ESSENTIAL ARGININE RESIDUE IN ACETYLCHOLINESTERASE FROM *TORPEDO CALIFORNICA**

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1. Introduction

Acetylcholinesterase (EC 3.1.1.7) has been the subject of many kinetic studies [2]. However, neither the kinetic mechanisms nor the amino acid residues involved in substrate binding with the exception of the activated serine residue [2] could be elucidated completely from these studies. From kinetic and chemical modification data histidine [3] and tyrosine [4] residues have been proposed to have functional roles in the catalytic mechanism and are located near or within the active centre of the enzyme.

A carboxyl residue different from that within the anionic part of the active centre was localized [5] within one of the peripheric anionic sites [2]. But nothing is known about the function of the basic amino acids like lysine and arginine. Since lysine as well as arginine are able to bind negatively charged substrates and also stabilize conformations we have investigated the role of basic amino acids in the catalytical mechanism of acetylcholinesterase by chemical modification of the arginine residues with both butanedione and phenylglyoxal, which are arginine-specific reagents. The chemical modifications were also carried out in the presence of the effectors *N*-methylpyridinium-2-aldoxime iodide, flaxedil, edrophonium, hexamethonium and decamethonium, because Changeux [6] observed an activation effect of peripheric bound ligands, probably caused by allosteric effects [4,7]. The location of the modified arginine residues and their participation in the catalytic mechanism is discussed.

* This work has been described in a preliminary report [1]

2. Experimental

Acetylcholinesterase was prepared from the electric organs of *Torpedo californica* delivered deep frozen by the Pacific Biomarine Labs, Venice, California [8]. The '11 S' or tryptic species was further purified by a single step affinity chromatography using the strong inhibitor phenyltrimethylammonium bromide coupled to Sepharose 4B [9]. A highly active enzyme preparation (specific activity about 3000 I. U./mg) containing 5% impurity was obtained.

Specific activity was measured at 25°C in 50 mM phosphate buffer, pH 7.2, with acetylthiocholine (500 μM) as the substrate according to [10]. Protein concentration was determined by the Folin method using serum albumin as standard. The amino acids were hydrolyzed in 6 M HCl for 24 h at 110°C and analyzed on the Biotronik amino acid analyzer using both short and long columns according to [11]. Chemical modification by butanedione or by phenylglyoxal was carried out at 25°C in phosphate-borate buffer (5 mM phosphate, 25 mM borate, 50 mM sodium chloride), pH 7.0, at a protein concentration in the incubation mixture of 10–20 μg/ml. Enzymatic activity was measured at various intervals under standard test conditions by the addition of 5 μl of the incubation mixture to the standard assay. Neither butanedione nor phenylglyoxal affected the enzymatic activity under standard assay conditions.

Butanedione (freshly distilled before use), phenylglyoxal, *N*-methylpyridinium-2-aldoxime iodide and decamethonium bromide were obtained from Fluka (Buchs, Switzerland). Acetylthiocholine and dithionitrobenzoic acid were purchased from Serva (Heidelberg, FRG), flaxedil from Böhringer (Ingelheim, FRG), and hexamethonium bromide

from Sigma (St Louis, USA). Buffer substances, all of analytical grade, were products of E. Merck (Darmstadt, FRG). Edrophonium bromide was synthesized by quaternization of *N,N*-dimethyl-*m*-aminophenol (Fluka, Buchs, Switzerland) with ethyl bromide (Merck) in acetone, according to [12].

The circular dichroism spectra of native and modified acetylcholinesterase (dissolved in phosphate-borate buffer pH 7.0) were obtained with a Cary 60 spectrophotometer (Varian, Darmstadt, FRG) equipped with a CD-device (6002). Ultracentrifugation analyses were performed in an analytical ultracentrifuge Spinco E from Beckman Instruments (München, FRG) at 20°C and 60 000 rev./min in phosphate-borate buffer pH 7.0, also used for the chemical modification. The protein concentration was 0.2–0.4 mg/ml.

3. Results

Whereas phenylglyoxal has almost no effect on enzyme activity during the period of incubation, butanedione inactivates the enzyme over the same time period. The time course of this inactivation is presented in fig.1.

Butadione inactivation is concentration-dependent. After 90 min, the enzyme is inactivated to about 80% in the presence of 4.6 mM butanedione. Longer incubation, even in the presence of higher concentrations of butanedione, does not cause complete inactivation of the enzyme and a residual activity of 5–10% always remains. The inactivation, however, is not reversible. Dialysis against butanedione- and borate-free buffer, dilution of samples with buffer, free of borate and butanedione, or incubation with 0.1 M NH_2CO could not restore the enzymatic activity.

Incubation with butanedione in the presence of effectors, as shown in fig.2, demonstrates that butanedione acts specifically because *N*-methylpyridinium-2-aldoxime iodide and flaxedil protect the enzyme almost completely against inactivation by this reagent, whereas the other effectors studied have weaker but significant effects. Edrophonium which binds specifically with a high affinity to the active centre, protects to a greater extent than the bifunctional compounds hexamethonium and decamethonium which show the weakest protection.

The amino acid analyses of the native enzyme protein and the samples treated with butanedione and phenylglyoxal in the absence or presence of *N*-methylpyridinium-2-aldoxime iodide (table 1) reveal that only arginine residues are modified. Phenylglyoxal modifies three arginine residues with a maximum activity loss of 15%. Butanedione modifies four arginine residues out of a possible thirtyone with

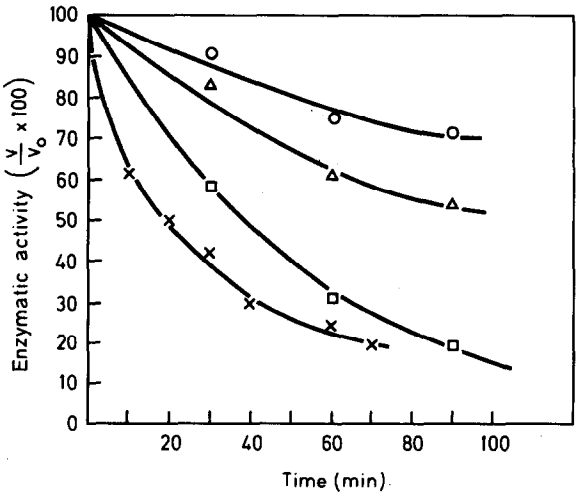

Fig.1. Time course of the loss of enzymatic activity of acetylcholinesterase after incubation with butanedione (○, 1.2 mM; △, 2.3 mM; □, 4.6 mM; ×, 6.9 mM). v_0 is the initial rate of the control, v that of the modified enzyme.

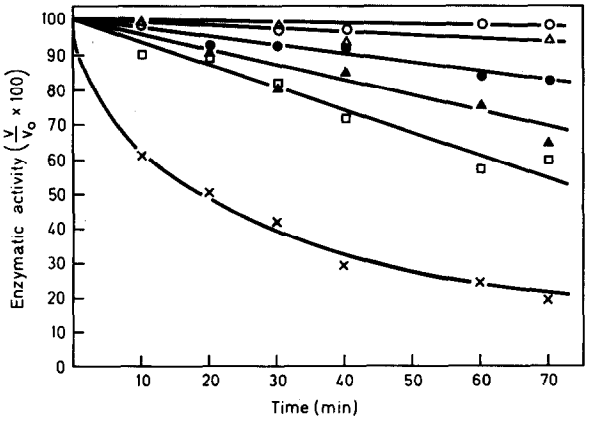

Fig.2. Influence of effectors on the time course of the inactivation of acetylcholinesterase by butanedione (5.6 mM) Incubation in the presence of edrophonium (●), hexamethonium (□), decamethonium (▲), flaxedil (△) and *N*-methylpyridinium-2-aldoxime iodide (○). Concentration of the effectors in each case 100 μM. × is the control in the absence of effector. v_0 is the initial rate of the control, v that of the enzyme incubated with butanedione and effector.

Table 1
Arginine content of acetylcholinesterase after modification with butanedione and phenylglyoxal

Sample	mol Arg/mol polypeptide chain	Enzymatic activity (%)
Control	31 ± 0.4	100
+ Butanedione, 5,6 mM	27.3 ± 0.2	27
+ Butanedione, 5.6 mM, and *N*-phenylpyridinium-2-aldoxime iodide, 100 μM	28.7 ± 0.4	98
+ Phenylglyoxal, 5 mM	28.2 ± 0.6	83

Amino acid analysis was performed (six analyses in each case) after incubation during 90 min under the same conditions as in fig.2, followed by dialysis against phosphate-borate buffer, pH 7.0 (two changes, 12 h) and distilled water (6 h)

more than 70% loss of activity. In the presence of *N*-methylpyridinium-2-aldoxime iodide, however, only three arginine residues without inactivation are modified, as was obtained with phenylglyoxal as the chemical modifier. From this it can be concluded that *N*-methylpyridinium-2-aldoxime iodide protects one arginine against modification and that this arginine residue is essential for the enzymatic activity.

The Michaelis constant of the enzyme for acetylthiocholine remains almost unchanged after modification (60 μM compared to 50 μM for the unmodified enzyme), but V_{max} decreases significantly from 3030 I.U./mg to 150 I.U./mg. However, sedimentation analysis shows that modification by butanedione changes $s_{20,w}$ from 10.5 S for the native enzyme to 10.0 S, indicating a small effect on the conformation. Incubation with butanedione in the presence of *N*-methylpyridinium-2-aldoxime iodide has less effect ($s_{20,w}$ 10.3 S). The decrease of the enzymatic activity caused by butanedione modification is accompanied by a decrease of circular dichroism amplitude at 229 nm. The data obtained from both, circular dichroism measurements and the sedimentation analysis indicate a conformational change caused by the modification of one arginine residue which can be protected by *N*-methylpyridinium-2-aldoxime iodide and flaxedil, since the modification in the presence of *N*-methylpyridinium-2-aldoxime iodide causes only a very small reduction of the amplitude at 229 nm.

4. Discussion

N-Methylpyridinium-2-aldoxime iodide and flaxedil protect the enzyme against inactivation by butanedione, edrophonium – although a very specific ligand for the active centre – is less effective, but affords better protection than either hexamethonium or decamethonium.

The protection effect might be due to one or both of the following reasons: (a) Steric hindrance. If the ligand is bound at the site where modification occurs, less inactivation should be observed. (b) If the ligand L induces a conformational change of the enzyme E such that the reactive residue becomes inaccessible, the reaction can be prevented according to the equilibrium of $E + L \rightleftharpoons EL$.

The effects of *N*-methylpyridinium-2-aldoxime iodide and flaxedil can be ascribed to both or either of these reasons, but a conformational change is most probable. When we consider the effects of all effectors used, their one common factor (edrophonium [13], hexamethonium and decamethonium [16], flaxedil [6,14] and *N*-methylpyridinium-2-aldoxime iodide [14–16]) is that they bind to the anionic part of the active centre in the presence of high ionic strength buffers used in our experiments. For this reason, a possible conformational change must be produced by binding to the anionic part of the active centre. This is indicated not only by sedimentation analysis, but also by circular dichroism measurements. But the binding of a quaternary ammonium group alone, like those of decamethonium, is not sufficient to achieve the conformational change which is necessary to induce the protection of the arginine residue. The three ligands with the best protection against inactivation, flaxedil, *N*-methylpyridinium-2-aldoxime iodide and edrophonium, all possess an aromatic ring.

Therefore, both ionic and hydrophobic interactions have to act together to produce a conformational change necessary for complete protection of the arginine residues in the active centre. It could be imagined that these compounds which contain both ionic as well as hydrophobic moieties act as a 'clamp' which distorts the conformation. In this connection, hydrophobic areas which can interact with aromatic ligands have been shown to be near to the anionic part of the active centre of the electric eel enzyme [17].

Summarizing our results, we can conclude that one of the four modified arginine residues near the anionic part of the active centre is protected against modification by a conformational change induced by binding of an aromatic compound to that site. Perhaps the inability of phenylglyoxal to yield the same result as butanedione is also caused by this effect. Modification of this arginine residue has an effect on the enzyme conformation and seems to influence the enzymatic activity only by this conformational change and not by direct influence on the catalytic mechanism.

Acknowledgements

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