, the enzyme activity was unchanged.

After chloroform extraction of the enzyme preparation, the lipid content of the sample decreased, whereas the protein content rose (Table I). The effect of

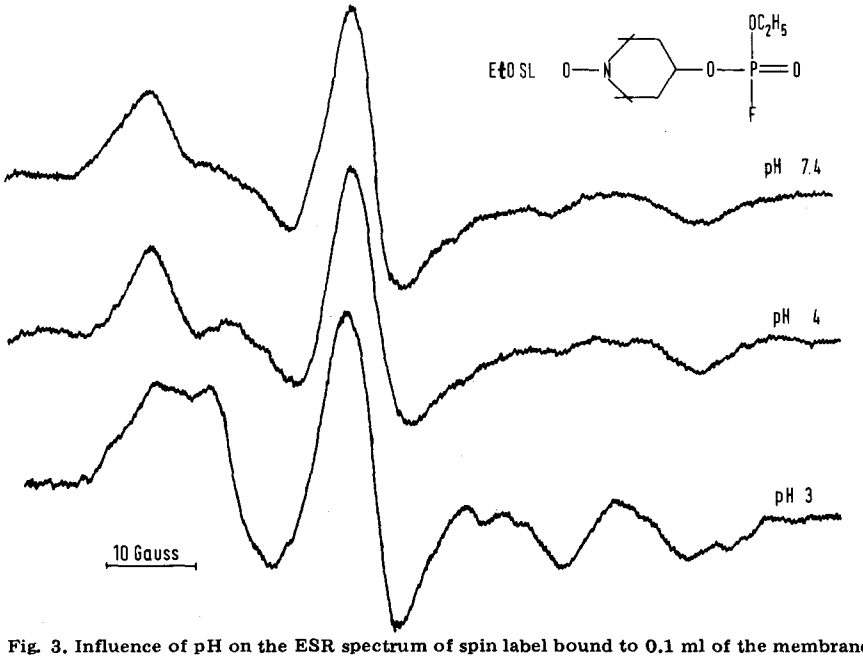

Fig. 3. Influence of pH on the ESR spectrum of spin label bound to 0.1 ml of the membrane acetylcholinesterase of *Torpedo marmorata* electric organ; the enzyme preparation was incubated for 30 min with 2.5 μM 1-oxyl-2,2,6,6-tetramethyl-4-piperidinyletoxyphosphonofluoridate, and the unreacted reagent washed away; free active serine concentration in the sample was approx. 1 μM and that of spin labeled active serines approx. 4 μM; ν = 9280 mHz; H = 3240—3320 G; at room temperature.

TABLE III

INFLUENCE OF pH

The influence of pH on the enzyme activity of membrane bound acetylcholinesterase of *Torpedo marmorata* electric organ *

Preincubated at pH (30 min)	% activity at pH 7.4 (1 mM ACh)
7.4	100**
6.0	100
5.0	83
4.0	43
3.0	0

* Values represent average of two or three determinations.
** Control.

chloroform extraction on the ESR spectra is reflected in a fast rotation of EtOSL molecules with $\tau_c \approx 10^{-9}$ s as it is in the case of 4 M urea (Fig. 2, curve c), though urea probably denatures proteins directly whereas chloroform extraction removes lipids. The enzyme activity of the chloroform-extracted membrane acetylcholinesterase was only about 5% of control.

The effect of temperature was recorded in the range from 0° to 60°C. From 0° to 40°C no change in the ESR lineshape was observed, but at 50°C the EtOSL molecules displayed a higher degree of motional freedom; after 10 min of incubation at this temperature membrane acetylcholinesterase was devoid of activity.

As has already been mentioned, the motion of EtOSL molecules bound to membrane acetylcholinesterase active centers is strongly restricted. All the investigated physico-chemical factors have the same influence on membrane acetylcholinesterase in the sense that at least two fractions of spin label molecules with different motional freedoms appear in the spectrum and that the more flexible fraction of spin label molecules increases with the decreasing enzyme activity.

To find out if the two populations characterized by different spin label motions were due to the progressive unfolding of the enzyme or to the removal of acetylcholinesterase from the membrane after treatment with such strong denaturants as are urea, chloroform, low pH and raised temperature, the purified acetylcholinesterase Worthington (code ECHP OIA) was labeled by the same procedure as membrane acetylcholinesterase, except that the enzyme preparation was washed out by alternative sedimentation with saturated ammonium sulphate solution and resuspension in elasmobranch medium. The spectra were the same as in the case of membrane acetylcholinesterase, indicating that the restricted motion is due to the immediate environment of spin label molecules and that the fraction of spin label molecules which exhibit free rotational motion is due to the unfolding of the active site.

Furthermore, in all cases only drastic influences causing irreversible unfolding of proteins have markedly affected the spin label motional freedom decreasing the correlation time from 10^{-7} to approximately 10^{-9} s. Spin label rotation is restricted by steric hindrances from surrounding groups supporting the suggestion that the active serine of membrane acetylcholinesterase is

located in a pocket of the enzyme surface and that, consequently, the serine spin label is buried in it. The above suggestion might be objected to because an association might occur between the piperidinyl part of the spin label molecule and the anionic binding site located in acetylcholinesterase close to the active serine. Nevertheless, in the presence of d-tubocurarine or phenyltrimethylammonium, τ_c remains unchanged even when the concentration of the compounds is as high as 1 mM. Since both substances react with the anionic sites of acetylcholinesterase, it is less probable that constraints on the spin label before protein denaturation are mainly due to an association between the anionic site and the piperidinyl part of the spin label molecule.

The discrepancy between the present results obtained with EtOSL and those of other authors obtained with MeSL [5,7] might be due to the similar, but still different, serine spin label used. For this reason MeSL was synthetised and the shape of its spectra was found to be similar to that of the spectra of EtOSL (Fig. 1), except that $A_{max} = 60 \pm 0.7$ G ($n = 5$). Experiments with MeSL were also run in the presence of urea at different concentrations; with this spin label no change in the ESR was observed until the urea concentration was 4 M; at higher concentrations, the changes were similar to those obtained with EtOSL.

Further reasons for the discrepancy between the results obtained by Morrisett et al. [5] and those obtained by the present authors might be due to differences in the membrane acetylcholinesterase preparations or in the spin labeling procedures. Experiments to elucidate these problems will be published in a separate paper.

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