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COMPENSATIONAL PHENOMENA IN REACTIVATION OF DIMETHYL- AND DIETHYLPHOSPHORYL BUTYRYLCHOLINESTERASES

I-CHENG WANG and PHILLIP E. BRAID

Health Effects Division, Bureau of Chemical Hazards, Environmental Health Directorate, Health and Welfare Canada, Ottawa, Ontario K1A OL2 (Canada)

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Summary

The thermodynamic and kinetic parameters for spontaneous and oxime reactivation of dimethyl- and diethylphosphoryl butyrylcholinesterases (acylcholine acyl-hydrolase, EC 3.1.1.8) are reported. The enthalpy and entropy changes in both the binding (ΔH^0 and ΔS^0) and the dephosphorylation steps (ΔH^* and ΔS^*) were found to be coupled, resulting in a minor variation in free energy changes (ΔG^0 and ΔG^*). While neither enthalpies nor entropies alone bore any relationship with the kinetic parameters K_D and k_R, the changes of free energies (ΔG^0 and ΔG^*) correlated linearly with the logarithmic values of the dissociation constants (K_D) and bimolecular rate constants (k_R/K_D), respectively. Compensation plots of entropies versus enthalpies gave straight lines with compensation temperatures of 275 K for the binding 260 K for the dephosphorylation. Spontaneous reactivation of dimethyl phosphoryl butyrylcholinesterase was investigated at various pH values and three temperatures. It implicated two catalytic sites with values of pK_i of 9.4 and 7.5, and heats of ionisation of 5.3 and 9.6 kcal · mol^{-1}, respectively. Possible conformational alteration of the inhibited enzyme arising from the binding of oximes is discussed.

Introduction

Cholinesterases (acylcholine acyl-hydrolases, EC 3.1.1.8) are inhibited by organophosphates through phosphorylation of their esteric sites:

$$(RO)_2POX + E \rightleftharpoons (RO)_2POE + X^- . \quad (1)$$

The inhibited enzyme may undergo spontaneous reactivation (Eqn. 2) or may be regenerated by nucleophilic agents called reactivators (Eqn. 3):

$$E \cdot I + H_2O \rightarrow E + P \quad (2)$$

$$E \cdot I + R \overset{K_D}{\rightleftharpoons} E \cdot I \cdot R \xrightarrow{k_R} E + P, \quad (3)$$

where EI is the inhibited enzyme, R the reactivator, E the regenerated enzyme, P the product formed, K_D the dissociation constant of the $E \cdot I \cdot R$ complex, and k_R the rate of breakdown of the complex.

A considerable amount of kinetic work has been done on the basis of the mechanism just mentioned, but the details are still by no means understood. This is especially so regarding the effect of temperature. In a number of investigations [1—4] the activation energies for the breakdown of the complexes have been determined, but it is difficult to assess the significance of the results either in terms of the variation of the phosphoryl group or the structural aspects of the reactivators. For the binding step only in one instance were the standard enthalpies and entropies determined; namely the reactivation of sarin inhibited acetylcholinesterases by α, ω-bis(4-hydroxyiminomethylpyridinium)-2-*trans*butene dibromide [3]. There has been little systematic study of the variation of such factors as temperature and pH. Such study might be expected to throw valuable light on the mechanism of reactivation, since it would be expected to yield information about the thermodynamics of the process as well as the chemical nature of the catalytic groups involved.

In the present communication the results from thermodynamic and kinetic studies for the reactivation of dimethyl and diethyl phosphoryl butyrylcholinesterases by five oximes are presented. Changes of entropy and enthalpy for both binding and dephosphorylation steps are found to be coupled, resulting in minor variation of the free enrgy changes. Studies of pH dependence indicate that the reactivation is catalyzed by two reactive sites. The bound oximes appear to induce a conformational change of the inhibited enzyme.

Materials and Methods

Partially purified horse serum butyrylcholinesterase (I.C.N. Life Sciences) was used in all experiments. Its K_m and V_m values were 0.64 ± 0.05 mM and 10.0 ± 0.5 μmol/min per mg, respectively; they were determined by pH-stat at pH 7.4 and 25° C under a current of nitrogen gas, using butyrylcholine as substrate in the concentration range between 0.33—3.3 mM. The enzyme yielded a single peak on Sephadex G-200 (fine) gel filtration at pH 7.4 and 25° C.

Reactivators were commercial products with the exception of two derivatives of pyridine aldoxime methiodide (3-PAM and 4-PAM) which were prepared by refluxing the respective aldoximes with methyl iodide. The reactivators were purified by repeated recrystallization from aqueous ethanol. Their melting points were: 2-PAM(Aldrich, 220—221° C; ref. 5, 218—220° C), isonitrosacetophenone (NAP, Aldrich, 131—132° C, ref. 5, 124—126° C), *N,N'*-trimethylenebis(Pyridinium-4-aldoxime)dibromide (TMB-4, K&K Lab. 230—232° C; ref. 6, 222° C), 3-PAM (157—158° C; ref. 5, 152—154° C), and 4-PAM (176—177° C; ref. 5, 171—173° C). Diethyl 4-nitrophenyl phosphate (Paraoxon, Koch-Light) and 1-chloro-1-diethylcarbamoyl-1-propen-2-yl-dimethyl-phosphate (Phosphamidon, Ciba-Geigy) were practical and analytical grades, respectively.

The enzyme solution (12 mg/ml) was prepared in 50 mM boric acid/borate

buffer containing 50 mM NaCl at pH 9.0. High pH was chosen to prevent ageing. The enzyme solution was incubated overnight at 4°C by 0.3 mM Phosphamidon and 0.02 mM Paraoxon, respectively, resulting in 90% inhibition. Excess inhibitor was removed by Sephadex G-25 (coarse) gel filtration.

The enzyme activity was determined spectrophotometrically, using benzoylcholine (Calbiochem.) as substrate at pH 7.4 and 25°C as described by Kalow [7]. Benzoylcholine remained stable in the presence of small amounts of reactivator. Change of absorbance at 240 nm was recorded on a slave recorder whereby a 0—0.1 absorbance range of the DK-2 Beckman spectrophotometer was expanded to the full width of a 10-inch chart. The fraction of reactivation was calculated on the basis of total inhibited enzyme concentration available for reactivation.

The standard enthalpy (ΔH^0) and the free energy (ΔG^0) of binding are given by:

$$\log K_D = \frac{\Delta H^\circ}{2.303RT} + \text{Constant}$$

and

$$\Delta G^0 = 2.303\, RT \log K_D,$$

where K_D is the dissociation constant of the reactivator-inhibited enzyme complex. Values of K_D and k_R were obtained from plots of the reciprocals of k_{app} and the molar concentrations of the reactivator according to the equation [4]:

$$\frac{1}{k_{app}} = \frac{1}{k_R} + \frac{K_D}{k_R(R)},$$

where k_{app} is the apparent first-order rate constant. The lines were calculated by the method of least squares. The standard deviations (S.D.) were found from the equations [3]:

$$\text{S.D.}(\Delta G^\circ) = 2.303RT\frac{\text{S.D.}(K_D)}{k_R}$$

and

$$\text{S.D.}\ (\Delta H^0) = 2.303\, R \cdot \text{S.D. (slope)}.$$

Values of standard entropy changes were calculated from the equation:

$$\Delta S^\circ = \frac{\Delta H^\circ - \Delta G^\circ}{T}.$$

Entropy of activation, ΔH^*, for the breakdown of the complex was calculated from the Arrhenius activation energy E_a. The energy of activation was obtained in the usual way by plotting the logarithms of rate constants against reciprocals of temperatures. Entropy of activation, ΔS^*, was calculated from the equation [8]:

$$\frac{\Delta S^*}{4.576} = \log k_R - 10.753 - \log T + \frac{E_a}{4.576T},$$

where k_R is the rate constant of breakdown of the complex. Because both ΔS^0 and ΔS^* varied little with temperature, average values with S.D. are reported.

Results

The reactivators were one neutral and four quaternary oximes. The structural difference of the inhibited enzyme was studied with dimethyl- and diethylphosphoryl butyrylcholinesterases, respectively. In Tables I and II are shown the reactivation parameters K_D and k_R as outlined in Eqn. 3. The results indicate that for the reactivation of dimethylphosphoryl butyrylcholinesterase the relative efficiencies of binding (K_D) and rates of breakdown of the complexes (k_R) for three monoquaternary oximes were in the order 2-PAM, 4-PAM, 3-PAM: 1.0, 1.5, 3.1 and 1.0, 0.16, 0.095, respectively. For the reactivation of diethylphosphoryl enzyme, the orders were 1.0, 0.87, 2.3 for K_D and 1.0, 0.05, 0.029 for k_R. It is seen that K_D runs in reverse order to that of k_R as expected, but change of k_R within a reactivator series is more drastic than is the change of K_D, indicating that the orientation of the oximino group to the pyridinium ring appears to be a major contributing factor to k_R while the quaternary nitrogen atom is the driving force for the binding. TMB-4, being a biquaternary compound, is characterized by a lower binding capacity (K_D) and higher intrinsic rate constant (K_R) in comparison with 2-PAM. Isonitrosoacetophenone is a neutral oxime; its K_D value was higher due to the absence of quaterbary nitrogen, while k_R was in the vicinity of that for 4-PAM (Tables I and II).

The structural differences in the two inhibited enzymes arising from altering the alkyl group of the phosphoryl moiety are also evident. While the affinity of monoquarterny oximes for the diethylphosphoryl enzyme was stronger than that for its dimethyl counterpart, the latter was dephosphorylated at a faster rate than was the former (Table II).

Effects of temperature on the binding and dephosphorylation

The Arrhenius plots describing the relationship between the dissociation constants (K_D) and temperatures indicated that the binding process of the reactivation was exothermic (Fig. 1) and that there was a transition temperature in the range of 25—30°C for the reactivation of dimethylphosphoryl enzyme by 2-PAM and 3-PAM, and for diethyl phosphoryl enzyme by 2-PAM and TMB-4 (Table I). The standard enthalpy (ΔH^0) and entropy (ΔS^0) for the binding of the reactivators to the inhibited enzymes were found to be coupled and sensitive to the structural changes of both the reactivators and the inhibited enzymes. But neither ΔH^0 nor ΔS^0 could be correlated with the values of K_D. Variation in free energy changes (ΔG^0) for a series of reactivators studied, however, was found to be a linear logarithmic function of K_D, as shown in Fig. 2. Since the change of ΔG^0 for the series of reactivators investigated was small, a linear relationship in a compensation plot of ΔH^0 versus ΔS^0 was observed (Fig. 3). The slope of this plot is expressed in Kelvins and is called compensation temperature. The value of 275 ± 9 K so obtained falls into the range of 250—315 K reported for similar plots for a variety of protein reactions [9].

The temperature effects of the dephosphorylation step operate in a similar

TABLE I

DISSOCIATION AND THERMODYNAMIC CONSTANTS FOR THE BINDING OF OXIME REACTIVATORS TO DIMETHYL- AND DIETHYLPHOSPHORYL BUTYRYLCHOLINESTERASES AT pH 7.4

Inhibited enzyme		Reactivator	$K_D \times 10^3$ (mM)	Temperature range (°C)	$-\Delta H^\circ$ (kcal · mol^{-1})	$-\Delta H^\circ$ (kcal · mol^{-1})	$-\Delta S^\circ$ (kcal · degree^{-1} · mol^{-1})	Transition temperature (°C)
Dimethyl-phosphoryl butyrylcho-linesterase	1.	2-PAM	0.63 ± 0.05	14—30	6.0 ± 0.4	4.5 ± 0.1	4.9 ± 0.2	30
			2.60 ± 0.45 *	30—36	40.2	3.7 ± 0.3	118	
	2.	TMB-4	0.97 ± 0.10	14—37	7.5 ± 0.7	4.1 ± 0.1	11.6 ± 0.3	
	3.	4-PAM	0.97 ± 0.09	12—35	4.4 ± 0.8	4.1 ± 0.1	0.84 ± 0.08	
	4.	3-PAM	1.94 ± 0.15	12—25	30.2 ± 3.0	3.7 ± 0.1	89.1 ± 0.1	25
			2.84 ± 0.36 *	25—35	6.3 ± 0.5	3.6 ± 0.2	8.7 ± 0.1	
	5.	INAP	3.34 ± 0.11	12—37	11.8 ± 1.1	3.4 ± 0.1	28.3 ± 0.1	
Diethyl-phosphoryl butyrylcho-linesterase	6.	4-PAM	0.47 ± 0.05	12—35	8.2 ± 0.7	4.6 ± 0.1	12.3 ± 0.2	
	7.	2-PAM	0.54 ± 0.03	12—30	15.2 ± 0.2	4.0 ± 0.1	38.6 ± 0.1	30
			4.66 ± 0.17 *	30—37	39.7	3.3 ± 0.1	117	
	8.	3-PAM	1.25 ± 0.34	25—37	4.0 ± 1.2	4.6 ± 0.4	—1.5 ± 0.5	
	9.	TMB-4	1.94 ± 0.08	12—30	11.6 ± 1.0	3.8 ± 0.1	26.4 ± 0.2	30
			8.20 ± 0.71 *	30—37	34.6	3.0 ± 0.1	101	

* The thermodynamic data without S.D. values were calculated from two temperatures at 36 or 37°C respectively.

TABLE II

KINETIC AND ACTIVATION PARAMETERS FOR THE DEPHOSPHORYLATION STEP AT pH 7.4 AND 25°C

Inhibited enzyme		Reactivator	$K_R \times 10^2$ (min^{-1})	k_R/K_D (M^{-1} · min^{-1})	ΔH^* (kcal · mol^{-1})	ΔG^* (kcal · mol^{-1})	$-\Delta S$ (kcal · degree^{-1} · mol^{-1})
Dimethyl-phosphoryl butyrylcho-linesterase	1.	2-PAM	2.21 ± 0.22	35.1	13.8 ± 1.5	22.1 ± 1.5	28.1 ± 0.4
	2.	TMB-4	3.14 ± 0.16	30.9	12.7 ± 1.5	22.3 ± 1.5	31.1 ± 0.2
	3.	4-PAM	0.36 ± 0.01	3.7	9.5 ± 0.5	23.2 ± 0.5	46.1 ± 0.1
	4.	INAP	0.30 ± 0.04	1.53	17.6 ± 0.9	23.3 ± 0.9	19.2 ± 0.1
	5.	3-PAM	0.21 ± 0.03	1.08	12.6 ± 0.4	23.8 ± 0.4	37.7 ± 0.2
Diethyl-phosphoryl butyrylcho-linesterase	6.	2-PAM	0.95 ± 0.05	17.6	14.6 ± 1.0	22.5 ± 1.0	29.3 ± 0.2
	7.	TMB-4	1.44 ± 0.05	7.4	12.3 ± 1.5	22.7 ± 1.5	34.1 ± 0.3
	8.	4-PAM	0.055 ± 0.005	1.1	13.0 ± 1.8	24.3 ± 1.8	38.6 ± 0.1
	9.	3-PAM	0.028 ± 0.008	0.22	12.9 + 1.2	23.9 ± 1.2	36.8 ± 0.5

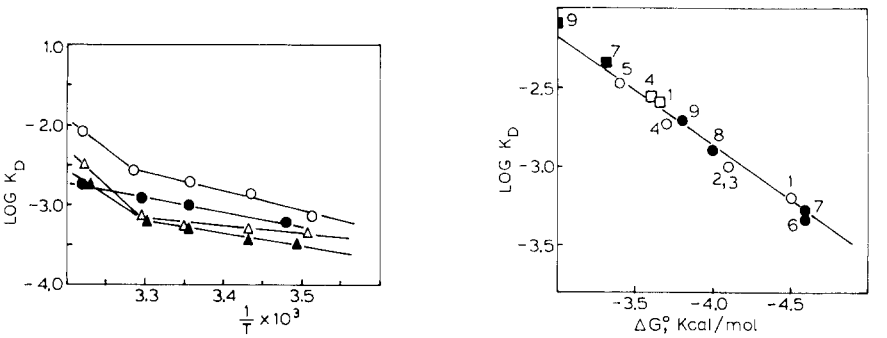


Fig. 1. Temperature dependence of the dissociation constants for the reactivation of dimethylphosphoryl butyrylcholinesterase by 2-PAM (▲) and TMB-4 (●), and for those of diethyl phosphoryl enzyme by 2-PAM (△) and TMB-4 (○), respectively.

Fig. 2. Dependence of the dissociation constants for the binding of oximes to the phosphorylbutyrylcholinesterase on ΔH^0 at pH 7.4. The values are those of Table I. Open and full circles refer to the values for the reactivation of dimethyl and diethyl phosphoryl enzyme at 25°C, and the squares for those at 37°C, respectively.

way. Thus the activation parameters, ΔH^* and ΔS^*, were found to be coupled but neither ΔH^* nor ΔS^* alone could account for the dephosphorylation rate. Variation in the change of free energies (ΔG^*) for a series of reactivators may be roughly correlated with the logarithmic values of the bimolecular rate constants (k_R/K_D), as shown in Fig. 4. A compensation plot of ΔH^* and ΔS^* also yielded a straight line with a compensation temperature approximately at 260 ± 39 K (Fig. 3).

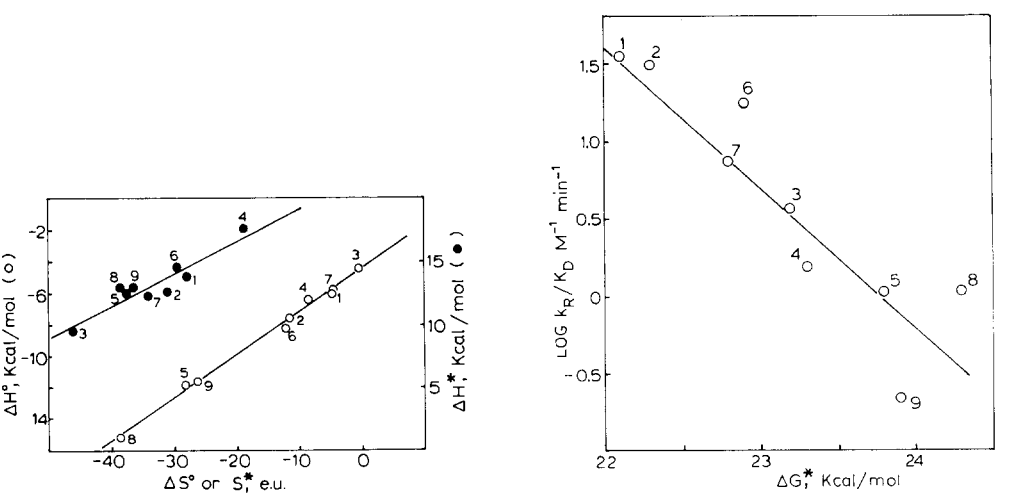


Fig. 3. Compensation plots using the data of Table I for the binding of the reactivators to the phosphoryl butyrylcholinesterase and Table II for the dephosphorylation step at pH 7.4 and 25°C. The lines were drawn according to the method of least squares. e.u., kcal · degree^{-1} · mol^{-1}.

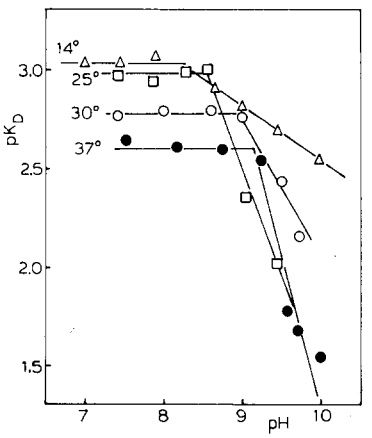
Fig. 4. Dependence of the bimolecular rate constants (k_R/K_D) for the oxime reactivation on ΔG^* at pH 7.4 and 25°C. The values are those of Table II.

Effects of pH

1. On binding. The effect of pH was studied with the reactivation of diethylphosphoryl butyrylcholinesterase by 2-PAM. The dependence of pK_D on pH at temperatures 14, 25, 30, and 37°C is shown in Fig. 5. The inflection points at pH 8.3, 8.6, 9.0, and 9.2, respectively, may be regarded as the apparent ionisation constants (pK_i) for the binding site of the inhibited enzyme, because the reactivation takes place between the protonated form of the inhibited enzyme and the anion of the reactivator. The ionizing group may be ascribed to an ammonium group on account of the range of pK_i values observed. The pK_i values increased with increase of temperature, an effect which is contrary to the normal one for ammonium group. The abnormality of the temperature effect will be discussed later.

2. On spontaneous reactivation. The inhibited enzyme may be regenerated in a buffer medium and the rate of reactivation depends on the nature of the alkyl group of the phosphoryl moiety. In the present investigation the rates of spontaneous reactivation for the dimethylphosphoryl butyrylcholinesterase at various pH values were measured at temperatures below 25°C in order to minimize the effect of ageing. The results are shown in Fig. 6. The catalytic group with an ionisation constant of 9.4 (based on pH at the mid-point of the two sides of the curve) and the heat of ionisation (ΔH_i) of 5.3 kcal · mol^{-1} may be assigned to the phenolic hydroxyl group of tyrosine (see Fig. 7). This result substantiates the similarity of the catalytic group of this enzyme with that of acetylcholinesterase, being in agreement with the data reported by Hovanec and Lieske [10], and roughly in accord with a pK_i value of 9.8 given by Reiner and Aldridge [11] for the latter enzyme. The catalytic group on the other side of the pH profile has a pK_i of 7.5 and ΔH_i of 9.6 kcal · mol^{-1}, which may be ascribed to an ammonium group. This pK_i value is slightly higher than that of 6.9 given by Reiner and Aldridge [11] for bovine acetylcholinesterase.

The effect of pH on the activation parameters is shown in Fig. 8. The free



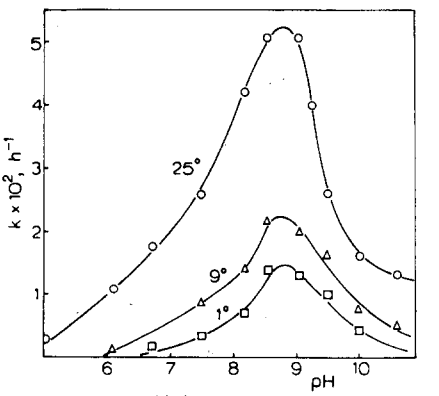


Fig. 5. Dependence of the dissociation constants, K_D, on pH at different temperatures.

Fig. 6. Rate vs. pH profile for the spontaneous reactivation of dimethylphosphoryl butyrylcholinesterase. *k* refers to the first order rate constants.

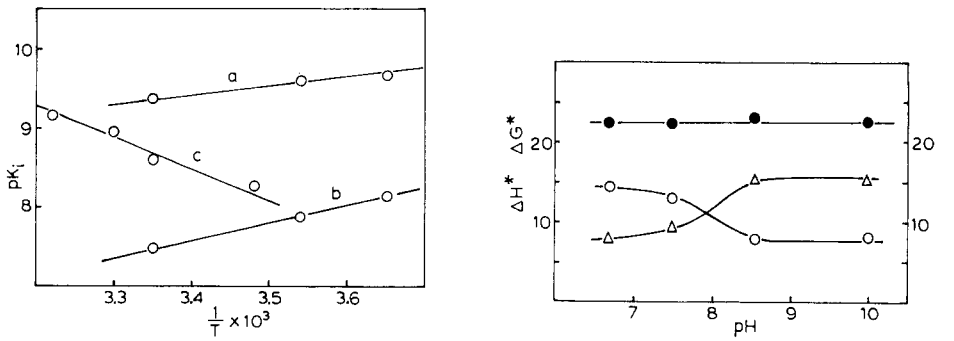


Fig. 7. The effect of temperature on the apparent ionisation constants (pK_i) of the catalytic groups responsible for the reactivation of dimethylphosphoryl butyrylcholinesterase. (a) Phenolic hydroxyl group, $pK_{i,25°C}$ = 9.4 (9.6) and ΔH_i = 5.3 (6.0) kcal · mol^{-1}; (b) α-ammonium group, $pK_{i,25°C}$ = 7.5 (7.6 — 8.4) and ΔH_i = 9.6 (10.0—13.0) kcal · mol^{-1}; and (c) pK_i for the binding site of diethyl phosphoryl butyrylcholinesterase evaluated from the pK_D vs. pH curves of Fig. 5. The values in parentheses refer to those found in literatures (see Steinhardt, J. and Beychok, S. (1964) in The Proteins (Neurath, H., ed.), Vol. 2, Chapt. 8, Academic Press, New York).

Fig. 8. Free energies, enthalpies, and entropies of activation in the spontaneous reactivation of dimethylphosphorylbutyrylcholinesterase. ΔH^* (○), $-T\Delta S^*$ (△), and ΔG^* (●) in kcal · mol^{-1}.

energies of activation change very little with pH whereas the enthalpies and entropies of activation change appreciably but compensated each other so that the free energies do not reflect these changes. ΔH^* for the phenolic group was considerably lower than that for the ammonium group. The average value of ΔG^* has been determined as 22.4 ± 0.4 kcal · mol^{-1} which is similar to that obtained for the oxime reactivation (Table II).

Discussion

The enthalpy-entropy compensation phenomenon has been observed in a considerable number of instances in reactions of small molecules [12] as well as proteins [9]. Unlike the linear free-energy relationship in which the entropy changes remain the same [13], the enthalpy changes in a compensation process are accompanied by corresponding entropy changes. While exact compensation between ΔH and ΔS leads to a very small effect on ΔG, a partial compensation between ΔH and ΔS is of such a nature that their influence on ΔG would allow one to interpret the effect of structural changes on rates, i.e., on ΔG. The reactivation process appears to fall into the latter category since variation in kinetic parameters, K_D and k_R/K_D, were found to be a linear function of ΔG^0 and ΔG^*, respectively. (Figs. 2 and 4).

The compensation phenomenon is generally explained in terms of solute-solvent interaction [9,12]. Water, which before binding is associated with and stabilizes both the inhibited enzyme and the reactivator, may be displaced during the binding process. On the other hand, release of water from the protein may be on occasion followed by rehydration of the bound ligand [14]. In studies of the effects of substituents on the activities of organic acids in water, Hepler [15] suggested that the thermodynamic parameters ΔH^0 and ΔS^0 could be the sum of the external (hydration) and internal (chemical reaction, or ion-

ion interaction) contributions and the classic equation $\Delta G^0 = \Delta H^0 - T\Delta S^0$ may be expanded to:

$$\Delta G^0 = (\Delta H^0_{int} + \Delta H^0_{ext}) - T(\Delta S^0_{int} + \Delta S^0_{ext}). \quad (4)$$

ΔH^0_{int} may be obtained from the compensation plot according to the relationship $\Delta H^0 = \Delta H_0 + T_c \Delta S^0$ [14,15], where T_c refers to the compensation temperature. Thus when $\Delta S^0 = 0$, $\Delta H_0 = \Delta H_{int} = -4.36$ kcal · mol^{-1} (Fig. 3), which represents the invariant, uncompensated force originating from the solute-solute interaction. The value so obtained is similar to that of -4.15 kcal · mol^{-1} given for the binding of cycloalkyl tetramethylammonium salts on bovine acetylcholinesterase [14]. It is seen that ion-ion interaction (-4.36 kcal · mol^{-1}) contributes virtually all of the free energy for binding. Substituting the values of ΔH^0_{int} and the compensation temperature into Eqn. 4 one obtains

$$\Delta G^0 = -4.36 + \Delta H^0_{ext} - 275\Delta S^0_{ext}. \quad (5)$$

Eqn. 5 allows the constants ΔH^0_{ext} and ΔS^0_{ext} to be readily evaluated. For example, the values of ΔH^0 and ΔS^0 for the binding of 2-PAM to dimethyl phosphoryl butyrylcholinesterase are -5.95 kcal · mol^{-1} and -4.90 kcal · degree^{-1} · mol^{-1} (Table I), respectively. ΔH^0_{ext}, therefore, has a value of -1.59 ($= -5.95 + 4.36$) kcal · mol^{-1} but it is compensated by the term of $T\Delta S^0_{ext}$ which it is $-275 \cdot 4.9$ i.e. -1.35 kcal · mol^{-1}. These values correspond to one molecule of water being involved in the binding process, since the freezing of one molecule of water is characterized by a ΔH^0 of -1.4 kcal · mol^{-1} and a ΔS^0 of -5.3 kcal · degree^{-1} · mol^{-1}. It is calculated that there were averages of 2 and 4 molecules of water involved in the reactivation of dimethyl- and diethyl-phosphoryl butyrylcholinesterases at temperatures below 30°C and 24 molecules above 30°C. The number of water molecules involved, however, bore no relationship to the values of K_D. It is to be noted that the thermodynamic parameters for 3-PAM (No. 4) appeared abnormal in that they were higher at temperatures below 25°C than those above 25°C. No explanation for this discrepancy could be offered.

Since the observed entropies (ΔS^0) were negative values, water-releasing ability of the oxime in the binding process will not be reflected in the relative values of ΔH^0 and ΔS^0. The negative entropies can only be interpreted as a sign of rehydration of the bound reactivators. Thus 2-PAM has a net structure-making effect on water when in bound state. Since reorganization of the water structure around the reactive site involves bond-making and -breaking of the hydrogen bond, it gives rise to a question of conformational aspects of the inhibited enzyme. Evidence gathered from the present investigation appears to favor a conformational transition. Firstly there are four cases for which the Arrhenius plotes were non-linear when the reactivations were carried out at temperatures above 30°C (Fig. 1 and Table I). The existence of a transition temperature is generally regarded as direct evidence of conformational change since the macroscopic state of protein may be determined by either enthalpy or entropy. The entropy value is particularly interesting because of its close relationship with the volume as well as the phase changes, and therefore is used as an index of the integrity of folding of proteins [9]. In Table I it is seen that

ΔS^0 values vary from 3- (No. 7) to 24-fold (No. 1) in these two temperature zones and the magnitude of the changes is large enough to suggest a conformational transition. Secondly, the values of pK_i for the binding site of the inhibited enzyme increase with increase of temperature (Fig. 7c) in contrast to a decrease as required by an ammonium group. The acidity of the binding site is therefore weakened at higher temperature and the binding capacity is correspondingly reduced (Table I). The abnormality of the temperature effect may very well arise from the conformational changes in the inhibited enzyme induced by bound or unbound 2-PAM in the binding step. The heat of ionisation could not be determined precisely without paying due consideration to the conformational changes. There is also indirect evidence which lends support in favor of conformation alteration. Kuhnen [16] reported that the biquaternary reactivators such as Toxogonin and TMB-4, have an activating or inhibitory effect on the enzyme acetylcholinesterase and explained the effects in terms of the presence of an allosteric site in the enzyme. Conformation modification arising from allosteric binding of quaternary ammonium ligands such as curare has been a subject of much discussion [17,18]. In a biophysical analysis of the system acetylcholinesterase-tetramethylammonium ligands, Belleau and Lavoie [14] concluded that the muscarinic activator ligands have a strong modulating influence on a phase transition in "ice-like" water molecules within the area of the enzyme binding cleft thereby causing a conformational alteration. Oximes of pyridinium compounds may not be comparable with muscarinic agents in a strict sense, but the similarity in being a quaternary nitrogen salt and their thermodynamic process, i.e., compensation phenomenon, tempts one to form the same conclusion.

We therefore conclude that the reactivation process, like many other reactions involving small molecules and proteins in water, may consist of a chemical part and a solvation (compensation) part processes. These two processes are coupled. The compensation process can only be revealed through studies of the temperature effects. When the reactivation is studied at one temperature, the solvation process is hidden. From the thermodynamic point of view enthalpy-entropy compensation may be a major physiologically important property of proteins in both equilibrium and rate processes.

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