Effect of Temperature and pH on Carbamoylation and Phosphorylation of Serum Cholinesterases

THEORETICAL INTERPRETATION OF ACTIVATION ENERGIES IN COMPLEX REACTIONS

By VERA SIMEON and ELSA REINER

*Institute for Medical Research, Yugoslav Academy of Sciences and Arts,
Zagreb, Croatia, Yugoslavia*

and C. A. VERNON

Department of Chemistry, University College London, London W.C.1, U.K.

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1. The effect of temperature and pH was studied on the kinetics of inhibition of horse serum and human serum cholinesterase by four organophosphorus compounds and five carbamates. 2. For all compounds, and at each pH and temperature, the inhibition followed the kinetics of a bimolecular reaction with the inhibitor in excess, and with a negligible concentration of the Michaelis complex. 3. The second-order rate constants (k_a) for inhibition of human serum cholinesterase by one organophosphate and one carbamate increased from 5° to 40°C with an apparent activation energy of 46 kJ/mol (11 kcal/mol). 4. The k_a constant for inhibition of horse serum cholinesterase increased with temperature from 5° to 30°C, and then decreased from 30° to 40°C. The theoretical interpretation of such an unusual effect of temperature is derived. 5. The increase of k_a with pH (human serum cholinesterase) followed the dissociation curve for a single group on the enzyme (pK 7.5). 6. Rate constants for decarbamoylation (k_{+3}) were determined, and the time-course of inhibition was calculated from the k_a and k_{+3} constants.

Organophosphorus compounds and carbamates acylate the active site of cholinesterases and the reaction proceeds through at least one intermediate:

where EH, EHAB and EA are the free enzyme, the Michaelis complex and the acylated enzyme respectively, and AB stands for the inhibitor. If the concentration of EHAB is only a small fraction of the total enzyme concentration eqn. (1) simplifies to:

where k_a is the second-order rate constant of acylation. The above reactions have been shown to apply to both acetylcholinesterase (EC 3.1.1.7) and cholinesterase (EC 3.1.1.8). With carbamates fewer data on cholinesterase are available (Myers, 1952; Goldstein & Hamlish, 1952; Main, 1964) than on acetylcholinesterase (Wilson *et al.*, 1960, 1961; Main & Hastings, 1966; Reiner & Aldridge, 1967) and there are no data on the effect of temperature and pH on carbamoylation of cholinesterase. We have studied the kinetics of inhibition of serum cholinesterase by carbamates and organophosphorus com-

pounds with the particular aim of establishing the extent of formation of reversible intermediates in the reaction.

Materials and Methods

Enzyme preparation

Purified human serum cholinesterase (Kabi AB, Stockholm, Sweden) and purified horse serum cholinesterase (Sigma Chemical Co., St. Louis, Mo., U.S.A., and a preparation given by Dr. Lj. Kamarić, Institute of Pathophysiology, University of Ljubljana, Ljubljana, Yugoslavia) were used. Stock solutions were made up in buffer or water (with 1% gelatin) and kept at 4°C for no longer than 2–3 days. Dilutions were prepared in buffer immediately before use. The enzyme concentrations during assay varied from 30 to 160 µg/ml and from 6 to 16 µg/ml for the human and the horse preparation respectively.

For some experiments native human plasma and native horse serum were also used as cholinesterase preparations. These were stored at 4°C for up to several weeks, and the concentrations during assay were 30 µl/ml for both preparations.

Table 1. *Chemical names and formulae of inhibitors*

2-Isopropoxyphenyl <i>N</i> -methylcarbamate	$(\text{CH}_3)_2\text{CH}-\text{O}-\text{C}_6\text{H}_4-\text{O}-\text{CO}-\text{NH}(\text{CH}_3)$
3-Isopropylphenyl <i>N</i> -methylcarbamate	$(\text{CH}_3)_2\text{CH}-\text{C}_6\text{H}_4-\text{O}-\text{CO}-\text{NH}(\text{CH}_3)$
Bromide of 3-trimethylaminophenyl <i>N</i> -methylcarbamate (monomethylneostigmine)	$(\text{CH}_3)_3\text{N}^+-\text{C}_6\text{H}_4-\text{O}-\text{CO}-\text{NH}(\text{CH}_3) \text{Br}^-$
3-Isopropylphenyl <i>NN</i> -dimethylcarbamate	$(\text{CH}_3)_2\text{CH}-\text{C}_6\text{H}_4-\text{O}-\text{CO}-\text{N}(\text{CH}_3)_2$
Methyl sulphate of 3-trimethylaminophenyl <i>NN</i> -dimethylcarbamate (neostigmine)	$(\text{CH}_3)_3\text{N}^+-\text{C}_6\text{H}_4-\text{O}-\text{CO}-\text{N}(\text{CH}_3)_2 \text{CH}_3-\text{SO}_4^-$
Di-(2-chloroethyl)-3-chloro-4-methylcoumarin-7-yl phosphate (haloxon)	$(\text{ClC}_2\text{H}_4-\text{O})_2\text{PO}-\text{O}-\text{C}=\text{CH}-\text{C}=\text{C}-\text{CH}=\text{CH}$ $\begin{array}{c} \text{O}-\text{CO}-\text{CCl}=\text{C}-\text{CH}_3 \\ \text{O}-\text{CO}-\text{CCl}=\text{C}-\text{CH}_3 \end{array}$
Methyl sulphate of diethyl 3-trimethylaminophenyl phosphate (phosphostigmine)	$(\text{C}_2\text{H}_5-\text{O})_2\text{PO}-\text{O}-\text{C}_6\text{H}_4-\text{N}^+(\text{CH}_3)_3 \text{CH}_3-\text{SO}_4^-$
Methyl sulphate of diethyl methyl <i>NN</i> -diethyl- <i>N</i> -methyldiethylthiophosphate (compound PTCh)	$(\text{C}_2\text{H}_5-\text{O})(\text{CH}_3)\text{PO}-\text{S}-[\text{CH}_2]_2-\text{N}^+(\text{CH}_3)(\text{C}_2\text{H}_5)_2 \text{CH}_3-\text{SO}_4^-$
Dimethyl <i>p</i> -nitrophenyl phosphate (compound Me-E600)	$(\text{CH}_3-\text{O})_2\text{PO}-\text{O}-\text{C}_6\text{H}_4-\text{NO}_2$

Inhibitors

The monomethylcarbamates, dimethylcarbamates and organophosphorus compounds used as inhibitors are listed in Table 1. Stock solutions (20 or 50 mm) of neostigmine, monomethylneostigmine and phosphostigmine were prepared in water, haloxon and compound Me-E600 in ethanol, compound PTCh in propylene glycol and the other compounds in dimethylformamide. All solutions were stored at 4°C except monomethylneostigmine, which is unstable and was prepared immediately before use. Further dilutions were prepared in water or buffer immediately before use.

Buffers

Phosphate buffers (0.1 and 0.5 M) were obtained by titrating 0.1 M- or 0.5 M- NaH_2PO_4 with 0.1 M- or 0.5 M- Na_2HPO_4 respectively to pH 7.4 at the required temperature. Phosphate-pyrophosphate buffer (0.02 M) was obtained by titrating 0.02 M- $\text{Na}_2\text{H}_2\text{P}_4\text{O}_7$ with 0.02 M- Na_3PO_4 to the required pH at 25°C.

Enzyme activity

The method of Ellman *et al.* (1961) was used with acetylthiocholine as substrate. The final substrate concentration was 1.0 mM, except when otherwise stated. The thiol reagent (5,5'-dithiobis-2-nitrobenzoate) was added immediately before or together with the substrate. The extinction was measured at 412 nm on a Beckman spectrophotometer (model DU) or Unicam spectrophotometer (SP.500) in 1.0 cm cuvettes thermostatically maintained at the given temperature.

Enzyme inhibition at pH 7.4

Enzyme and inhibitor (3.0 ml total volume) were incubated in 0.1 M-phosphate buffer for a given time before the substrate (0.3 ml) was added. The extinction was read at 15 s intervals for 1.0–2.0 min, the first reading being taken 30 s after the substrate had been added.

To obtain the degree of reversible inhibition, the enzyme was added to a mixture of substrate and inhibitor, and the activity was measured as described above.

The increase in extinction was linear with time for both inhibited and control enzyme samples.

Effect of pH on enzyme inhibition

Enzyme and inhibitor were incubated for a given time in 0.02 M-phosphate-pyrophosphate buffer at a given pH. At suitable time-intervals 0.5 M-phosphate buffer (containing substrate and the thiol reagent) was added to bring the pH to 7.4, at which pH the enzyme activity was always measured. Details of the procedure were as described by Reiner & Aldridge (1967).

Spontaneous reactivation at pH 7.4

Enzyme and inhibitor were incubated for about 15 min to obtain 80–90% cholinesterase inhibition. Then the enzyme-inhibitor solution was diluted 500-fold with 0.1 M-phosphate buffer. At suitable time-intervals 3.0 ml samples were withdrawn, and the activity of the enzyme was determined as described above under 'Enzyme inhibition at pH 7.4'. The concentrations of the inhibitor during reactivation were

such that after dilution the inhibitor did not inhibit more than 5% of the free enzyme during the reactivation time.

Results and Discussion

Kinetics of inhibition

The compounds listed in Table 2 were studied as inhibitors of purified horse serum cholinesterase. The experiments were done in 0.1M-phosphate buffer, pH 7.4, at 25°C.

To obtain the second-order rate constants of inhibition (k_a), four or five different inhibitor concentrations were used and for each concentration the degree of inhibition was determined for at least three different times of incubation. An example of the results is presented in Fig. 1. For a given inhibitor concentration the rate of inhibition follows first-order kinetics (Fig. 1a), and the first-order rate constants (k) are linear functions of the inhibitor concentration (Fig. 1b). The inhibition thus shows the characteristics of a bimolecular reaction with one

Table 2. Second-order rate constants (k_a) for inhibition of purified horse serum cholinesterase by some carbamates and organophosphates measured in 0.1M-phosphate buffer, pH 7.4, at 25°C

The s.e.m. values are the standard errors of the slopes of the line obtained when first-order rate constants of inhibition k are plotted against the inhibitor concentrations.

Inhibitor	$10^{-4} \times k_a \pm \text{s.e.m.}$ ($\text{M}^{-1} \cdot \text{min}^{-1}$)	No. of expts.	Range of inhibitor concentrations (μM)
2-Isopropoxyphenyl <i>N</i> -methylcarbamate	0.36 ± 0.01	35	3–100
3-Isopropylphenyl <i>N</i> -methylcarbamate	12.4 ± 0.4	21	0.08–15
Monomethylnestigmine	17.7 ± 0.1	8	0.5–4
3-Isopropylphenyl <i>N</i> -dimethylcarbamate	1.52 ± 0.01	7	3–50
Neostigmine	10.6 ± 0.3	7	0.15–8
Haloxon	1520 ± 41	24	0.0056–0.056
Phosphostigmine	492 ± 7	17	0.1–0.5

Fig. 1. Inhibition of purified horse serum cholinesterase by haloxon measured in 0.1M-phosphate buffer, pH 7.4, at 25°C

(a) Plot of log [activity (%)] against time of inhibition. The inhibitor concentrations are shown against each line.
(b) Plot of the first-order rate constants k (i.e. slope of the inhibition lines in a), against the haloxon concentrations.

component in excess, and with the concentrations of the Michaelis complex negligibly small (eqn. 2):

$$\ln \left(\frac{[E_0]}{[E_0] - [EA]} \right) = k_a [AB] t \quad (3)$$

where $[E_0]$ is the total enzyme concentration. The k_a constants given in Table 2 were calculated from a plot of the first-order rate constants (k) against the inhibitor concentrations (cf. Fig. 1).

When monomethylcarbamates were used as inhibitors eqn. (3) was applicable only when the time of inhibition was short, and all k_a values listed in Table 2 were therefore calculated from experiments with inhibition times less than 10 min. When inhibition proceeded for a longer time, deviations from the kinetics described by eqn. (3) were observed and the reaction approached steady state after about 100–150 min (Fig. 2). Such kinetics are due to spontaneous reactivation of the monomethylcarbamoylated cholinesterase:

Rates of spontaneous reactivation were measured, and the calculated rate constants (k_{+3}) are given in Table 3.

When an inhibited enzyme is unstable so that reaction (4) occurs simultaneously with reaction (2)

the time-course of inhibition will be described by eqn. (5) (cf. Aldridge & Reiner, 1972):

$$[EA] = \frac{k_a [E_0] [AB]}{k_a [AB] + k_{+3}} \{1 - \exp[-(k_a [AB] + k_{+3}) t]\} \quad (5)$$

Insertion of values for k_a and k_{+3} from Tables 2 and 3 into eqn. (5) allowed calculation of the theoretical curves in Fig. 2, which approximate to the experimental points. A similar agreement is found for the time-course of inhibition by both 2-isopropoxyphenyl *N*-methylcarbamate and 3-isopropylphenyl *N*-methylcarbamate. Monomethylneostigmine is unstable and therefore long times of inhibition could not be studied.

Eqn. (5) will approximate to eqn. (3) if k_{+3} is much smaller than $k_a [AB]$. This is the case with monomethylcarbamates when inhibition is followed over a short period of time, and consequently high concentrations of AB must be used. For inhibition with dimethylcarbamates eqn. (3) was valid up to reaction times of 150 min and with relatively low inhibitor concentrations, because k_{+3} for the dimethylcarbamylated horse serum cholinesterase is smaller than for the monomethylcarbamylated enzyme (Table 3). Horse serum cholinesterase phosphorylated by haloxon did not reactivate spontaneously within 1 h; diethylphosphorylated horse serum cholin-

Fig. 2. Inhibition of purified horse serum cholinesterase by 2-isopropoxyphenyl *N*-methylcarbamate measured in 0.1 M-phosphate buffer, pH 7.4, at 25°C

The inhibitor concentrations are: (a) $\circ$, 3.0 μM ; (b) $\bullet$, 5.0 μM ; (c) $\triangle$, 10.0 μM . The points are the experimental results and the curves have been calculated from eqn. (5), by using $k_a = 3.6 \times 10^3 \text{ M}^{-1} \cdot \text{min}^{-1}$ and $k_{+3} = 4.3 \times 10^{-3} \text{ min}^{-1}$ (Tables 2 and 3).

Table 3. Rate constants of spontaneous reactivation (k_{+3}) of carbamoylated human and horse serum cholinesterase

The reactivations of horse serum cholinesterase and human serum cholinesterase were carried out in 0.1 M-phosphate buffer and 0.02 M-phosphate-pyrophosphate buffer respectively.

Enzyme preparation	Carbamoyl group	$10^3 \times k_{+3}$ (min^{-1})	No. of expts.	Temp. (°C)
Purified horse serum	-CO-NH-CH ₃	1.2	1	10
	-CO-NH-CH ₃	4.3	9	25
	-CO-NH-CH ₃	7.9	1	40
Purified human serum	-CO-NH-CH ₃	3.7	1	25
Purified horse serum	-CO-N(CH ₃) ₂	0.71	5	25
	-CO-N(CH ₃) ₂	1.7	2	40
Purified human serum	-CO-N(CH ₃) ₂	3.3	3	25

esterase is also stable (Davison, 1955) and consequently eqn. (3) will be applicable to haloxon and phosphostigmine at any time of inhibition.

Of the carbamates studied there are two pairs of compounds with the same leaving group, one compound a monomethyl- and the other a dimethyl-carbamate: monomethylneostigmine and neostigmine, 3-isopropylphenyl *N*-methylcarbamate and 3-isopropylphenyl *N*-dimethylcarbamate (Table 2). In both instances the monomethyl derivative is a better inhibitor. The same is true for inhibition of acetylcholinesterase by these compounds (Reiner & Aldridge, 1967). However, this is not generally true for any pair of mono- and di-methylcarbamates (cf. Metcalf, 1971).

The kinetics of inhibition of serum cholinesterase by phosphostigmine indicates that a measurable concentration of a Michaelis complex was not present, in contrast with the progressive inhibition of acetylcholinesterase by the same compound (Reiner & Aldridge, 1967). Also, the kinetics of inhibition of serum cholinesterase by haloxon shows no evidence of a reversible complex, which is present in the reaction of acetylcholinesterase with haloxon (Aldridge & Reiner, 1969).

The Michaelis complex between EH and AB, with any of the inhibitors studied in the present work, did not show up in the kinetics of progressive inhibition; the first-order rate constants were therefore always linear functions of the inhibitor concentration. However, for a given compound, the inhibition lines obtained with the highest inhibitor concentration did not always pass through the origin [i.e. $\log(100\% \text{ activity}) = 2.00$] but intercepted the ordinate somewhere between $\log(100\% \text{ activity})$ and $\log(85\% \text{ activity})$ (cf. Fig. 1*a*). The same intercept was obtained by either extrapolating the inhibition lines to zero time, or by adding the enzyme to a mixture of substrate and inhibitor (cf. 'Enzyme inhibition at pH 7.4' in the Materials and Methods section). This 'intercept' has

the characteristics of a reversible complex, as proved in the following two ways. When inhibition was measured with 10 mM-substrate (instead of 1.0 mM) the slope of the inhibition line was the same (i.e. the same rate of progressive inhibition was obtained) but no intercept was present. Further, when the enzyme-inhibitor mixture was incubated for a certain time and then diluted 60-fold (by adding a large volume of substrate) the slope of the inhibition line was again the same, but no intercept was present. [For details of these two procedures see Aldridge & Reiner (1969).] An intercept of the same order was also obtained when the activity was measured with other substrates (1.0 mM-2-nitrophenyl butyrate or 1.0 mM-*n*-butyrylthiocholine).

The nature of a reversible complex that occupies only a small fraction of the total enzyme concentration is difficult to study. Whenever an enzyme forms a reversible complex (Michaelis complex or any other complex) this complex will show up in the kinetics of progressive inhibition in such a way that the first-order rate constants will not be linear with the inhibitor concentration (Aldridge & Reiner, 1972). However, when the inhibitor occupies only 10% or less of the total enzyme concentration, the deviations from linearity will be small and difficult to observe. Further, it is not possible to study the effect of substrate on the degree of reversible inhibition, which would help to elucidate the nature of the complex. Such studies have been possible in the reaction between haloxon and acetylcholinesterase where the 'intercept inhibition' was very large, and from the effect of substrate it was concluded that haloxon reacts in an allosteric site of acetylcholinesterase (Aldridge & Reiner, 1969). This reaction does not seem to occur with haloxon and serum cholinesterase within the concentration range used in our experiments (Table 2); higher haloxon concentrations could not be applied because the rates of inhibition were too fast to be measured accurately.

Effect of pH

The effect of pH on inhibition of purified human serum cholinesterase was studied in 0.02M-phosphate-pyrophosphate buffer at 25°C. At all pH values and for all inhibitors the time of inhibition was less than 10 min. Under these conditions the reactions followed the kinetics defined by eqn. (3), from which the rate constants k_a were calculated. The results are summarized in Fig. 3 and Table 4.

The rate constants increase with pH and reach a maximum at about pH 9.5. The increase follows a theoretical curve for the dissociation of a single group on the enzyme with $pK_1 = 7.5$. Inhibition by each of

Fig. 3. Effect of pH on inhibition of purified human serum cholinesterase

k_a ($M^{-1} \cdot \text{min}^{-1}$) is the second-order rate constant of inhibition obtained at 25°C in 0.02M-phosphate-pyrophosphate buffer. The curve is calculated from a dissociation constant $pK_1 = 7.5$. Inhibitors: ●, neostigmine; ▲, 3-isopropylphenyl *N*-methylcarbamate; ■, 3-isopropylphenyl *N*-dimethylcarbamate; ○, compound Me-E600; △, compound PTCh.

the five compounds follows the same theoretical curve, irrespective of whether the inhibitor was an organophosphate or a carbamate, a positively charged or an uncharged compound. In this respect serum cholinesterase is different from acetylcholinesterase. Inhibition of acetylcholinesterase by positively charged organophosphorus compounds and carbamates requires two groups on the enzyme, pK_1 6.2 and pK_2 10.25; inhibition of acetylcholinesterase by uncharged organophosphates and carbamates requires only one group, pK_2 10.25 (Reiner & Aldridge, 1967). Such differences between the two enzymes might indicate that their catalytic mechanisms are different.

The rates of inhibition by a given compound of human serum cholinesterase (Table 4) and horse serum cholinesterase (Table 2) are of the same order of magnitude.

Effect of temperature

The effect of temperature was studied in 0.1M-phosphate buffer at pH 7.4 over the temperature range 10–40°C. Most experiments were done on purified horse serum cholinesterase, but for some experiments native human and horse serum and purified human serum cholinesterase were also used. The inhibitors were two organophosphorus compounds (haloxon and phosphostigmine) and two carbamates (neostigmine and 3-isopropylphenyl *N*-methylcarbamate).

The activities of the enzyme sample without inhibitor remained constant during the experiment at any temperature; i.e. no thermal inactivation was observed for any enzyme preparation within the temperature range used.

At any given temperature, the kinetics of inhibition followed eqn. (3) for each enzyme preparation and each inhibitor. In all experiments the time of inhibition was 10 min or less. The effect of temperature on the k_a value differed for horse serum and human serum cholinesterase.

The second-order rate constants of inhibition (k_a)

Table 4. Second-order rate constants of inhibition (k_a) of purified human serum cholinesterase at optimal pH (9.0–10.0) measured in 0.02M-phosphate-pyrophosphate buffer at 25°C

Inhibitor	$10^{-4} \times k_a$ ($M^{-1} \cdot \text{min}^{-1}$)	No. of expts.	Range of inhibitor concentrations (μM)
3-Isopropylphenyl <i>N</i> -methylcarbamate	17.8	5	0.5–2
3-Isopropylphenyl <i>N</i> -dimethylcarbamate	2.0	5	4–16
Neostigmine	37	2	0.4–1.6
Compound Me-E600	2.7	4	4–16
Compound PTCh	1780	4	0.01–0.05

Fig. 4. Effect of temperature on the second-order rate constants of inhibition (k_a) for (a) purified horse serum cholinesterase and (b) purified human serum cholinesterase measured in 0.1 M-phosphate buffer, pH 7.4

of purified horse serum cholinesterase increased with temperature up to 30°C, but when the temperature increased further the rate constants decreased (Fig. 4a). Inhibition by neostigmine was also measured with a different method (pH-stat method with 10 mM-acetylcholine as substrate) and the results were the same. The effect of temperature on inhibition of native horse serum cholinesterase by neostigmine was also the same as that on the purified preparation. Such an effect of temperature is unusual.

However, the effects of temperature on the inhibition of human serum cholinesterase (purified and native) by haloxon and neostigmine were different (Fig. 4b). The k_a values increased with temperature and the increase followed the Arrhenius law. The energies of activation for inhibition of the purified human serum cholinesterase are 44.4 kJ/mol (10.6 kcal/mol) and 46.9 kJ/mol (11.2 kcal/mol) respectively. The same activation energies are also found for the inhibition of acetylcholinesterase by organophosphates (Aldridge, 1953; Aharoni & O'Brien, 1968; Davison, 1955) and carbamates (Winteringham & Fowler, 1966).

The theoretical basis for the unusual effect of temperature on the inhibition of horse serum cholinesterase is outlined in the Appendix. Eqn. (1) involves a model in which the apparent second-order rate coefficient k_a is a function of three rate coefficients characterizing the individual steps. It is shown

that negative values of the apparent activation energy can arise if:

$$E_{-1} > (E_{+1} + E_{+2})$$

where the three quantities represent the activation energies of the individual steps. It is further shown that the apparent activation energy for the overall process will be positive at low temperatures, where the intermediate EHAB is not at equilibrium with reactants, and will become negative at higher temperatures when effective equilibrium between intermediate and reactants will be established. In an important sense the observed behaviour is an indirect proof of the existence of a complex between enzyme and inhibitor of the 'Michaelis' type. Direct proof would be difficult since the observed kinetic form entails that the concentration of the intermediate is low compared with the concentrations of reactants.

It is also shown that one possible physical interpretation of the origin of the unusual temperature-dependence of the rate is that a large entropy change occurs on the formation of the intermediate EHAB. This might reflect changes in solvation produced by a considerable conformational change in the protein.

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APPENDIX

Theoretical Interpretation of the Activation Energies in Complex Reactions

For any chemical reaction (unimolecular or bimolecular) proceeding by one stage, the rate constant can be expressed in the form:

$$k_1 = A_1 \cdot \exp(-E_1/RT) \quad (1)$$

where A_1 is sensibly independent of temperature and E_1 is the so-called activation energy. For reactions proceeding in one stage only, E_1 always has a positive value. The functional dependence of k_1 on T is obtained by taking logarithms, which gives the conventional Arrhenius plot.

Some reactions proceed via an intermediate that remains at equilibrium with the reactants throughout the course of the reaction:

If the concentration of XY is small compared with the concentrations of X and Y (i.e. the equilibrium lies to the left), the reaction will show second-order kinetics and the apparent rate constant k_a will be given by:

$$k_a = \frac{k_{+1}}{k_{-1}} \cdot k_{+2} \quad (3)$$

If all the rate constants are replaced by eqn. (1) we have:

$$A_a \cdot \exp(-E_a/RT) = \frac{A_{+1} \cdot A_{+2}}{A_{-1}} \cdot \exp[-(E_{+2} + E_{+1} - E_{-1})/RT] \quad (4)$$

This reaction will obey the Arrhenius equation, but the apparent activation energy E_a has no simple

significance since it is made up of three terms all of which are unknown:

$$E_a = (E_{+2} + E_{+1} - E_{-1}) \quad (5)$$

The quantity $(E_{+1} - E_{-1})$ is equal to the standard enthalpy change (ΔH^θ) for the formation of the intermediate XY , hence:

$$E_a = E_{+2} + \Delta H^\theta \quad (6)$$

The value of E_{+2} must be positive but ΔH^θ can be either positive or negative. Consequently, although a plot of $\log k_a$ against $1/T$ will always be a straight line, the value of E_a could be negative and the reaction rate would steadily decrease as the temperature is raised. The reason such behaviour is not actually observed is that the requirement that equilibrium between intermediate and reactants lies to the left is, for most cases of interest, equivalent to the requirement that the standard free energy change (ΔG^θ) for the formation of the intermediate be positive. On the other hand, E_a can be negative only if the corresponding standard enthalpy change be large and negative. Such a situation is, in principle, possible, but is most unlikely in reactions involving small molecules. What is not possible with this model is for the apparent activation energy to have a positive value over one temperature range and a negative one over another.

The reactions involved in the combination of an enzyme with inhibitors of the type studied in the

present work can be formulated as in eqn. (1) in the main paper. Making the usual assumption that $[AB] \gg [E_0]$ and that the intermediate is at steady state, the expression for the reciprocal of the apparent second-order rate coefficient is:

$$\frac{[E_0][AB]}{v} = \frac{1}{k_a} = \frac{[AB]}{k_{+2}} + \left(\frac{k_{-1} + k_{+2}}{k_{+2} \cdot k_{+1}} \right) \quad (7)$$

where v is the initial velocity of the reaction.

$$E_{-1} > (E_{+1} + E_{+2}) \quad (11)$$

$$\left| \frac{A_{-1}}{A_{+2}} (E_{+1} + E_{+2} - E_{-1}) \cdot \exp[(E_{+1} + E_{+2} - E_{-1})/RT] \right| > \left| E_{+1} \cdot \exp(E_{+1}/RT) \right| \quad (12)$$

The inequality (12) rearranges to the more convenient form:

$$\left| \frac{A_{-1}}{A_{+2}} \frac{(E_{+1} + E_{+2} - E_{-1})}{E_{+1}} \right| > \left| \exp[(E_{-1} - E_{+2})/RT] \right| \quad (13)$$

The reactions reported here all showed second-order kinetics: consequently the concentrations of the intermediates must be low compared with the concentrations of the reactants. This means that the first term on the right-hand side of eqn. (7) can be neglected and the reciprocal of the apparent second-order rate coefficient is given to a good approximation by:

$$\frac{1}{k_a} = \frac{k_{-1}}{k_{+2} \cdot k_{+1}} + \frac{1}{k_{+1}} \quad (8)$$

It has been found that the apparent activation energies for some of the reactions are positive below about 30°C but become negative as the temperature increases. To explain this unusual behaviour it is necessary to write k_a as a function of temperature. Substitution of eqn. (1) into eqn. (8) gives the required expression:

$$\frac{1}{k_a} = \frac{A_{-1}}{A_{+2} A_{+1}} \cdot \exp[(E_{+1} + E_{+2} - E_{-1})/RT] + \frac{1}{A_{+1}} \cdot \exp(E_{+1}/RT) \quad (9)$$

The temperature-dependence of rate coefficients is usually examined from the functional relationship between $\log k$ and absolute temperature. It is immediately apparent that this procedure would lead to algebraically intractable expressions with eqn. (9). In fact this is true of all equations describing enzymic processes, since these always contain expressions in which rate coefficients are added. The way to avoid this difficulty is to examine the functional dependence of the reciprocal of the rate coefficient and absolute temperature. The required expression is easily found by differentiating eqn. (9), i.e.:

$$\frac{d(1/k_a)}{dT} = - \frac{A_{-1}}{A_{+2} \cdot A_{+1}} \frac{(E_{+1} + E_{+2} - E_{-1})}{RT^2} \cdot \exp[(E_{+1} + E_{+2} - E_{-1})/RT] - \frac{E_{+1}}{A_{+1} \cdot RT^2} \cdot \exp(E_{+1}/RT) \quad (10)$$

Now the condition that the apparent activation energy for the whole process should be negative [i.e. $d(\log k_a)/dT$ be negative] is equivalent to the condition that $d(1/k_a)/dT$ be positive. It is immediately apparent that such behaviour is possible, since although the second term on the right-hand side of eqn. (10) must always be negative the first may be either positive or negative. The quantity $d(1/k_a)/dT$ will, in fact, be positive given the two inequalities (11) and (12):

The two inequalities (11) and (13) therefore represent the necessary and sufficient conditions for a reaction conforming to eqn. (1) (in the main paper) and obeying second-order kinetics to show negative values of the apparent activation energy over some temperature range. It is important to note that if the inequality (13) is true at some temperature T_2 it must cease to be true at some lower temperature T_1 , since the left-hand side is virtually independent of temperature whereas the right-hand side increases as the temperature decreases if, as required for the inequality to be true anywhere, $E_{-1} > E_{+2}$. Hence the value of the apparent activation energy will become negative, if at all, only when the temperature is raised, and this, of course, is what is observed. In fact, if the inequality (11) is true then there must be a temperature above which the inequality (13) is also true. This temperature, which may not be experimentally accessible, is the point at

which the sign of the apparent activation energy changes from positive to negative.

It is important to note that when the inequality (13) is false but the inequality (11) is true, i.e. over that part of the temperature range for which the apparent activation energy is positive, the intermediate EHAB cannot be at equilibrium with respect to EH and AB. This is seen by writing eqn. (8) in the form:

$$\frac{1}{k_a} = \frac{k_{-1} + k_{+2}}{k_{+2} \cdot k_{+1}} \quad (14)$$

and noting that the condition for EHAB to be effectively at equilibrium with reactants is:

$$k_{-1} \gg k_{+2} \quad (15)$$

This has the effect of removing the second term from the right-hand side of eqn. (8): consequently the sign of $d(1/k_a)/dT$ is now wholly determined by the sign of the quantity $(E_{+1} + E_{+2} - E_{-1})$, and if the inequality (11) is true the apparent activation energy is negative at all temperatures.

Above the temperature at which the sign of the apparent activation energy changes, the concentration of the intermediate EHAB becomes close to the equilibrium concentration, since the inequality (11) requires that:

$$E_{-1} \gg E_{+2} \quad (16)$$

and, consequently, the inequality (15) must become true as the temperature is raised sufficiently. The standard enthalpy change for the formation of EHAB is given by:

$$\Delta H^\theta = E_{+1} - E_{-1} \quad (17)$$

and must therefore have a negative sign, i.e. the reac-

tion must be exothermic. From inequality (11) and eqn. (17) we have:

$$|\Delta H^\theta| > |E_{+2}| \quad (18)$$

This inequality entails that when the intermediate EHAB is sensibly at equilibrium with reactants the decrease in its concentration as the temperature is raised, as a consequence of the shift of the equilibrium to the left, more than offsets increased breakdown to products. Hence the overall reaction slows down as the temperature increases.

The analysis cannot be taken further in general terms, and it is necessary to consider some likely values for the various quantities. The values of E_{+1} and E_{+2} cannot be less than about 16 kJ/mol (4 kcal/mol): hence E_{-1} cannot be less than about 40 kJ/mol (10 kcal/mol) for the inequality (11) to be true. With these values $\exp[(E_{-1} + E_{+2})/RT]$ is in the range 10^4 – 10^5 ; hence the inequality (13) is satisfied only if the ratio A_{-1}/A_{+2} is 10^5 – 10^6 . This is, of course, equivalent to saying that the entropies of activation of the two reactions (i.e. breakdown of EHAB to products or to initial reactants) must be very different. It is therefore possible, although by no means entailed by the analysis, that one of these steps is associated with a large entropy change arising from a conformational change in the protein.