BIOORGANIC CHEMISTRY 14, 222–227 (1986)

Leaving Group Effects in Binding and Reaction Steps of Acetylcholinesterase Inhibition by *O,O*-Diethylthiophosphates[1]

JAAK JÄRV AND AAVO AAVIKSAAR

Department of Organic Chemistry, Tartu State University, Kingissepa 14/16, Tartu 202400, and Department of Biochemistry, Institute of Chemical Physics and Biophysics, Academy of Sciences of the Estonian SSR, P.O. Box 670, Tallinn 200026, Estonian SSR, USSR

Received April 16, 1985

Affinity and phosphorylation constants for the inhibition of acetylcholinesterase by a series of *O,O*-diethylthiophosphates, $(C_2H_5O)_2P(O)SX$, where $X = C_nH_{2n+1}$ (n = 4–8), $(CH_2)_nSC_2H_5$ (n = 2–6), and $(CH_2)_nS^+(CH_3)C_2H_5$ (n = 3–6), have been measured at 25°C and pH 7.0 in order to quantify the specificity-determining factors in the binding and reaction steps of the process. In contrast to the influence of the leaving group of the acetic ester substrates, where the hydrophobicity of the substituent affects the binding and acetylation steps equally, it has been found that the hydrophobicity of the leaving group of the nonionic organophosphorus inhibitor affects only the binding in the enzyme's active site whereas the rate of the phosphorylation reaction is governed solely by the inductive effect of substituent X. A common phosphorylation rate constant logarithm–σ_X^* relationship holds for the cationic inhibitors and their nonionic analogs; however, an extra effect of the cationic group in X appears in the dissociation constant for the enzyme–inhibitor complex and points to the putative influence of the enzyme's anionic site in the binding step. The data obtained suggest that the hydrophobic cleft for the binding of the inhibitors' leaving group is not "shut" in the transition state of the phosphorylation of the enzyme's active site, in contrast to the acetylation reaction in which the closed hydrophobic slit in the transition state is shown to stabilize the complex.

INTRODUCTION

In acetylcholinesterase-catalyzed hydrolysis of acetic esters, $CH_3C(O)X$, the hydrophobicity of the substrate leaving group has been shown to have equal effect on the free energies of substrate binding and enzyme active site acetylation (*1*). In terms of the correlation equations

$$pK_s^X = pK_s^0 + \varphi_b\pi_X \quad [1]$$

and

$$\log k_2^X = \log k_2^0 + \varphi_a\pi_X + \rho^*\sigma_X^*, \quad [2]$$

it appears that $\varphi_b = \varphi_a$, where φ and ρ^* are the intensity factors of the hydropho-

[1] Preliminary results have been reported at the Third Congress of the Hungarian Pharmacological Society, Budapest, 1979.

0045-2068/86 $3.00

bic and inductive effects of substituent X, and constants K_s and k_2 correspond to the reaction

$$E + S \overset{K_s}{\rightleftharpoons} ES \xrightarrow{k_2} EA + P_1 \xrightarrow{k_3} E + P_2. \quad [3]$$

In accordance with the explanation of the same phenomenon in chymotrypsin reactions (*2*), it has been suggested (*1*) that the appearance of the hydrophobic interaction as a specificity factor in the bond-breaking step of acetylcholinesterase acetylation points to a conformational change in the enzyme molecule during activation, consisting of the "shutting" of the hydophobic slit in the active center of the enzyme in the transition state of the reaction. The conformational change makes the activated complex with the closed slit energetically more favorable by a factor $\varphi_a\pi_X$ than the complex with the open slit, thereby increasing the hydrophobic selectivity of the enzyme's active center.

Because the leaving group of the organophosphorus inhibitors has been shown to bind in the acetylcholinesterase active center in a hydrophobic area outside the binding cleft (*3*) of the substrate's leaving group it is of interest whether the equality between φ_b and φ_a might be characteristic of the interaction between the inhibitors' leaving group and the active surface of the enzyme as well, i.e., whether the above-mentioned conformational change may also concern this particular hydrophobic area.

For this purpose we have determined the affinity and phosphorylation constants K_s and k_2 for the inhibition of acetylcholinesterase according to [3] with $k_3 = 0$ (*4*) by a series of *O,O*-diethylthiophosphates, $(C_2H_5O)_2P(O)SX$, where $X = C_nH_{2n+1}$ (n = 4–8), $(CH_2)_nSC_2H_5$ (n = 2–6), and $(CH_2)_nS^+(CH_3)C_2H_5$ (n = 3–6).

EXPERIMENTAL

The kinetic measurements and chemicals used have been described in previous studies (*5, 6*). Acetylcholinesterase from cobra *Naja naja oxiana* venom was purified by gel filtration on a Sephadex G-50 column as described in (*7*). The synthesis and properties of the organophosphorus inhibitors used have been described in (*8*). The nonionic inhibitors were purified of possible contaminants of high anticholinesterase activity by using a special chromatographic procedure (*9*).

Enzyme inhibition was carried out under pseudo-first-order conditions where $[E]_0 \ll [S]_0$. At zero time the enzyme and inhibitor solutions were mixed in a temperature-controlled vessel of a pH-stat (pH121/BAT15/B705, USSR), containing 5 ml of 0.15 M KCl, pH 7.5, at 25°C. At appropriate time intervals 0.1–0.2 ml of this reaction mixture was added to 5 ml of the enzyme assay solution in a temperature-controlled vessel of a second pH-stat (TTT1/ABU1/SBR2, "Radiometer," Denmark), and the residual velocities of the substrate hydrolysis were measured at pH 7.5 (2.3 mM acetylcholine iodide, 0.15 M KCl, 25°C).

The pseudo-first-order rate constants k_{obs} were calculated from $\ln v_t$ versus t plots,

$$\ln v_t = \ln v_0 - k_{obs} \cdot t, \quad [4]$$

TABLE 1

KINETIC CONSTANTS OF ACETYLCHOLINESTERASE INHIBITION BY ORGANOPHOSPHORUS COMPOUNDS $(C_2H_5O)_2P(O)SX$ IN 0.15 M KCl SOLUTION AT pH 7.0 AND 25.0°C

X	$10^3 K_s$ (M)	k_2 (sec^{-1})
C_4H_9	2.6 ± 0.8	$(2.1 \pm 0.7) \times 10^{-3}$
C_5H_{11}	2.0 ± 0.9	$(3.2 \pm 0.9) \times 10^{-3}$
C_6H_{13}	0.72 ± 0.29	$(2.9 \pm 1.4) \times 10^{-3}$
C_7H_{15}	0.36 ± 0.20	$(3.3 \pm 0.8) \times 10^{-3}$
C_8H_{17}	0.27 ± 0.18	$(3.2 \pm 1.9) \times 10^{-3}$
$(CH_2)_2SC_2H_5$	2.1 ± 0.9	$(1.6 \pm 1.0) \times 10^{-2}$
$(CH_2)_3SC_2H_5$	1.2 ± 0.5	$(1.0 \pm 0.6) \times 10^{-2}$
$(CH_2)_4SC_2H_5$	0.68 ± 0.19	$(7.3 \pm 2.3) \times 10^{-3}$
$(CH_2)_5SC_2H_5$	0.24 ± 0.12	$(2.7 \pm 1.1) \times 10^{-3}$
$(CH_2)_6SC_2H_5$	0.15 ± 0.10	$(2.1 \pm 1.5) \times 10^{-3}$
$(CH_2)_3S^+(CH_3)C_2H_5$	0.35 ± 0.03	$(5.2 \pm 0.4) \times 10^{-1}$
$(CH_2)_4S^+(CH_3)C_2H_5$	0.133 ± 0.008	$(1.7 \pm 0.1) \times 10^{-2}$
$(CH_2)_5S^+(CH_3)C_2H_5$	0.077 ± 0.07	$(6.5 \pm 0.3) \times 10^{-3}$
$(CH_2)_6S^+(CH_3)C_2H_5$	0.045 ± 0.003	$(4.2 \pm 0.4) \times 10^{-3}$

where v_t denotes the residual activity of the enzyme after incubation time t. The reactions were observed for at least two half-life periods. The kinetic constants k_2 and K_s were obtained from the straight lines in the coordinates $1/k_{obs}$ and 1/[S], in accordance with the linear transformation of

$$k_{obs} = \frac{k_2[S]}{K_s + [S]}. \qquad [5]$$

The hydrophobicity constants π for substituents X were calculated additively, taking $\pi = 0.5$ for $-CH_2-$, $\pi = 1.05$ for $-SC_2H_5$ (*10*), and $\pi = -4.35$ for $-S^+(CH_3)C_2H_5$ (*11*). The inductivity constants σ^* were calculated as described in (*5, 6*), being derived from the σ^* values 0.56 for $-CH_2SC_2H_5$ and 4.40 for $-CH_2S^+(CH_2)C_2H_5$ (*12*). For alkyl groups $-C_nH_{2n+1}$ at $n \geq 3$, σ^* was taken to be zero within the error limits [cf. (*1*)]. All calculations and statistical treatment of the experimental data were performed on a Nairi-2 computer in Department of Organic Chemistry, Tartu State University.

RESULTS

The values obtained for the kinetic parameters K_s and k_2 are listed in Table 1. The limited solubility of the compounds explains the low precision of these parameters for nonionic inhibitors. However, the results obtained agree reasonably well with the second-order rate constants $k_i = k_2/K_s$, determined earlier under the conditions $[S] \ll K_s$ (*5*). For the cationic compounds solubility was not the critical point and the separation of the k_2 and K_s values was precise.

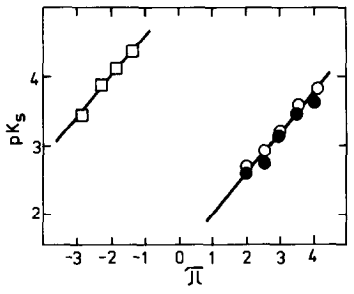

FIG. 1. Plot of pK_s versus π_X for the interaction of acetylcholinesterase and *O,O*-diethylthiophosphates $(C_2H_5O)_2P(O)SX$, where X = C_nH_{2n+1} (●), $(CH_2)_nSC_2H_5$ (○), and $(CH_2)_nS^+(CH_3)C_2H_5$ (□).

Figure 1 shows that the reversible binding step of the nonionic inhibitors in the enzyme's active center is governed by a hydrophobic interaction. According to Eq. [1] a common relationship holds for both types of compounds, those containing alkyl and those containing nonionic electronegative substituents X. The correlation parameters of Eq. [1] for this subseries of inhibitors were $\varphi_b = 0.58 \pm 0.05$ and $pK_s^0 = 1.42 \pm 0.14$ ($n = 10$, $r = 0.980$, SD = 0.102). Figure 1 also shows the experimental points for cationic compounds which give a separate line with a similar slope. Thus the deviations of the pK_s values of the cationic inhibitors from Eq. [1] can be taken into account by an extra term, θ_b, in Eq. [1]:

$$pK_s^{X^+} = pK_s^0 + \varphi_b\pi_X + \theta_b. \qquad [6]$$

The average θ_b value for all the cationic inhibitors was found to be 3.7, for the nonionic inhibitors, $\theta_b = 0$.

For the enzyme phosphorylation step, the reactivity of the inhibitors depends only on the inductive effect and can be described by Eq. [2] with $\varphi = 0$:

$$\log k_2^X = \log k_2^0 + \rho^*\sigma_X^*. \qquad [7]$$

Figure 2 shows that the log k_2 versus σ^* plot provides a common linear relationship for the inhibitors with both ionic and nonionic leaving groups, yielding $\rho^* = 4.0 \pm 0.35$, and $\log k_2^0 = -2.67 \pm 0.08$ ($n = 9$, $r = 0.97$, SD = 0.17). The k_2 values for the inhibitors with alkyl substituents X remain unchanged in the error limits and are close to the k_2^0 value found using Eq. [7]. This result is in agreement with the σ^* constants for the alkyl groups which are equal to zero.

DISCUSSION

The data obtained show that the hydrophobicity of the leaving group is an important structural factor in the noncovalent binding of both nonionic and cationic inhibitors in the active center of acetylcholinesterase. The intensity factor $\varphi_b = 0.6$ for the inhibitors is comparable to $\varphi_b = 0.8$ for the interaction of the acetic ester substrate's leaving groups with the enzymes active surface (*1*). The

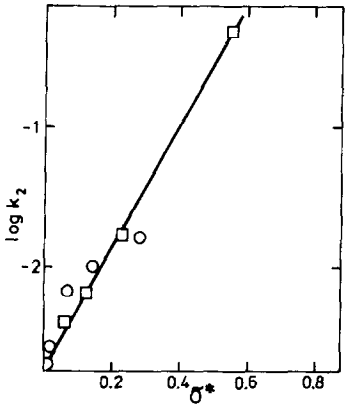

FIG. 2. Plot of log k_2 versus σ^* for acetylcholinesterase active site phosphorylation by *O,O*-diethylthiophosphates $(C_2H_5O)_2P(O)SX$, where X = $(CH_2)_nSC_2H_5$ (○) and $(CH_2)_nS^+(CH_3)C_2H_5$ (□).

difference between the two hydrophobic binding areas manifests itself in the reaction step. For the leaving group of the inhibitors, φ_a is clearly zero, and no interrelationship between binding and phosphorylation is observed within the reaction series studied. Thus, stabilization of the transition state by hydrophobic interaction is not a general property of hydrophobic binding areas in enzyme active centers but rather seems to be a specific feature of the definite hydophobic clefts predetermined for selectivity enhancement in appropriate parts of substrate molecules. Earlier a similar conclusion was drawn for butyrylcholinesterase-catalyzed reactions (*13*). An appropriate model for the hydrophobic binding regions in the active sites of cholinesterases has been given in this journal (*14*).

The intensity of the influence of the leaving group's hydrophobic and inductive effects on the second-order rate constants of acetylcholinesterase active center phosphorylation by nonionic *O,O*-diethylthiophosphates has been determined previously (*5*) on the basis of

$$\log k_{II}^X = \log k_{II}^0 + \rho^*\sigma_X^* + \varphi\pi_X \qquad [8]$$

with $\log k_{II}^0 = -1.12 \pm 0.14$, $\rho^* = 3.96 \pm 0.22$, $\varphi = 0.57 \pm 0.05$ ($n = 10$, $r = 0.992$, SD = 0.078). Agreement of these data with the φ_b and ρ^* values obtained in the present work on the basis of Eqs. [1] and [2] points to the fact that k_{II} can be represented as k_2/K_s, and Eq. [8] can be given as the sum of Eqs. [1] and [2]:

$$\log(k_2/K_s)^X = \log(k_2/K_s)^0 + \rho^*\sigma_X^* + (\varphi_a + \varphi_b)\pi_X. \qquad [9]$$

Coefficient φ in Eq. [8] turns out to be φ_b since $\varphi_a = 0$.

To take into account the deviations of log k_{II} for the cationic *O,O*-diethylthiophosphates from Eq. [8], the additional term θ was introduced in (*6*). In doing so, it was assumed that the extra term described the effect of the enzyme's anionic site by compensating for the energetically unfavorable transfer of the charged group from water to the hydrophobic region of the enzyme's active site (*6*). The results of the present work support this assumption as the extra effect appears

only in the noncovalent binding step. The putative mechanism for the anionic site phenomenon seems to involve ion-pair formation between the cationic group of the ligand and the anionic group of the enzyme, and has been discussed elsewhere (*14*).

REFERENCES

1. JÄRV, J., KESVATERA, T., AND AAVIKSAAR, A. (1976) *Eur. J. Biochem.* **67,** 315.
2. AAVIKSAAR, A., PARIS, J., AND PALM, V. (1971) *Org. React.* **8,** 817.
3. JÄRV, J., AAVIKSAAR, A., GODOVIKOV, N., AND LOBANOV, D. (1977) *Biochem. J.* **167,** 823.
4. ALDRIDGE, W. N., AND REINER, E. (1972) *Front. Biol.* **26,** 56.
5. JÄRV, J. L., AAVIKSAAR, A. A., GODOVIKOV, N. N., AND LOBANOV, D. I. (1976) *Bioorgan. Khim.* **2,** 978.
6. JÄRV, J. L., AAVIKSAAR, A. A., GODOVIKOV, N. N., AND LOBANOV, D. I. (1977) *Bioorgan. Khimiya* **3,** 268.
7. RABA, R., AAVIKSAAR, A., RABA, M., AND SIIGUR, J. (1979) *Eur. J. Biochem.* **96,** 152.
8. JÄRV, J. L., AAVIKSAAR, A. A., LOBANOV, D. I., AND GODOVIKOV, N. N. (1976) *Izv. Akad. Nauk. SSSR, Ser. Khim.* 426.
9. JÄRV, J., AND AAVIKSAAR, A. (1976) *Proc. Acad. Sci. Estonian SSR* **25,** 168.
10. HANSCH, C., LEO, A., UNGER, S. H., KIM, K. H., AND LIEN, E. J. (1973) *J. Med. Chem.* **16,** 1207.
11. SIKK, P., AAVIKSAAR, A., AND ABDUVAKHABOV, A. (1977) *Proc. Acad. Sci. Estonian SSR* **26,** 242.
12. JÄRV, J. L., AAVIKSAAR, A. A., GODOVIKOV, N. N., AND MOROZOVA, N. A. (1972) *Org. React.* **10,** 243.
13. LANGEL, Ü., AND JÄRV, J. (1978) *Biochim. Biophys. Acta* **525,** 122.
14. JÄRV, J. (1984) *Bioorg. Chem.* **12,** 259.