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Acetylcholinesterase Inhibition by Alkanesulfonylchlorides: Allosteric Regulation by Tetraalkylammonium Ions

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Acetylcholinesterase inhibition by *n*-alkanesulfonylchlorides $C_nH_{2n+1}SO_2Cl$ at $n = 1–4$ and its regulation by tetraalkylammonium ligands $(C_nH_{2n+1})_4N^+$ at $n = 1–4$ was investigated at the level of the second-order rate constants at several temperatures. The influence of the tetraalkylammonium ions upon the sulfonylation reaction ranges from activation to inhibition and depends on the structure of both reagents. The tetraethylammonium ion was the most effective and the tetrabutylammonium ion the weakest accelerator. The acceleration of the reactions of the more active alkanesulfonylchlorides turned out to be greater. The influence of temperature revealed that changes in the activation entropy make larger contributions to the acceleration effects observed than do changes in the activation enthalpy. The structure–activity relationships of the inhibition of acetylcholinesterase suggest that the chemically inert alkyl groups of alkanesulfonylchlorides hinder this reaction because of some spatial restrictions. The acceleration effect can be related to the weakening of these hindrances as a result of a conformational change induced by the anionic site-located ligand. This change seems to be localized near the esteractic center and its influence declines sharply, becoming negligible in the case of propyl and butyl groups. The results obtained point to the interaction between two subsites which bind the acyl part and leaving group of ester substrates in the active center. It is suggested that similar interaction between different subsites is also important in the case of acetylcholinesterase interaction with choline esters.

INTRODUCTION

Methanesulfonylfluoride is supposed to sulfonylate the catalytic serine residue in the esteratic center of acetylcholinesterase (*1*). This reaction proceeds through the formation of the reversible complex which is followed by the bond-breaking step of enzyme sulfonylation, as has been experimentally shown by Pavlic and Wilson (*2*).

Under the second-order conditions this reaction can be accelerated by various cations (*3, 4*). The latter phenomenon has been explained by the formation of the ternary complex of the enzyme with the sulfonylating agent and the alkylammonium ligand, which are supposed to interact with the esteratic and the anionic sites of the enzyme, respectively (4). The influence of the structure of the alkylammonium ions upon the acceleration phenomenon has been studied by Pavlic (*3, 4*), Belleau and DiTullio (*5*), and Krupka (*6*), who have used methanesulfonylfluoride as a sulfonylating reagent. On the other hand, however, there are no experimental data about the influence of the structure of the sulfonylating reagent upon the

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effect of acceleration, which could give information about the structure and functioning of the esteratic center.

With the present study we have filled this gap by investigating acetylcholinesterase inhibition by a series of *n*-alkanesulfonylchlorides $C_nH_{2n+1}SO_2Cl$ at $n = 1–4$ in the presence of *n*-tetraalkylammonium ions $(C_nH_{2n+1})_4N^+$ at $n = 1–4$. Owing to the limited solubility of alkanesulfonylchlorides the values of the dissociation and catalytic constants for the inhibition reaction cannot be estimated with sufficient precision to perform their structure–activity analysis. For that reason the following analysis is based on the second-order rate constants, which correspond to the ratio of the rate and dissociation constants and therefore reflect both the noncovalent binding and the bond-breaking steps.

EXPERIMENTAL

Acetylcholinesterase (EC 3.1.1.7) from cobra (*Naja naja oxiana*) venom, purified by affinity chromatography, was generously donated by Dr. Raivo Raba (Institute of Chemical and Biological Physics of the Academy of Sciences of the Estonian SSR). All the chemicals and methods used in the study of the sulfonylation reaction of the enzyme have been described previously (*7*). Tetramethylammonium bromide and tetraethylammonium chloride (Reakhim, USSR) were recrystallized from an ethanol–acetone mixture. *n*-Tetrapropylammonium bromide (Aldrich, USA) and *n*-tetrabutylammonium bromide (EGA-Chemie, West Germany) of analytical grade were used without purification. The activity of acetylcholinesterase was measured spectrophotometrically according to Ellmann *et al.* (*12*) on a Perkin–Elmer 402 spectrophotometer, using 1 mM acetylthiocholine as a substrate in 0.15 M K-phosphate buffer at pH 7.50, 25°C.

The enzyme sulfonylation reaction was followed by a decrease in the acetylcholinesterase activity. The second-order rate constants of the enzyme inhibition in the absence and in the presence of tetraalkylammonium salts were calculated according to the following formula:

$$k_a = -\frac{1}{[I]} \ln \frac{v_t}{v_0}, \qquad [1]$$

where v_0 is the initial enzyme activity, v_t is its activity at time t, and [I] is alkanesulfonylchloride concentration.

The influence of the alkylammonium salts on the acetylcholinesterase sulfonylation reaction was analyzed according to the following reaction scheme:

$$\begin{aligned} E + I &\xrightarrow{k_i} E' \\ E + A &\rightleftarrows EA \\ EA + I &\xrightarrow{k_A} E' + A, \end{aligned} \qquad [2]$$

where E stands for enzyme, I for alkanesulfonylchloride, and A for the tetraalkylammonium salt. The apparent rate constant of the inhibition reaction k_A depends

upon the concentration of the ligand A according to the following equation (*4*):

$$k_a = \frac{k_i + (k_A/K_A)[A]}{1 + [A]/K_A} . \quad [3]$$

This equation is valid under the second-order conditions of the enzyme inhibition reaction while the concentration of the tetraalkylammonium salts can be varied above the value of K_A. In the present study the ligand concentration exceeded the K_A value up to three times. This was sufficient for a reliable estimation of the kinetic parameters k_A and K_A of Eq. [3].

The experiments were performed at different temperatures from 5 to 35°C where spontaneous inactivation of the enzyme was negligible. The results were analyzed according to the Arrhenius equation:

$$\log k_A = \log A + E_a/(2.303*R*T), \quad [4]$$

and the activation parameters $H^{\ddagger}$ and $S^{\ddagger}$ were calculated. All the calculations were performed on a IBM PC/XT computer by means of linear and nonlinear least-squares programs.

RESULTS

The influence of the tetraalkylammonium salts on the acetylcholinesterase sulfonylation reaction followed the hyperbolic function [3] that allowed us to calculate the inhibition rate constants k_A and the dissociation constants K_A for the enzyme-ligand complex EA (Fig. 1). The results obtained at 25°C are illustrated in Figs. 2 and 3. The data shown in Fig. 2 were independent of the alkanesulfonylchloride used and the mean values of the constants K_A were in good agreement with the K_i values derived from the inhibition of the acetylcholinesterase-catalyzed hydrolysis of acetylthiocholine. This result confirms that in both of these cases the same binding site for tetraalkylammonium ions is involved.

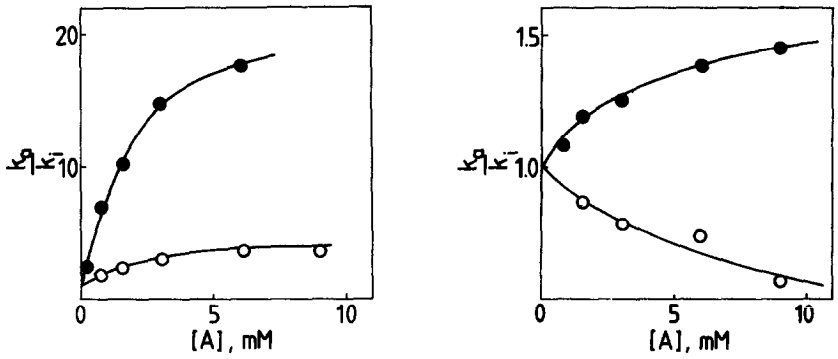

FIG. 1. The effects of tetramethylammonium (○) and tetraethylammonium (●) ions on acetylcholinesterase reaction with ethanesulfonylchloride (left) and propanesulfonylchloride (right) in 0.15 M phosphate buffer, pH 7.5, t = 25°C.

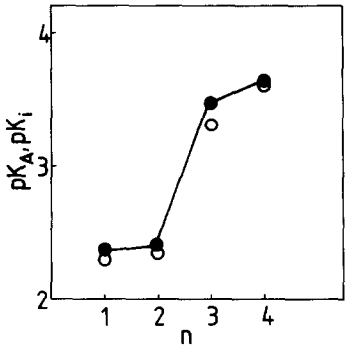

FIG. 2. Dependence of pK_A and pK_i values on the structure of tetraalkylammonium ions $(C_nH_{2n+1})_4N^+$ as determined from the sulfonylation (○) and substrate hydrolysis (●) reactions.

It can be seen from Fig. 2 that the increase in the length of the *n*-alkyl groups of tetraalkylammonium ions facilitates their binding with the enzyme active site. At the same time there is no simple linear free-energy relationship between the binding effectiveness (pK_A or pK_i) and the length (or hydrophobicity) of the alkyl groups of tetraalkylammonium ions. This indicates that hydrophobicity is not the only factor that determines the effectiveness of ligand binding.

Enzyme specificity is characterized by the reactivity of *n*-alkanesulfonylchlorides toward acetylcholinesterase. The data in Fig. 3 show that the binding of tetraalkylammonium ions with acetylcholinesterase has no effect upon the main features of the specificity of this enzyme. It can be seen that the increase in the length of the alkyl groups of alkanesulfonylchlorides decreases their reactivity in the case of all the enzyme–ligand complexes studied as well as in the case of the free enzyme. These data also show that the formation of the complex EA acceler-

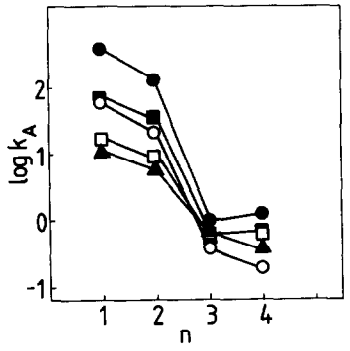

FIG. 3. The effect of alkanesulfonylchloride $C_nH_{2n+1}SO_2Cl$ on the logarithms of the second-order rate constants of inhibition of free acetylcholinesterase (▲) (data from Ref. (7)) and the enzyme complexes with tetramethylammonium (○), tetraethylammonium (●), tetrapropylammonium (□), and tetrabutylammonium (■) ions.

TABLE 1

Reactions of Alkanesulfonylchlorides $C_nH_{n+1}SO_2Cl$ with Free Acetylcholinesterase and Enzyme–Ligand Complexes in 0.15 M Potassium Phosphate Buffer at pH 7.5 and 25°C

Ligand	K_A (M^{-1} s^{-1})			
	$n = 1$	$n = 2$	$n = 3$	$n = 4$
$(CH_3)_4N^+$	60.6 ± 1.3	23.3 ± 0.1	0.4 ± 0.14	0.22 ± 0.03
$(C_2H_5)_4N^+$	384 ± 13	139 ± 6	1.9 ± 0.07	1.5 ± 0.16
$(C_3H_7)_4N^+$	68 ± 1.3	40 ± 1	0.78 ± 0.04	0.66 ± 0.12
$(C_4H_9)_4N^+$	17.8 ± 0.5	10 ± 0.6	0.7 ± 0.04	0.7 ± 0.1
Free enzyme[a]	13.1 ± 1	6.0 ± 0.2	0.7 ± 0.03	0.4 ± 0.03

[a] Data taken from Ref. (7).

ates the enzyme sulfonylation reaction in the case of almost all ligands. Only tetramethylammonium ions inhibited the enzyme sulfonylation reaction by propane- and butanesulfonylchlorides incompletely.

The rate constants k_A obtained for the enzyme–ligand complexes in the present study and the rate constants k_i given for the free enzyme in our previous report (7) allow us to quantify the effects of the tetraalkylammonium salts on acetylcholinesterase inhibition by making use of the coefficients $\alpha = k_A/k_i$. The dependence of α upon the ligand structure revealed clear maxima at the tetraethylammonium ion ($n = 2$) for all alkanesulfonylchlorides, whereas the accelerating effects of the tetramethyl- and tetrapropylammonium ions were similar (Fig. 4). The influence of the tetrabutylammonium ion was the weakest, despite the fact that this ligand binds with the enzyme most effectively of all the compounds studied.

On the other hand, the results obtained clearly show that the enzyme reaction with active alkanesulfonylchlorides are more accelerated than the reaction with

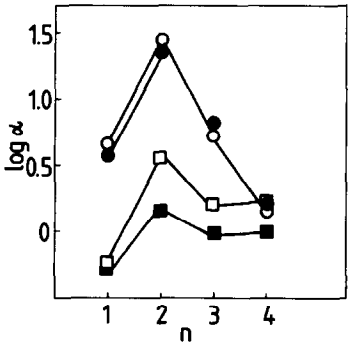

FIG. 4. The dependence of acceleration effect upon the structure of tetraalkylammonium ions $(C_nH_{2n+1})_4N^+$ for methanesulfonylchloride (○), ethanesulfonylchloride (●), propanesulfonylchloride (□), and butanesulfonylchloride (■).

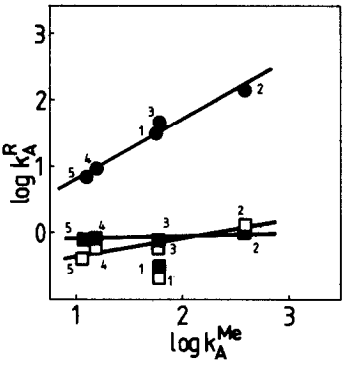

FIG. 5. Plot of log k_A values for acetylcholinesterase inhibition with ethanesulfonylchloride (●), propanesulfonylchloride (□), and butanesulfonylchloride (■) versus log k_A values for methanesulfonylchloride: 1, complex with $(CH_3)_4N^+$; 2, $(C_2H_5)_4N^+$; 3, $(C_3H_7)_4N^+$; 4, $(C_4H_9)_4N^+$; 5, no ligand.

less active alkanesulfonylchlorides. The linear dependence of the log k_A values of methanesulfonylchloride on those of ethanesulfonylchloride gives intercept = -0.18 ± 0.14, slope = 0.91 ± 0.08, $R = 0.99$. In the case of propane- and butanesulfonylchlorides the effects of the ammonium ligands were much weaker and the slopes of the linear dependencies were close to zero. Moreover, the points for tetramethylammonium ions deviate remarkably from the linear dependencies for these two alkanesulfonylchlorides, showing protection of the enzyme against sulfonylation (Fig. 5).

The effect of temperature on the reaction of alkanesulfonylchlorides with acetylcholinesterase and its complexes with tetraalkylammonium ions was found to follow Eq. [4] (Fig. 6), which enabled us to calculate the appropriate activation parameters. The results obtained are listed in Fig. 7 as an isokinetic plot in coordinates $\Delta H^{\ddagger}$ versus $\Delta S^{\ddagger}$. These data reveal that the acetylcholinesterase reaction

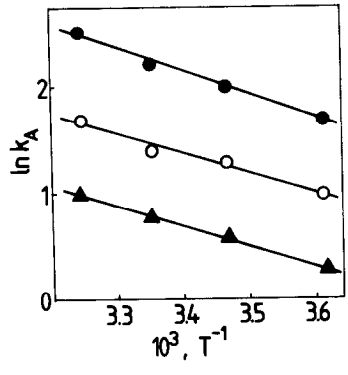

FIG. 6. Arrhenius plots for ethanesulfonylchloride reaction with the free enzyme (△) and its complexes with tetramethylammonium (○) and tetraethylammonium (□) ions.

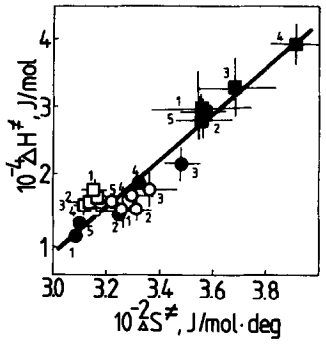

FIG. 7. Isokinetic plot for acetylcholinesterase inhibition with methanesulfonylchloride (○), ethanesulfonylchloride (●), propanesulfonylchloride (□), and butanesulfonylchloride (■): 1, complex with $(CH_3)_4N^+$; 2, $(C_2H_5)_4N^+$; 3, $(C_3H_7)_4N^+$; 4, $(C_4H_9)_4N^+$; 5, no ligand.

with alkanesulfonylchlorides under the second-order conditions can be treated as a series of isokinetic reactions and the mechanism of sulfonylation of all the enzyme–ligand complexes remains similar to that of the free enzyme.

DISCUSSION

The influence of the tetraalkylammonium ions upon the sulfonylation reaction is diverse, ranging from activation to inhibition, and depends on the structure of both the reagents. Even in the case of the largest tetraalkylammonium ions and alkanesulfonylchloride molecules used there was no protection of the esteratic site of the enzyme against sulfonylation, which points to the absence of direct competition between these molecules for the same binding area of the enzyme. This is in agreement with our earlier findings suggesting that the variable alkyl group of the alkanesulfonylchlorides is oriented away from the anionic center (*7*). Protection of the enzyme by tetramethylammonium ions against propane- and butanesulfonylchlorides was incomplete, decreasing the rate constant about twofold. These facts point to the allosteric regulation mechanism of the sulfonylation reaction by tetraalkylammonium ligands.

The following regularities are characteristic of the structure–activity relationships of this allosteric regulation phenomenon. Firstly, the effect of the tetraalkylammonium ligands on the rate of the sulfonylation reaction is not directly related to their binding effectiveness. This conclusion follows from the data shown in Figs. 2 and 3. It can be seen (Fig. 4) that the constant α always has the highest value in the case of the tetraethylammonium ion, independent of the alkanesulfonylchloride structure, while the dissociation constants for these ligands decrease systematically as the length of the alkyl substituent increases. Secondly, the acceleration effect is directly related to the reactivity of alkanesulfonylchlorides as the reactions of more active alkanesulfonylchlorides are more accelerated. This leads to an increase in the specificity of acetylcholinesterase

against these irreversible inhibitors. For example, if in the case of the free enzyme the ratio of the highest and the lowest values of the rate constants for the series of the alkanesulfonylchlorides used was 35, then in the case of the enzyme–tetraethylammonium complex this ratio was equal to 275. Thirdly, the same set of specificity-determining factors seem to govern the sulfonylation reaction of the free enzyme and the enzyme–ligand complexes. The linear-free-energy relationship shown in Fig. 5 allows us to assume that the intensity of a single factor is altered depending on the ligand bound to the enzyme.

The conclusion drawn above about the isokinetic nature of the sulfonylation reaction of the enzyme in the presence of tetraalkylammonium ligands differs from that arrived at by Pavlic (*8*) about the isoenthalpic nature of the acceleration of the acetylcholinesterase reaction with methanesulfonylfluoride. However, the present results also reveal that the changes in the activation entropy make larger contributions to the acceleration effects observed. Thus, it can still be concluded that the acceleration phenomenon can be related to the conformational changes in the enzyme molecule caused by the binding of ligands to the anionic site. As the tetraethylammonium ion gives the greatest effect, the acceleration phenomenon observed can be explained by the ligand fitting into the cavity of the enzyme anionic site, which leads to conformational transition. Large ions probably do not fit into the cavity, which explains the decrease in their acceleration effect or its absence. On the other hand, the binding energy of the tetramethylammonium ions or any other less hydrophobic ions cannot be sufficient to induce a full-scale conformational transition. Somewhat anomalous behavior of tetraethylammonium ions has also been observed earlier in an analogous situation by Roufogalis and Thomas (*13*). They suggest that part of the binding energy is spent on bringing about a conformational change in the enzyme.

The acceleration effects of inorganic cations, found by Pavlic (*4*) in the case of the reaction of methanesulfonylfluoride with acetylcholinesterase, seem to follow the same regularity. In this paper it has been shown that the larger and thus the more "hydrophobic" inorganic cations, if characterized by their partition coefficients for the water–octanol system (*9*), have stronger influence on the inhibition reaction.

It should be mentioned that the proposed allosteric regulation mechanism of acetylcholinesterase activity in the sulfonylation reaction is also in agreement with the results obtained by Belleau and DiTullio (*5*). They have shown in the case of the reaction of methanesulfonylfluoride that also within a series of the ammonium cations $(CH_3)_3N^+R$ with various normal and cycloalkyl groups an optimal ligand structure can be found for the acceleration effect. In their case the most effective accelerating ligand was the cyclohexyltrimethylammonium ion. Those results indicate that the varied alkyl group of these ions is not oriented toward the esteratic center or otherwise the ligand would overlap the esteratic site and the sulfonylation reaction would be inhibited. This situation where the ligands can cause different acceleration effects in spite of the fact that their variable alkyl substituents are not oriented toward the esteratic center was also taken as an indication of a conformational change at the esteratic site induced by an anionic site-located ligand (*5*).

Pavlic has explained the acceleration phenomenon of the acetylcholinesterase reaction with methanesulfonylfluoride, caused by the anionic site-located cationic ligands, as a change in the hydration of the catalytic serine residue in the active center of the enzyme (*6*). The acceleration is assumed to occur through the dehydration of the nucleophilic OH-group at the esteratic site of the enzyme, thus enabling faster formation of the reversible complex of the inhibitor molecule and the enzyme (*8*). In this model the acceleration effect is connected only with the interactions between the sulfonyl group of the inhibitor and the hydroxyl group of the serine residue.

However, our studies of the present series of *n*-alkanesulfonylchlorides suggest that the alkyl substituent is crucial in the acceleration phenomenon. In view of the structure–activity relationships of the acetylcholinesterase inhibition it has been suggested that the chemically inert alkyl groups hinder this reaction because of some spatial restrictions at either the noncovalent binding step or the covalent reaction step or simultaneously for both of them (*7*). Therefore, the acceleration effect discussed above can be related to the weakening of these hindrances resulting from conformational transition of the enzyme active center, caused by ligand binding to the anionic site. This change seems to be localized near the esteratic center and its influence declines sharply with increase in the distance, becoming negligible in the case of propyl and butyl derivatives of the sulfonylating agent.

The alkyl groups of alkanesulfonylchlorides were shown to be localized in the same region that accommodates the acyl part of ester substrates (*7*). Thus, the influence of cationic ligands upon the sulfonylation reaction can be used to model the effect brought about by the cationic group of choline esters upon their hydrolysis by cholinesterases. In the case of acetylcholine this possibility was discussed by Pavlic (*11*). The present results confirm this hypothesis, but in addition they point to the possibility that the cationic group of choline esters can govern enzyme sensitivity against the structure of the acyl part of substrates, thus making acetic esters the specific substrates of acetylcholinesterase.

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