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Chloroacetone as an active-site-directed inhibitor of the aliphatic amidase from *Pseudomonas aeruginosa*

Michael R. HOLLAWAY, Patricia H. CLARKE and Tamara TICHO
Department of Biochemistry, University College London, Gower Street, London WC1E 6BT, U.K.

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1. Chloroacetone (I) was shown to be an active-site-directed inhibitor of the aliphatic amidase (EC 3.5.1.4) from *Pseudomonas aeruginosa* strain PAC142. 2. This inhibitor reacted with the enzyme in two stages: the first involving the reversible formation of an enzymically inactive species, EI, and the second the formation of a species, EX, from which enzymic activity could not be recovered. 3. Different types of kinetic experiment were conducted to test conformity of the reaction to the scheme:

$$E + I \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} EI \xrightarrow{k_{+2}} EX$$

A computer-based analysis of the results was carried out and values of the individual rate constants were determined. 4. No direct evidence for a binding step before the formation of EI could be obtained, as with $[E]_0 \ll [I]_0$ the observed first-order rate constant for the formation of EI was directly proportional to the concentration of chloroacetone up to 1.2mM (above this concentration the reaction became too rapid to follow even by the stopped-flow method developed to investigate fast inhibition). 5. The value of k_{+1} exhibited a bell-shaped pH-dependency with a maximum value of about $3 \times 10^3 M^{-1} \cdot s^{-1}$ at pH6 and apparent pK_a values of 7.8 and about 4.8. 6. The values of k_{-1} and k_{+2} were similar and changed with the time of reaction from values of about $3 \times 10^{-3} s^{-1}$ (pH8.6) at short times to about one-sixth this value for longer periods of incubation. In this respect the simple reaction scheme is insufficient to describe the inhibition process. 7. The overall inhibition reaction is rapid, whether it is considered in relation to the expected chemical reactivity of chloroacetone, the rate of reaction of other enzymes with substrate analogues containing the chloromethyl group, or the rate of the amidase-catalysed hydrolysis of *N*-methylacetamide, a substrate that is nearly isosteric with chloroacetone. 8. Acetamide protected the amidase from inhibition by chloroacetone, and the concentration-dependence of the protection gave a value of an apparent dissociation constant similar to the K_m value for this substrate. 9. Addition of acetamide to solutions of the species EI led to a slow recovery of activity. Recovery of active enzyme was also observed after dilution of a solution of EI in the absence of substrate. 10. The species EI is considered not to be a simple adsorption complex, and the possibilities are discussed that it may be a tetrahedral carbonyl adduct, a Schiff base (azomethine) or a complex in which the enzyme has undergone a structural change. The species EX is probably a derivative in which there is a covalent bond between a group in the enzyme and the C-1 atom of the inhibitor.

The inducible aliphatic amidase (acylamido-hydrolase, EC 3.5.1.4) from *Pseudomonas aeruginosa* provides an attractive subject for the study of enzyme mechanism and specificity, as it is possible to generate mutant bacteria that produce amidases with altered catalytic properties (Clarke, 1974). This amidase catalyses the hydrolysis of short-chain amides such as acetamide and propion-amide, and acts also, although more slowly, with butyramide and longer-chain amides as substrates (Kelly & Clarke, 1962). Methyl acetate and other aliphatic esters are poorer substrates than are

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the corresponding amides (McFarlane *et al.*, 1965; Hollaway & Ticho, 1979).

In addition, amidase catalyses acyl transfer from amides or, less readily, esters to hydroxylamine, i.e.:

$$RCONH_2 + NH_2OH + H^+ \rightarrow RCONHOH + \overset{+}{N}H_4$$

Active-site-directed inhibitors containing a chloromethyl ketone function have proved valuable in the study of enzymic mechanisms (e.g. see Glazer, 1976). A well-known example is provided by 1-chloro-4-phenyl-3-L-tosylamidobutan-2-one ('toluene-*p*-sulphonyl-L-phenylalanine chloromethyl ketone'), which reacts with chymotrypsin (Schoellmann & Shaw, 1963) to give a covalently modified enzyme in a reaction that exhibits saturation kinetics (Glick, 1968).

For the bacterial amidase, an obvious choice of an active-site-directed inhibitor is chloroacetone, and the present paper describes the results of an investigation of the reaction of the enzyme with this compound. The results indicate that chloroacetone does react rapidly with amidase to give an inactive product in a reaction that involves at least two steps, the first of which is reversible. The characteristics of the reaction indicate that this compound can be regarded as an effective active-site-directed inhibitor.

Materials and methods

Purification of the amidase

Preparations of the amidase were made by starting from 300g batches of harvested cells of *Pseudomonas aeruginosa* PAC142 (Smyth & Clarke, 1975; Brown & Clarke, 1972), grown as described by Brammar & Clarke (1964). The method of purification was that described by Brown *et al.* (1969), with the following modifications: (i) in the first step of the purification, 1g of streptomycin sulphate was added for every 30g of protein; (ii) after the heat-treatment and centrifugation steps, first $(NH_4)_2SO_4$ was added to the supernatant to give 40% saturation, then, after centrifugation at 1800*g* for 40min, the supernatant was adjusted to 50% saturation with $(NH_4)_2SO_4$, centrifuged as before and the supernatant adjusted to 65% saturation. The apparent pH value (glass electrode) was maintained at 7.2 throughout by suitable additions of 2M-NH_3. The harvested precipitate from this stage was dissolved in a buffer comprising 0.1M-Tris, 0.15M-KCl, 1mM-EDTA and 2mM-dithiothreitol (adjusted to pH7.2 with HCl) and dialysed overnight against the same buffer.

The final stage of the preparation involved ion-exchange chromatography on a 25cm × 10cm column of DEAE-Sephadex A-50. Typically, 3.3g of protein from the 65%-saturation-$(NH_4)_2SO_4$ step, in 150ml of the dialysis buffer, was applied to the column, which has been equilibrated with the same pH7.2 buffer. Amidase transferase activity (see below) was eluted as a single peak at a concentration of about 0.35M in the linear salt gradient. Within this peak each fraction gave a specific transferase activity of 1500 ± 125 units/mg.

Fractions from the column that contained enzymic activity and were more than 97% homogeneous by disc gel electrophoresis (see below) were pooled, and the enzyme was precipitated by addition of $(NH_4)_2SO_4$ to 70% saturation. After centrifugation this fraction was stored at −10°C.

Disc gel electrophoresis

Disc gel electrophoresis on 7% polyacrylamide gels was performed as described by Brown *et al.* (1973), with 15–35μg samples of protein. Gels were stained for protein by overnight immersion in 0.2% (w/v) Coomassie Brilliant Blue in a solvent of methanol/acetic acid/water (3:1:6, by vol.) and destained by washing in the same solvent.

Peak fractions from the column stage of the enzyme preparations gave single bands at a loading of 15μg/gel, but at 35μg/gel very faint minor bonds were discernible. Densitometric scans of the destained gels obtained with a Joyce–Loebl mk. IIIB microdensitometer indicated that the main band constituted more than 98% of the total protein stain.

Protein determinations

Dry-weight measurements were made of known volumes of amidase solutions of known absorbance at 280nm. Each sample was heated at 110°C until the weight did not change significantly over a period of 24h. By this method it was found that a solution of the enzyme containing 1mg/ml gives an absorbance at 280nm of 1.26 ± 0.02 (S.E.M. for six measurements). Molar concentrations of enzyme solutions were calculated on the basis of a mol.wt. of 240000 (Gregoriou & Brown, 1979).

Assays of enzymic activity

During the purification of the amidase, enzymic activity was determined by the single-time-point amidase transferase assay as described by Brammar & Clarke (1964). One unit of transferase activity is defined as the amount of enzyme catalysing the formation of 1μmol of acylhydroxamate/min under the standard assay conditions at 37°C. Typical enzyme preparations gave specific activities of about 1500 units/mg by this method.

To record continuous time courses of amide hydrolase activity with either ethyl acetate or acetamide as substrate, a pH-stat was used to follow the proton release associated with these reactions.

With ethyl acetate as substrate the assay medium comprised 5ml of a solution containing 1mM-EDTA, 0.1M-NaCl and 0.05M- or 0.1M-ethyl

acetate, and the reaction was initiated by addition of a suitable volume of amidase solution. The volume of 14mM-NaOH required to maintain the pH value of the assay mixture (usually 7.00 or 8.66) was monitored by using a Metrohm (Herisau) Combititrator comprising an E353B Potentiometer, E473 Impulsomat and Dosimat, modified to give a 0.2ml delivery of solution for a full-scale deflexion on the chart recorder, and equipped with an AG 9100 combination glass electrode. Chart deflexions were calibrated in μmol of H_3O^+ ion by measurements of the pen deflexion on addition of known volumes of standard HCl solutions to the assay mixture.

By using this method it was shown that the initial velocity of the reaction was proportional to enzyme concentration in the range employed in the present study and proportional to ethyl acetate concentration up to 0.4M. The K_m value for this substrate is therefore greater than 0.4M. The k_{mol}/K_m value was found to be $200 \pm 4\,M^{-1}\cdot s^{-1}$ (linear regression at pH7.0 and 25°C, and k_{mol} is defined as the molecular activity of the enzyme, i.e. the number of substrate molecules transformed/s per molecule of enzyme).

Measurement of acetamide hydrolase activity was carried out by employing a similar pH-stat assay. However, assays were performed at pH8.66, as there is negligible proton release at pH7.0 [The pK_a of NH_4^+ ion is 9.25 (Bates & Pinching, 1950).] The K_m value for acetamide hydrolase activity was found to be 0.8 ± 0.2mM (eight observations) at pH8.66 and 25°C, which is close to the value of 0.83mM given by Findlater & Orsi (1973) for pH7.2 and 25°C.

Other assays involving measurement of proton release in the ester or amide hydrolase reaction were carried out by following the change in absorbance in a reaction mixture containing a pH indicator, *p*-nitrophenol or Cresol Red (see below).

Preparation of solutions of enzyme for inhibition studies

A short time before each experiment the required amount of stored enzyme was dissolved in a small volume of 50mM-potassium phosphate buffer containing 0.1M-KCl, 2mM-EDTA and 2mM-dithiothreitol. $(NH_4)_2SO_4$ and dithiothreitol were removed by passage through a Sephadex G-25 column equilibrated with the same buffer without dithiothreitol. The solutions of enzyme so prepared retained full enzymic activity for at least 24h.

Stopped-flow measurements of the time course of amidase inhibition by chloroacetone

The rapid loss of ester hydrolase activity on reaction with high concentrations of chloroacetone (i.e. 0.1mM and above) was followed by mixing an enzyme solution (syringe 1) with a solution of ethyl acetate and chloroacetone (syringe 2) in a Durrum–Gibson stopped-flow spectrophotometer (Durrum Instruments Corp., Palo Alto, CA, U.S.A.). Both solutions contained 50μM-*p*-nitrophenol, 1mM-EDTA, 0.1M-NaCl and sufficient potassium phosphate buffer (about 2mM) to restrain changes in the pH value of the reaction mixture to less than 0.1 unit. The pH of each solution was adjusted to pH7.1.

Photographs were taken of the oscilloscope recording of change in absorbance with time at 400nm. The negatives were projected on graph paper for drawing.

Controls were done in which enzyme, chloroacetone and substrate respectively were omitted. Other control reactions showed that the *p*-nitrophenol did not influence the enzymic hydrolysis, e.g. doubling the *p*-nitrophenol concentration did not alter the shape of the absorbance–time course, although the amplitude increased 2-fold.

Indicator-linked assays employing Cresol Red were performed also, with a conventional Cary 14 spectrophotometer. Cresol Red was found not to influence the time courses below a concentration of 0.1mM.

Inhibitor solutions

Solutions of redistilled chloroaceteone in methanol were made up immediately before experiments were conducted. The concentration of the inhibitor was adjusted so that the concentration of methanol in the inhibition mixtures did not exceed 2%, a concentration that was found not to affect the rate of either the ethyl acetate or acetamide hydrolase reactions.

The concentration of chloroacetone in stock solutions was determined by the following method. A known volume of the inhibitor solution was added to a solution of 3.6mM-2-mercaptoethanol in 50mM-potassium phosphate buffer, pH7.3, to give a final estimated concentration of 2mM. The concentrations of 2-mercaptoethanol remaining at different times were determined by removal of samples for reaction with 5,5′-dithiobis-(2-nitrobenzoic acid) (Ellman's reagent). [The absorbance of 412nm was divided by $\varepsilon_{412} = 13\,600\,M^{-1}\cdot cm^{-1}$ (Ellman, 1959) to give the concentration of the thiol compound.] The concentration of chloroacetone was calculated from the amplitude of the total decrease in thiol concentration.

From the time course a value of the second-order rate constant for the reaction of chloroacetone with 2-mercaptoethanol was calculated to be $0.31\,M^{-1}\cdot s^{-1}$ at pH7.30 and 25°C. There was no observable change in the concentration of chloroacetone in an aqueous solution at pH7 or 8.66 during 2h.

Materials

Fractions of chloroacetone (Aldrich Chemical Co., Gillingham, Kent, U.K.) distilling at 120 ± 1°C

were collected and stored in a glass-stoppered vessel in the dark. Ethyl acetate was purified by distillation at 76 ± 1°C. Acetamide (Koch–Light Laboratories, Colnbrook, Bucks., U.K.) was crystallized twice from ethanol; hydroxylamine, from the same supplier, was recrystallized twice from ethanol/water (3:1, v/v). *p*-Nitrophenol and Cresol Red were from Hopkin and Williams, Chadwell Health, Essex, U.K. Ellman's reagent from Sigma Chemical Co. (St. Louis, MO, U.S.A.) was recrystallized twice from ethanol/water (1:1, v/v). Other reagents were of AnalaR grade from BDH Chemicals (Poole, Dorset, U.K.) unless specified otherwise.

Theory

Rate equations arising from the kinetic scheme for the reaction of amidase with chloroacetone

In the Results section, Scheme 1 is employed to describe the inhibition of amidase by chloroacetone, and the following analysis is in terms of the species and symbols as defined there.

In different types of experiments enzyme and inhibitor were incubated for a given time (t_1), at which the formation of EI was then blocked, or greatly slowed, by dilution of the reaction mixture or addition of acetamide to give a concentration much greater than the K_m value.

The resulting samples were assayed for enzymic activity with either ethyl acetate or acetamide as substrate.

At the end of the incubation time t_1, solution of the appropriate differential equations gives the concentrations of the various enzymic species according to eqns. (1)–(3):

$$[EI]_{t_1} = k_{+1}[I]_0[E]_0(e^{P_1 t_1} - e^{P_2 t_1})/R \quad (1)$$

$$[EX]_{t_1} = \frac{k_{+1}k_{+2}[I]_0[E]_0}{R}\left(\frac{(e^{P_1 t_1}-1)}{P_1} - \frac{(e^{P_2 t_1}-1)}{P_2}\right) \quad (2)$$

$$[E]_{t_1} = [E]_0 - [EI]_{t_1} - [EX]_{t_1} \quad (3)$$

where $R = (A^2 - 4B)^{\frac{1}{2}}$, $P_1 = -A/2 + R/2$, $P_2 = -A/2 - R/2$, $A = (k_{+1}[I]_0 + k_{-1} + k_{+2})$ and $B = k_{+1}k_{+2}[I]_0$.

In the derivation of these equations the linearizing assumption that $[I]_0 \gg [E]_0$ has been made.

On dilution of the inhibition mixture at time t_1 the second-order reaction between species E and EI will be slowed relative to the first-order decays of EI to E and EX. Enzymic activity will then return to an extent that depends on the relative values of $k_{+1}[I]_d$, k_{-1} and k_{+1} (where $[I]_d$ is the concentration of inhibitor after dilution).

Solution of the differential equations arising from the new conditions after dilution gives eqns. (4)–(6) as the concentrations of EI, EX and E at time t_2 after dilution by a factor f:

$$f\cdot[EI]_{t_2} = [C\cdot e^{P_3 t_2} + (1-C)e^{P_4 t_2}][EI]_{t_1} \quad (4)$$

where $P_3 = -L/2 + Q/2$, $P_4 = -L/2 - Q/2$, $C = (P_4 + k_{-1} + k_{+2})/(P_4 - P_3)$, $L = (k_{+1}[I]_d + k_{-1} + k_{+2})$, $M = k_{+1}k_{+2}[I]_d$ and $Q = (L^2 - 4M)^{\frac{1}{2}}$. The expression for $[EX]_{t_2}$ is given by eqn. (5):

$$f\cdot[EX]_{t_2} = k_{+2}[EI]_{t_1}[C(e^{P_3 t_2} - 1)/P_3 + (1-C)(e^{P_4 t_2} - 1)/P_4] \quad (5)$$

where the symbols are those of eqn. (4). The expression for free enzyme is then given from the conservation eqn. (6):

$$f\cdot[E]_{t_2} = [EI]_{t_1} - f\cdot[EI]_{t_2} - f\cdot[EX]_{t_2} + [E]_{t_1} \quad (6)$$

The presence of any unreacted enzyme ($[E]_{t_1}$) will give a finite initial velocity in the enzymic assay, and the rate will then increase as some of the EI species reverts to free enzyme. The concentration of product ($[P]_{t_2}$) formed in the assay medium at time t_2 will be given by eqn. (7).

$$[P]_{t_2} = \frac{k_p[EI]_{t_1}\cdot a}{f}\cdot\left(t_2 + \frac{b}{P_3 a}(1 - e^{P_3 t_2}) + \frac{d}{P_4 a}(1 - e^{P_4 t_2})\right) \quad (7)$$

where $a = 1 - k_{+2}(C-1)/P_4 + k_{+2}C/P_3$, $b = -C\cdot(1 + k_{+2}/P_3)$ and $d = -(1-C)\cdot(1 + k_{+2}/P_4)$, where if $[S]_0 \gg K_m$ (the case in the assays with acetamide as substrate), k_p will be the molecular activity of the enzyme (k_{mol}), i.e. the catalytic-centre activity multiplied by the number of active sites per molecule. In the assays with ethyl acetate as substrate, $[S]_0 \ll K_m$, so k_p will be approximately equal to k_{mol}/K_m.

In some cases the dilution factors were so great that the term $k_{+1}[I]_d$ could be neglected in comparison with k_{-1} and k_{+2}. This allows simplification of eqns. (4) and (5) to eqns. (8) and (9) respectively:

$$[EI]_{t_2} = [EI]_{t_1}\cdot e^{-(k_{-1}+k_{+2})} \quad (8)$$

$$f\cdot[E]_{t_2} = [E]_{t_1} + \frac{k_{-1}}{(k_{-1}+k_{+2})}[EI]_{t_1}(-e^{-(k_{-1}+k_{+2})t_2}) \quad (9)$$

This also leads to eqn. (10) as a simplified form of eqn. (7).

$$[P]_{t_2} = k_p[E]_{t_1}\cdot t_2 + k_p\frac{k_{-1}}{(k_{-1}+k_{+2})}[EI]_{t_1}\left(t_2 + \frac{(e^{-(k_{-1}+k_{+2})t_2} - 1)}{(k_{-1}+k_{+2})}\right) \quad (10)$$

Eqns. (8)–(10) also apply to experiments in which the combination of enzyme and inhibitor was blocked by addition of substrate to a concentration much greater than the K_m value.

Processing of data comprising values of $[P]_{t_2}$, versus time, t_2, from experiments of this type was carried out in the following way by using the program FACSIMILE (Chance *et al.*, 1977) on an IBM 360 computer. Two separate code blocks, INITIAL and RESID, were set up. In code block INITIAL, eqns. (1)–(3) were programmed so that, given values of $[E]_0$, $[I]_0$, k_{+1}, k_{-1} and k_{+2}, the values $[E]_{t_1}$, $[EI]_{t_1}$ and $[EX]_{t_1}$ could be computed for any t_1 value. In code block RESID, eqn. (7) or (10), as appropriate, was programmed so that values of $[E]_{t_1}$ and $[EI]_{t_1}$ from code block INITIAL gave a value for $[P]_{t_1}$ at any time t_2, employing the same values of rate constants used in INITIAL and a given value for k_p, calculated from an experiment in which inhibitor was omitted. The program FACSIMILE gives the values of varied parameters, giving the best fit of theory and observation by the following procedure. The optimization routine minimizes the function RSQ, given by eqn. (11):

$$\mathrm{RSQ} = \sum_{j=1}^{m} R_j^2 = \sum_{j=1}^{m} \left(\frac{(v_j - u_j)}{r_i \cdot e} \right)^2 \qquad (11)$$

where the residuals for a given time-course, R_j, are given by the difference between the observed and calculated values of $[P]_{t_2}$ (respectively v_j and u_j) divided by the standard error of the curve ($r_i \cdot e$). The standard error is the product of r_i (the largest value of [P]) and e (the estimated error in the data, taken as 0.01 for all time courses given here).

χ^2 is given by the minimum value of RSQ, and the results presented here (Table 1) are expressed as χ^2 per degree of freedom, i.e. as $\chi^2/(M-P)$, where M is the number of data points and P the number of parameters varied during the optimization. Thus a value of $\chi^2/(M-P)$ of < 1 corresponds to a fit of the data to theory to within 1%. Values of rate constants are given ± S.D.

Some computer fits were carried out also by normal FACSIMILE programming, where numerical integration was employed in place of the analytical solutions of the above equations. The results obtained by this procedure were within 1% of those for the analytical method, so indicating that the assumptions in the latter approach (e.g. $[I]_0 \gg [E]_0$) did not introduce any significant error.

By inspection of eqn. (9) it can be seen that, as t_2 approaches infinity, the concentration of enzyme approaches $[E]_\infty$ given by eqn. (12).

$$f \cdot E]_\infty = [E]_{t_1} + \left(\frac{k_{-1}}{k_{-1} + k_{+2}} \right) [E]_{t_1} \qquad (12)$$

If a sufficient concentration of inhibitor $[I]_0$ is employed in the t_1 incubation period to satisfy the condition $k_{+1}[I]_0 \gg k_{-1}$, then $[E]_{t_1} \approx [E]_0 e^{-k_{+1}[I]_0 t_1}$. Combining this result with eqn. (12) gives eqn. (13) as the expression for the fraction of active enzyme recovered (F):

$$F = e^{-k_{+1}[I]_0 t_1} + \left(\frac{k_{-1}}{k_{-1} + k_{+2}} \right) e^{-k_{+2} t_1} \qquad (13)$$

Observed and calculated values of F are given in Table 1.

Results

Evidence is presented below that description of the inhibition of amidase (E) by chloroacetone (I) requires a kinetic model not simpler than Scheme 1. In this Scheme, species EI is a reversibly formed enzymically inactive complex between amidase and the inhibitor, and EX is an inactive product that is formed in an effectively irreversible reaction.

Formation of the EI complex (k_{+1} step)

The loss of enzymic activity on reaction with chloroacetone was monitored continuously by the following procedure. A solution of amidase in one syringe of the stopped-flow apparatus was mixed with the contents of the second syringe comprising a solution of ethyl acetate and chloroacetone. The concentration of inhibitor was more than 10 times that of the enzyme in each case. In this procedure the inhibition and assay of enzymic activity are observed continuously and simultaneously without the necessity for removing samples for assay, a procedure that is commonly employed but that can lead to dissociation of reversibly formed complexes (cf. Sluyterman, 1968).

It can be seen from the typical time courses of proton release in the presence of inhibitor, shown in Figs. 1(*a*) and 1(*b*), that, although the inhibitor did not alter the initial velocity of hydrolysis of the substrate, the rate decreased rapidly with time until there was an apparently complete loss of enzymic activity. (It was established that the reaction with chloroacetone gave a more than 99.9% loss of activity.) Curves such as those of Fig. 1 followed closely an exponential decay (with an apparent rate constant $k_{1(\mathrm{app.})}$) for more than 90% of the reaction if inhibitor concentrations greater than 0.3 mM were employed and the condition $[I]_0 \gg [E]_0$ was satisfied. Plots of $k_{1(\mathrm{app.})}$ against $[I]_0$ were linear (see

$$E + I \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} EI \xrightarrow{k_{+2}} EX$$

Scheme 1. *Kinetic model for the reaction of chloroacetone with amidase*

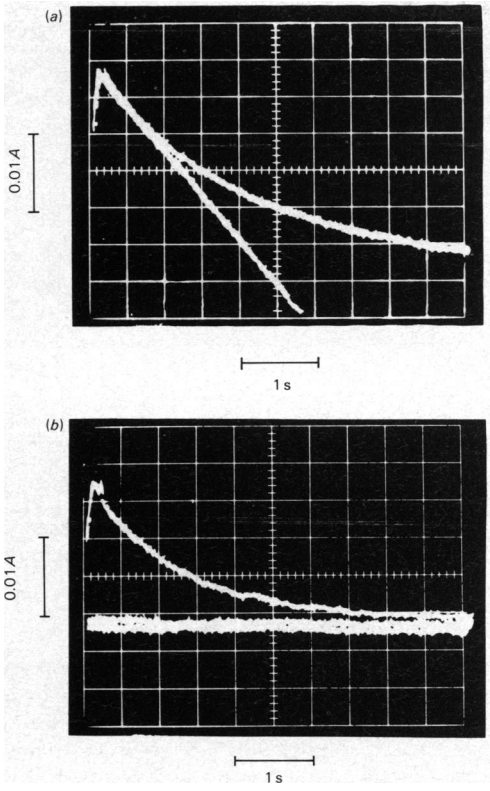


Fig. 1. *Stopped-flow time courses of the inhibition of ester hydrolase activity by chloroacetone*

A solution of amidase in syringe 1 of the stopped-flow apparatus was mixed with a solution containing ethyl acetate and chloroacetone (syringe 2). The final concentration of enzyme in the observation cuvette was 2.3 μM and that of ethyl acetate 0.05 M for both (*a*) and (*b*). The final concentrations of chloroacetone were 0.3 mM (*a*) and 0.6 mM (*b*), and photograph (*a*) shows also the linear time course for the control reaction where inhibitor was excluded. Each reaction mixture contained 50 μM-*p*-nitrophenol, 1 mM-EDTA, 0.1 M-NaCl and 2 mM-phosphate buffer. (Syringe contents were each adjusted separately to pH 7.1.) The absorbance changes correspond to a pH change of less than 0.004 unit.

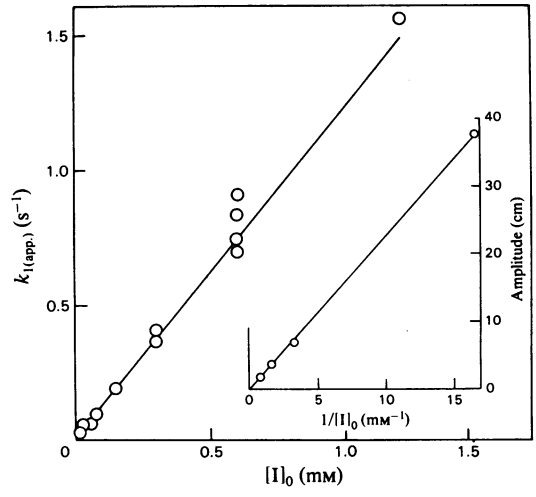


Fig. 2. *Dependence of the apparent first-order rate constant for reaction of amidase with chloroacetone on inhibitor concentration*

Values of $k_{1(app.)}$ for different inhibitor concentrations ($[I]_0$) were measured from time courses such as those of Fig. 1. Also shown (inset) is the dependence of the amplitude of the time course (number of cm from the point of mixing to the position of zero rate in the drawings of time courses) on the reciprocal of inhibitor concentration. The value of $k[E]_0$ (see eqn. 14) was 3.8 cm·s^{-1}. All conditions were as given in Fig. 1.

Fig. 2) and did not give a finite intercept on the ordinate, so it can be assumed that $k_{+1}[I]_0 \gg k_{-1}$ in the range of concentration of inhibitor employed. Under the conditions of the time courses of Fig. 1, $[S]_0 \ll K_m$, so the enzymic reaction will have a negligible effect on the inhibition reaction and the concentration of unreacted sites [E] at any time t will be given by eqn. (14):

$$[E] = [E]_0 \cdot e^{-k_{1(app.)}t} \qquad (14)$$

where $k_{1(app.)} = k_{+1}[I]_0$. The rate of the enzyme-catalysed reaction will be given by eqn. (15):

$$d[P]/dt = k \cdot [S]_0 \cdot [E] \qquad (15)$$

where $k = k_{mol}/K_m$. Substitution of eqn. (14) in eqn. (15) and integration given an expression for the time-dependence of the concentration of product (eqn. 16):

$$[P] = \frac{k \cdot [E]_0}{k_{1(app.)}} \cdot (1 - e^{-k_{1(app.)}t}) \qquad (16)$$

Values of $k_{1(app.)}$ were obtained by conventional plots of log ($[P]_\infty - [P]$) against time, where $[P]_\infty$ is the concentration of product release at the completion of the inhibition reaction.

The plot of $k_{1(app.)}$ against $[I]_0$ shown in Fig. 2 gives a slope of $k_{+1} = 1.24\ (\pm 0.09) \times 10^3 M^{-1} \cdot s^{-1}$. In addition, a value for this rate constant can be obtained from the dependence of $[P]_\infty$ on the concentration of inhibitor as, from eqn. (16), $[P]_\infty = k[E]_0/k_{+1} \cdot [I]_0$, so that a plot of $[P]_\infty$ against $1/[I]_0$ will give a straight line of slope $k[I]_0/k_{+1}$, where values of k and $[E]_0$ are known. Such a plot is given as an inset to Fig. 2, and the value of k_{+1} calculated from the slope is about $1.7 \times 10^3 M^{-1} \cdot s^{-1}$, in fair agreement with the value derived from the measurements of $k_{1(app.)}$.

pH-dependence of the value of k_{+1}. The values of k_{+1} at different pH values, given in Fig. 3, were determined from time-courses of loss of enzymic activity after addition of chloroacetone solutions to pH-stat assays with ethyl acetate as substrate ($[S]_0 \ll K_m$). The procedure involved the initiation of the ethyl acetate hydrolase reaction in the usual way at the required pH value (see the Materials and methods section) and, after the time courses had become linear, addition of a small volume of a solution of chloroacetone. The observed rate of hydrolysis decreased to give eventually that observed in the absence of enzyme. Analysis of the time courses of proton release was performed on the basis of eqn. (16) as described in the preceding section.

From the data of Fig. 3 it can be seen that there is a bell-shaped pH–k_{+1} profile with a pK_a value of 7.8 ± 0.1 associated with the alkaline limb. The maximum value of $k_{+1} = 3 \times 10^3 M^{-1} \cdot s^{-1}$ was at about pH 6, below which k_{+1} decreases to approach about half the maximum value at pH 4.8. The apparent pK_a of the acid limb is then about 4.8, although there were insufficient data to allow confidence limits to be placed at better than ± 0.3 pH unit.

It is noteworthy that the values of k_{+1} obtained by using the pH-stat method were about double those found from the stopped-flow experiments (cf. above and Fig. 3). The source of this 2-fold factor is obscure, but the difference could arise from the 100-fold difference in enzyme concentrations in the two types of experiment.

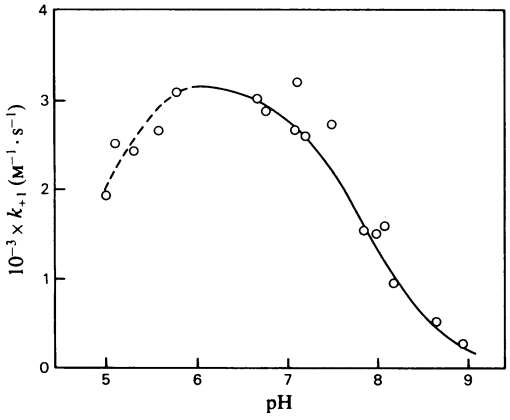


Fig. 3. *pH-dependence of the second-order rate constant* (k_{+1}) *for the reaction of amidase with chloroacetone*
Values of k_{+1} were determined at different pH values from continuous time courses of ethyl acetate hydrolysis after the addition of chloroacetone, by the procedure described in the text (logarithmic plots based on eqn. 15). Each reaction mixture (5 ml) contained 0.05 M-ethyl acetate, 1 mM-EDTA, 0.1 M-NaCl and 0.08 μM-amidase at 25°C. Chloroacetone was added to reaction mixtures after initiation of the pH-stat time course to give a final concentration in the range 1.2–4.8 μM.

Protection by acetamide of the inhibition of amidase by chloroacetone

Addition of chloroacetone to a final concentration of 24 μM during an assay with ethyl acetate as substrate ($[S]_0 \ll K_m$) led to a rapid loss of enzymic activity with a half-life for the inhibition of about 20–30 s (see Fig. 4*a*). However, addition of the inhibitor to the same final concentration during an assay with acetamide ($[S]_0 \gg K_m$) gave a relatively slow loss of activity with a half-life of about 5 min (Fig. 4*b*), thereby demonstrating that acetamide 'protects' the enzyme from reaction with chloroacetone.

These results should not be taken to indicate that ethyl acetate and acetamide are hydrolysed at different sites on the enzyme, as we have shown (Hollaway & Ticho, 1979) that there is a direct competition between ethyl acetate and acetamide in amidase hydrolase reactions.

The different substrates were employed to work under the two different conditions of $K_m \gg [S]_0$ and $K_m \ll [S]_0$ for technical reasons. The low sensitivity of the amide hydrolase assay and relatively low K_m value for acetamide made it difficult to work under conditions of $[S]_0 \ll K_m$, and the low solubility and high K_m value (> 0.4 M) of ethyl acetate renders it impossible to carry out assays with this substrate under the condition of $[S]_0 \gg K_m$.

The results of Fig. 4 were analysed quantitatively by a procedure employing the program FACSIMILE (see the Materials and methods section) in the following way. The data for the experiment where inhibitor was added after initiation of the assay (Fig. 4*b*) can be fitted to the analytical expression of eqn. (17):

$$[P] = \frac{k_p \cdot [E]_0}{k_{+1} \cdot [I]_0} \cdot (1 - e^{-k_{1(obs.)} t}) \qquad (17)$$

where $k_{1(obs.)} = k_{+1}[I]_0 \cdot [K_m/(K_m + [S]_0)$ and $k_p = k_{mol}[S]_0/K_s$, with the time, t, measured from the time of addition of the inhibitor. The derivation of eqn. (17) assumes that none of the intermediates in the enzymic reaction can react with chloroacetone, that $[I]_0 \gg [E]_0$, that $[S]_0 \gg [E]_0$ and that the concentration of substrate is constant during the inhibition time course.

The best fit of the data of Fig. 4(*b*) to eqn. (17) was obtained with $K_m = 1.8 \pm 0.1$ mM, a value that can be compared with that of 0.8 mM obtained by conventional steady-state methods (see the Materials and methods section and Findlater & Orsi, 1973).

Addition of the inhibitor to a solution of amidase before addition of acetamide led to a rapid decrease in initial velocity with the time of preincubation, t_1,

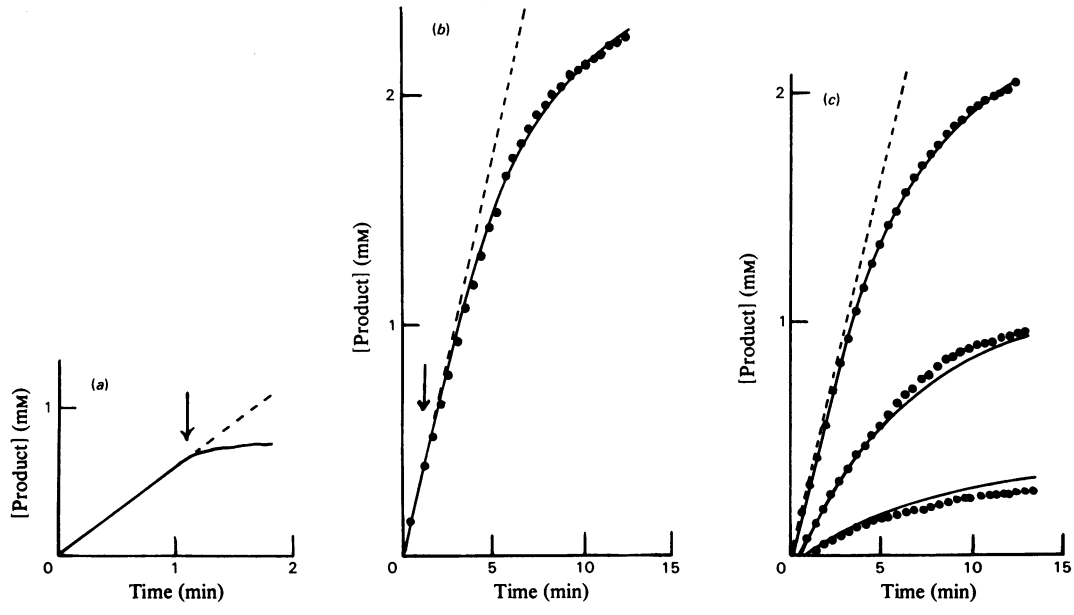


Fig. 4. *Protection of chloroacetone inhibition of amidase by acetamide*

(*a*) Time course of a pH-stat assay with ethyl acetate (0.1 M) as substrate, at pH 8.66. At the time indicated by the arrow a solution of chloroacetone was added to give a final concentration of 48 μM. The broken line gives the time course in the absence of inhibitor with the same concentration of enzyme (0.113 μM). (*b*) A similar experiment to that in (*a*) but with 16 mM-acetamide as substrate and 0.028 μM-enzyme. Chloroacetone was added to 48 μM at the time indicated by the arrow. The control rate is given by the broken line. The ● points are the observed values and the continuous line is the theoretical time course for $K_m = 1.8$ mM, $k_{+1} = 0.7 \times 10^3$ $M^{-1} \cdot s^{-1}$, $k_{-1} = 0.8 \times 10^{-3}$ s^{-1} and $k_{+2} = 0.6 \times 10^{-3}$. These values were computed by using the program FACSIMILE. χ^2 for the fit was 2.5 per degree of freedom. (*c*) As in (*b*), but enzyme and inhibitor were mixed at $t_1 = 5$, 30 and 60 s respectively before addition of acetamide. The ● points give the observed time courses and the continuous lines the computed curves for $K_m = 1.8$ mM, $k_{+1} = 0.7 \times 10^3 M^{-1} \cdot s^{-1}$, $k_{-1} = 0.8 \times 10^{-3} s^{-1}$ and $k_{+2} = 0.6 \times 10^{-3} s^{-1}$. The value of χ^2 for the overall fit of all curves fitted simultaneously was 2.9 per degree of freedom.

and a slower loss of activity as the enzymic reaction was in progress (Fig. 4*c*). Analysis of the time courses by using FACSIMILE programmed with eqns. (9) and (17) gave best-fit values for K_m of 1.8 ± 0.2 mM. (The computer fits were for an optimization run with all curves fitted simultaneously.) The best-fit computed value for k_{+1} was 0.70 ± 0.15 $mM^{-1} \cdot s^{-1}$, taking all curves from Figs. 4(*b*) and 4(*c*) together.

The computer fits of Figs. 4(*b*) and 4(*c*) exhibit minor but significant systematic deviations that could arise from non-equivalence of the active sites of the enzyme (see the Discussion section).

In other experiments (not shown) it was demonstrated that acetamide gave less protection at lower concentrations. For example, at pH 8.85 the half-life of inhibition of amidase by 120 μM-chloroacetone in the presence of 0.4 mM-acetamide was less than 20 s, but with 16 mM-acetamide the inhibition half-life was about 5 min.

Recovery of enzymic activity from amidase inhibited by reaction with chloroacetone

Recovery by addition of substrate. In Figs. 5(*a*) and 5(*b*) are shown time courses of hydrolysis of ethyl acetate and acetamide catalysed by amidase that had been incubated with chloroacetone for different times, t_1. The initial velocity of hydrolysis of ethyl acetate (Fig. 5*a*) decreased with t_1 in the way expected from the previous results, i.e. from the data of Fig. 5(*a*) the apparent second-order rate constant for the loss of enzymic activity is about $1.2 \times 10^3 M^{-1} \cdot s^{-1}$, in fair agreement with the value obtained from the stopped-flow experiments. In addition, the initial velocity in the acetamide hydrolase reaction decreased in parallel with that recorded in the assay with ethyl acetate (see Fig. 5*c*).

However, the time courses in the acetamide hydrolase assays exhibited a striking acceleration that was not apparent in the esterase reactions (compare Figs. 5*a* and 5*b*).

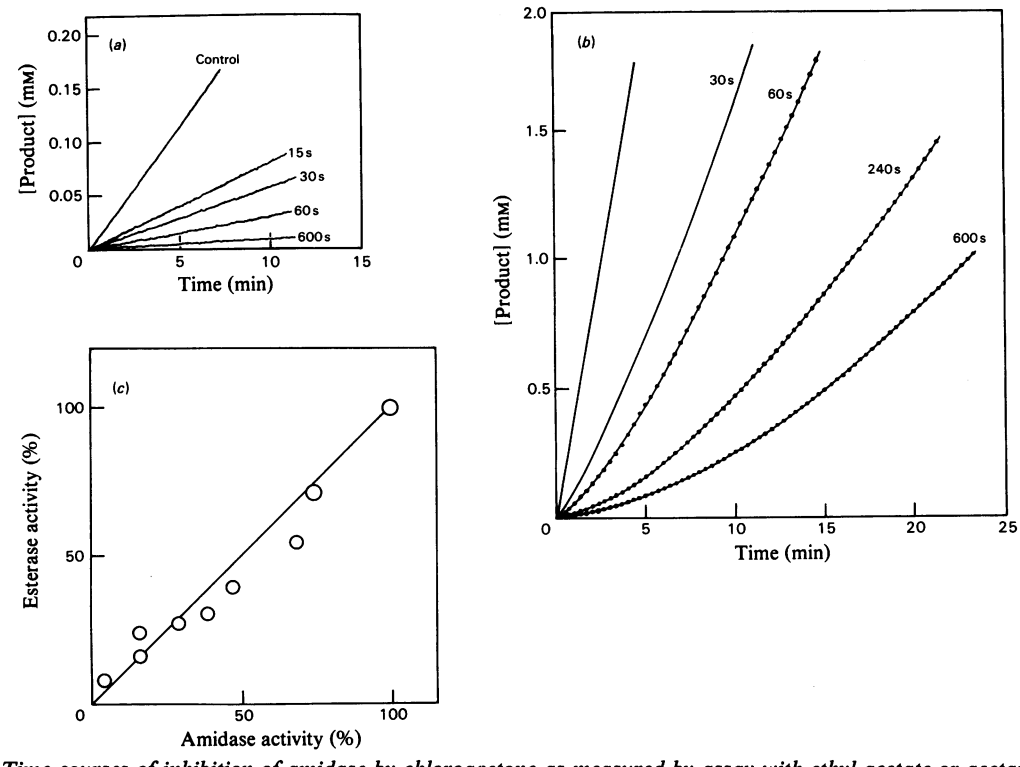


Fig. 5. *Time courses of inhibition of amidase by chloroacetone as measured by assay with ethyl acetate or acetamide as substrate*

Solutions containing 1.05 μM-amidase and 48 μM-chloroacetone were incubated for times t_1 (given in the diagrams) at pH 7.1 and 25°C in a 12 mM-phosphate buffer containing 1 mM-EDTA and 0.1 M-NaCl. Samples were removed, diluted 25-fold to the pH-stat assay medium and assayed with either ethyl acetate (0.1 M) or acetamide (8 mM) at pH 8.66 and 25°C. The assay time courses shown in (*a*) and (*b*) were with ethyl acetate and acetamide respectively as substrates. The continuous lines were drawn directly from the pH-stat time courses and the ● points are the computed best-fit curves obtained by using the program FACSIMILE. (*c*) shows the dependence of initial velocity in the ethyl acetate assay on that in the acetamide assay at the corresponding times of preincubation, t_1.

These observations can be interpreted in the simplest way in terms of Scheme 1. The decrease in initial velocity, which occurs at the same rate with both substrates, can be attributed to the formation of an inactive EI species. In the case of the assay with ethyl acetate it can be inferred that the substrate concentration was too low compared with the K_m value to perturb the distribution of enzyme species between E and EI towards the free enzyme.

Conversely, in the amidase hydrolase assay the concentration of acetamide was 10 times the K_m value, and this was sufficient to perturb the distribution towards the free enzyme, thereby giving rise to the time-dependent increase in velocity.

Employment of a higher concentration of acetamide in the assay mixture led to a more rapid and extensive recovery of amidase activity, even without dilution of the reaction mixture. This is illustrated by an experiment (see Fig. 6) in which a pH-stat assay with ethyl acetate as substrate was initiated and, after a linear time course was established, chloroacetone was added to a final concentration of 48 μM. The esterase activity decreased rapidly to reach less than 1% of the initial value 11 min after the addition of inhibitor. At this time (t_1), acetamide was added to a final concentration of more than 100 times the K_m value, whereupon acetamide hydrolase activity appeared, eventually giving 30% of the rate observed with the uninhibited enzyme. The esterase reaction would not have contributed to the time course under these conditions (see Hollaway & Ticho, 1979).

'Rescue' of enzymic activity from the inactive EI species depended on the relative concentrations of chloroacetone and acetamide in the solution. For example, a solution of enzyme was incubated with 214 mM-chloroacetone for 9 min and this led to a complete loss of enzymic activity in assays with acetamide at concentrations of the order of the K_m value. However, with 200 mM-acetamide (about 200

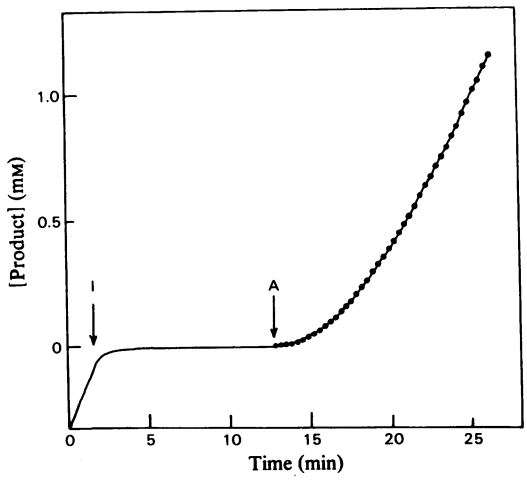


Fig. 6. *'Rescue' of enzymic activity by addition of 'saturating' concentrations of acetamide to inactive enzyme*

A pH-stat assay with 0.1 M-ethyl acetate as substrate was initiated at pH 8.66 and 25°C. The concentration of amidase was 37.8 nM. At the time indicated by the first arrow (I) chloroacetone was added to a final concentration of 48 μM. At the time indicated by the second arrow (A, 11 min after arrow 1) acetamide was added to a final concentration of 200 mM and the time course of acetamide hydrolysis was recorded. (Up to this time the continuous line gives the time course of ethyl acetate hydrolysis on an arbitrary concentration scale.) The ● points give the computed best-fit curve with values of $k_{+1} = 1.2 \times 10^3 M^{-1} \cdot s^{-1}$, $k_{-1} = 0.90 \times 10^{-3} s^{-1}$ and $k_{+2} = 0.55 \times 10^{-3} s^{-1}$ (see Table 1).

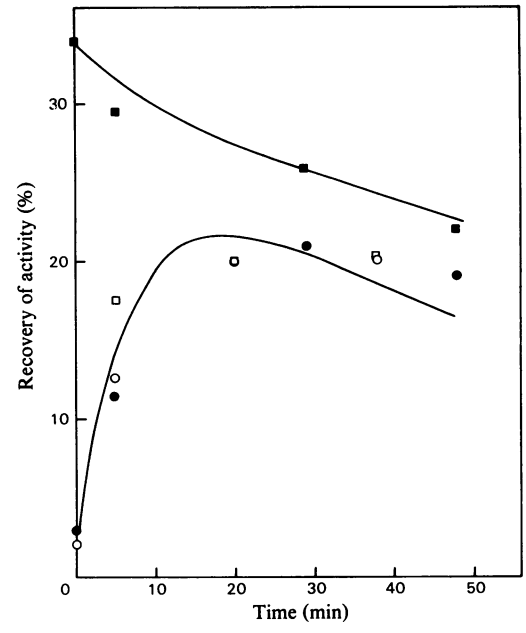


Fig. 7. *Recovery of amidase and esterase activities after dilution of a solution containing the inactive EI species*

A solution of amidase (6.5 μM) and chloroacetone (53 μM) in 25 mM-phosphate buffer, pH 7.1, containing 1 mM-EDTA and 0.1 M-NaCl was incubated at 25°C for 4 min, then diluted with 'assay medium' and left for different times before assay at pH 8.66 with 8 mM-acetamide or 0.1 M-ethyl acetate. The dilution factor was 200 for the assays with acetamide and 100 for the ethyl acetate assays. The ● and ■ points give the initial (●) and final (■) velocities for the assay with acetamide, and the ○ and □ points the corresponding velocities in the ethyl acetate assay. The values of the rate constants employed to generate the continuous (theoretical) lines were $k_{+1} = 0.7 \times 10^3 M^{-1} \cdot s^{-1}$, $k_{-1} = 0.83 \times 10^{-3} s^{-1}$ and $k_{+2} = 1.04 \times 10^{-3} s^{-1}$. These values were substituted in eqns. (1)–(3), (6) and (13).

times the K_m value) about 10% of the amide hydrolase activity returned.

The analytical treatment of equations arising from Scheme 1 for the experiments described in this section has been given in the Theory section, together with a description of the computer-based procedure for determination of the values of the rate constants. The simulated curves for product formation as a function of time obtained by this procedure are shown, together with the experimental observations, in Figs. 5(*b*) and 6, and the values of k_{-1} and k_{+2} (see Scheme 1) are given in Table 1.

The fits to all curves are all to within the assumed experimental error of 1% (i.e. χ^2 is less than 1 per degree of freedom). However, the best-fit values of the rate constants k_{-1} and k_{+2} (Table 1), which are well-determined, show a clear decrease with increasing time, t_1. That is, the recovery of enzyme activity is slower for longer times of incubation of enzyme with the inhibitor.

Similar experiments to those employing pH-stat assays were conducted in which acetamide hydrolase activity was measured after different times of incubation of amidase with chloroacetone by using the indicator-linked spectrophotometric assay with 16 mM-acetamide as substrate (see the Materials and methods section). The assay time courses exhibited the same type of acceleration phases as observed in the pH-stat assays and the data were analysed in the same way by using the computer program FACSIMILE. The best-fit values of the rate constants so obtained are given in Table 1. These experiments were conducted to provide a check of the results obtained by using pH-stat assays.

The absence of an acceleration in the assays with ethyl acetate (Fig. 5*a*) could be due to the 25-fold dilution being insufficient to take the concentration

Table 1. *'Best-fit' values of rate constants for the decomposition of the EI species (k_{-1} and k_{+2} of Scheme 1)*
The experimental details are given in the legends of the designated Figures. The pH values of inhibition mixtures (assays) for (*a*), (*b*), (*c*) and (*d*) were 7.10 (8.66), 8.66 (8.66), 7.10 (8.66) and 8.0 (8.0) respectively. $[I]_0$ is the concentration of chloroacetone in the preincubation mixture and $[I]_r$ that in the assay mixture. The unweighted least-squares best-fit values of rate constants are given ± S.D. and the measure of 'goodness of fit' as χ^2 per degree of freedom (computed by the program FACSIMILE). F is the fraction of enzymic activity recovered (see eqn. 13).

Type of experiment	$[I]_0$ (μM)	t_1 (s)	$[I]_r$ (μM)	10^3k_{-1} (s^{-1})	10^3k_{+2} (s^{-1})	χ^2 of fit per degree of freedom (no. of degrees of freedom)	$F_{obs.}$	$F_{calc.}$
(*a*) 'Rescue' with 8mM-acetamide (Fig. 5)	48	60	2	2.47 ± 0.32	3.82 ± 0.28	0.45 (38)	0.36	0.31
	48	240	2	0.758 ± 0.007	1.49 ± 0.02	0.16 (66)	0.24	0.24
	48	600	2	0.438 ± 0.017	0.867 ± 0.044	0.79 (57)	0.17	0.2
(*b*) 'Rescue' with 200mM-acetamide (Fig. 6)	12	600	12	2.40 ± 0.09	1.20 ± 0.06	0.25 (25)		
	48	60	48	2.80 ± 0.04	2.10 ± 0.08	0.14 (20)	0.57	0.52
	12	660	48	0.90 ± 0.02	0.55 ± 0.02	0.11 (38)	0.39	0.43
	48	840	48	1.12 ± 0.05	0.89 ± 0.05	0.31 (33)	0.28	0.26
	48	1800	48	0.83 ± 0.04	0.58 ± 0.02	0.60 (57)	0.24	0.21
	48	2700	48	0.54 ± 0.02	0.56 ± 0.01	0.40 (85)	0.13	0.11
(*c*) Dilution experiments:								
Assay with acetamide	53	240	0.26	1.12 ± 0.03	1.49 ± 0.04	0.85 (80)	0.34	0.3
Assay with ethyl acetate (Fig. 7)	53	240	0.53	0.83 ± 0.09	1.04 ± 0.09	0.43 (68)	0.11	
(*d*) 'Rescue' by dilution and assay with acetamide (indicator-linked assay). Inhibition and assay mixtures incubated at pH 8	24	30	0.24	9.36 ± 1.4	10.8 ± 1.8	0.86 (12)	0.65	0.33
	240	30	2.4	3.60 ± 0.02	6.19 ± 0.32	0.58 (20)	0.277	0.26
	24	1200	0.24	1.00 ± 0.04	0.47 ± 0.02	0.45 (20)	0.33	0.39

of free inhibitor sufficiently low for significant reversion to occur. Alternatively it is possible that, as the amidase is a hexameric enzyme, binding of the substrate to one subunit in a molecule could influence the rates of formation of E and EX from EI in the other subunits. To resolve these possibilities the experiments described in the next section were performed.

Recovery of enzymic activity by dilution of the amidase–chloroacetone complex (EI). The following experiment was conducted to determine whether dilution of a solution of the EI species in the absence of substrate would lead to a recovery of free enzyme.

A solution of 6.5μM-amidase and 53μM-chloroacetone was incubated for 4min at pH 7.1. At this time the enzymic activity with both acetamide and ethyl acetate had decreased to less than 5% of that of the control. It can be calculated from computed values of k_{+1}, k_{-1} and k_{+2} (see above, Table 1 and the Theory section) that the concentration of the EI species at this time would be about 80% of the total enzyme concentration and EX would be about 15%.

The reaction mixture was then diluted 100-fold by addition of the assay medium, pH 8.66 (see the Materials and methods section), so the free chloroacetone concentration was decreased to less than 0.53μM. Portions of the resulting solution were left for different times and then assayed for activity at pH 8.66 with ethyl acetate as substrate by using the pH-stat method (see the Materials and methods section).

The resulting time courses for ethyl acetate hydrolysis showed marked acceleration phases at short incubation times. However, with increasing times of incubation a finite initial velocity was observed, which after 20min reached a plateau at about 20% of the rate with uninhibited enzyme. At this time there was no acceleration phase in the assay time course.

The dependence of initial velocity and final velocity on time of incubation is shown in Fig. 7, which gives also the corresponding values when the inhibition mixture was diluted 200-fold after 4min and assayed with acetamide. In this case the recovery of activity at early incubation times was about 34%. This would at first sight seem to indicate that acetamide influenced the extent of recovery of activity by a process other than perturbation of the distribution of enzyme between the E and EI species. However, the detailed computer simulation studies described below indicate that the results in Fig. 7 are consistent with the simple perturbation hypothesis. Computer simulations of the time courses of recovery of activity immediately after dilution were

performed with the program FACSIMILE as described in the Theory section and best-fit values of k_{+1}, k_{-1} and k_{+2} were obtained (see the legend to Fig. 7 and Table 1). Substitution of these values of the rate constants in the analytical expressions appropriate for these experiments (see the Theory section) gave the theoretical curves for initial and final velocities drawn in Fig. 7. It can be seen from this Figure that the fit of observation to theory is sufficiently close to conclude that, if acetamide has any influence on the values of the rate constants of Scheme 1, then it must be relatively small. In these experiments different dilution factors were employed for the assays with the different substrates for technical reasons arising from the different rates of proton release in the two assays and the instrumental limitations imposed by the pH-stat.

Discussion

In principle an active-site-directed irreversible inhibitor should react rapidly and specifically to form a covalent bond with a functional group in the active centre of the target enzyme, and the inhibition should be protected against by the presence of substrates at concentrations in the range of their K_m values. Such inhibitors would be expected to have similar structures to that of a substrate for the particular enzyme. By these criteria the above data in conjunction with the following discussion indicate that chloroacetone is a good active-site-directed inhibitor for the aliphatic amidase from *Pseudomonas aeruginosa*.

This inhibition reaction is rapid, whether it is considered in relation to the expected chemical reactivity of chloroacetone, the rate of reaction of other enzymes with substrate analogues containing a chloromethyl group or the rate of reaction of amidase with a substrate analogue with a structure closely similar to that of chloroacetone. These relationships are discussed in the following text.

First, the formation of the 'irreversibly inhibited' species, EX, from amidase and chloroacetone via the reversibly formed complex, EI (see Scheme 1), occurs with an apparent second-order rate constant ($k_{2(app.)}$) of about $10^3\,M^{-1}\cdot s^{-1}$. The latter value can be calculated from the relation: $k_{2(app.)}=k_{+1}k_{+2}/(k_{-1}+k_{+2})$ by substitution of the values of k_{-1} and k_{+2} given in Table 1 and the values of k_{+1} given in the text. This rate constant is more than three orders of magnitude greater in value than that for the reaction of chloroacetone with 2-mercaptoethanol, a thiol compound that would be expected to be at least as reactive as any 'normal' side chain in a protein. The rate constant for the latter reaction under similar conditions is $0.31\,M^{-1}\cdot s^{-1}$ (see the Materials and methods section), and probably involves nucleophilic substitution at the chloromethyl group, as the value of the dissociation constants for addition of thiols to the carbonyl group would be too high for significant carbonyl addition to occur. For example, the value for the addition of 2-methoxyethanethiol to acetone is greater than 5 M (Lienhard & Jencks, 1966). In this respect, it is noteworthy that the rate of reaction with chloroacetone of the highly reactive thiolate anion of cysteine-149 in glyceraldehyde 3-phosphate dehydrogenase is more than two orders of magnitude lower than the corresponding reaction involving acetone (see Table 2).

If the species EX were to be a Schiff base (azomethine; see below) then the enzyme–inhibitor reaction also would be fast in comparison with chemical precedent, as, for example, the maximum value of the second-order rate constant for the reaction of acetone with hydroxylamine in aqueous solution is $1.8\,M^{-1}\cdot s^{-1}$ (Jencks, 1969).

Secondly, as can be seen from inspection of Table 2, the rate of irreversible inhibition of amidase by chloroacetone is rapid in comparison with the rates of inhibition of other enzymes by substrate analogues containing a chloromethyl function. Only the reaction of acetoacetyl-CoA thiolase with a chloromethyl ketone analogue closely similar to a relatively large substrate is faster overall than that of the reaction of amidase with chloroacetone. Comparison of the values of Table 2 reveals that the high overall rate of the amidase inhibition reaction derives mainly from 'pre-binding', as the first-order, presumed covalent-bond-formation, steps, for most of the reactions are as fast or faster than the k_{+2} step in the reaction involving amidase.

Thirdly, the value of $k_{cat.}/K_m$ for amidase action with *N*-methylacetamide as substrate is about $1.2\times10^3\,M^{-1}\cdot s^{-1}$ [cf. Hollaway & Ticho (1979) and Findlater (1974)], and this is close to the value for the second-order rate constant of about $10^3\,M^{-1}\cdot s^{-1}$ reported in the present paper for the reaction of amidase with chloroacetone. *N*-Methylacetamide is similar to chloroacetone in structure: the van der Waal's radius of the chloride group (0.18 nm; Bondi, 1964) is close in value to that of the methyl group (0.20 nm; Pauling, 1962), and the molecules are isoelectronic apart from a completed L shell in the chloride. This similarity should not be overstressed, however, as the considerable double-bond character of the carbon–nitrogen bond in the amide will give a geometry in the ground state somewhat different from that of the ketone.

The results given in the present paper show that acetamide protects amidase from inhibition by chloroacetone, but the value of the apparent dissociation constant for the substrate of about 1.5 mM determined from these experiments is nearly double that of the K_m value for this substrate (i.e. 0.8 mM; Findlater & Orsi, 1973). It is possible that the two

Table 2. *Kinetic parameters for the two-step inhibition of some enzymes by chloromethyl ketone analogues*

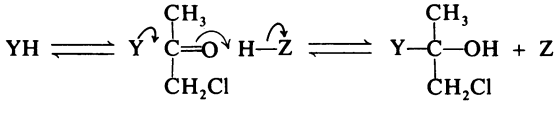
A two-step inhibition reaction is assumed with a rapid and reversible binding, characterized by an apparent dissociation constant K_I, followed by an 'irreversible first-order step with a rate constant k_{+2}. The overall apparent second-order rate constant for the reaction is k_{+2}/K_I.

Enzyme	Inhibitor	K_I (mM)	k_{+2} (s^{-1})	k_{+2}/K_I ($M^{-1} \cdot s^{-1}$)	Reference
Chymotrypsin	Chloromethyl ketone analogue of L-tosylphenylalanine	0.3	3×10^{-3}	10	Schoellmann & Shaw (1963); Glick (1968); Kezdy *et al.* (1967)
Trypsin	Chloromethyl ketone analogue of L-tosyl-lysine	—	—	5.6	Shaw *et al.* (1965)
Elastase	Chloromethyl ketone analogue of Ac-Pro-Ala	40	2×10^{-3}	0.05	Thompson & Blout (1973)
	Chloromethyl ketone analogue of Ac-Ala-Pro-Ala	0.85	3×10^{-2}	35	Thompson & Blout (1973)
	Chloromethyl ketone analogue of Ac-Pro-Ala-Pro-Ala	—	—	260	Thompson & Blout (1973)
Protease B (from *Staphylococcus griseus*)	Chloromethyl ketone analogue of Boc-Gly-Leu-Phe	—	—	82	Gertler (1974)
Glyceraldehyde 3-phosphate dehydrogenase	Chloroacetone	—	—	3.0	Byers (1977)
Leucyl-tRNA synthase	Chloromethyl ketone analogue of L-valine	7.5	5.7×10^{-2}	7.6	Silver & Laursen (1974)
Acetoacetyl-CoA thiolase	11-Chloro-10-oxoundecanoyl-CoA	0.0024	5.0×10^{-2}	2×10^4	Bloxham *et al.* (1978)
Amidase	Chloroacetone	$\sim 1 \times 10^{-3}$	0.5×10^{-3}–4.0×10^{-3}	$\sim 10^3$	This paper

values are not significantly different, but the discrepancy could indicate that one or more of the intermediates along the catalytic pathway can react with chloroacetone.

The nature of the chemical reactions involved in the different steps of Scheme 1 remains obscure. First, in considering the species EI, it seems that the k_{+1} and k_{-1} steps are slow compared with what would be expected for a simple adsorption–desorption process. Therefore it is tempting to propose that EI is a carbonyl addition compound involving the reaction of a nucleophilic group Y in the active site of the enzyme. In this case one way in which the bell-shaped pH–K_{+1} profile could arise is from an ascending limb concomitant with the formation of the nucleophilic Y species from an unreactive protonated YH species, and a descending limb involving loss of a proton from an assisting electrophilic group ZH (see Scheme 2). This Scheme is formally similar to that proposed by Poulos *et al.* (1976) to describe the first step in the inhibition of subtilisin by a chloromethyl ketone analogue. In the latter case, on the basis of X-ray crystallographic studies, the group Y was designated as a serine residue and ZH corresponded to a combination of an amide and a peptide grouping.

The plausibility of such an interpretation, however, is diminished by the observation that acetone is a competitive inhibitor of amidase action with a value of K_I of about 4×10^{-2} M (T. Ticho & M. R. Hollaway, unpublished work). Acetone and chloroacetone would not be expected to differ by so much in behaviour in addition reactions: for example, the equilibrium constants for hydration of the carbonyl group are 0.002 and 0.08 respectively (Lewis & Wolfenden, 1977).



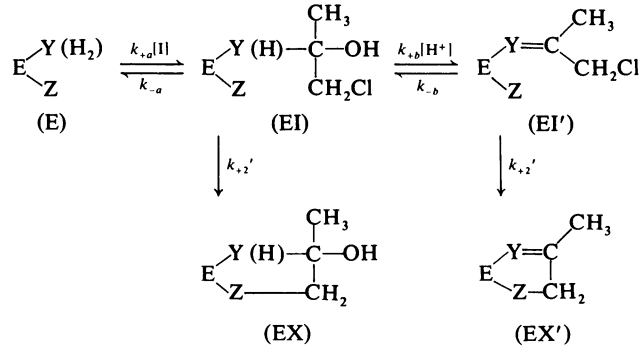
Scheme 2.

Another possibility, suggested by the bell-shaped pH–k_{+1} profile and reversibility of the formation of EI, is that this species could be a Schiff base formed by reaction of an amino group in the active site. Bell-shaped pH–rate profiles for the condensation reaction of amino compounds with ketones are observed commonly and have been attributed to a change in rate-limiting step with pH (Jencks, 1969). At low pH values addition of the amino group is considered to be rate-limiting, but as the amino group passes over to the reactive base form with increasing pH the dehydration of the carbinolamine tetrahedral adduct becomes rate-limiting, and, as this reaction is subject to specific acid catalysis, the descending limb of the pH–rate profile is generated (cf. Scheme 3).

Yet again, this interpretation becomes less attractive when it is considered that acetone is a rather poor competitive inhibitor of amidase action (see above). As acetone and chloroacetone react at similar rates with hydroxylamine to form oximes (S. Clarke & M. R. Hollaway, unpublished work), it seems unlikely that an amino residue in the active site of amidase would exhibit such widely different reactivity towards these compounds in a Schiff-base-forming reaction.

On similar grounds the possibility that the irreversibly formed enzymically inactive species EX is a Schiff base seems even less likely.

Some results obtained by Gregoriou & Brown (1979) may have some bearing on the present findings. It was shown that amidase from strain AI 3 of *Pseudomonas aeruginosa* is inhibited by urea,



Scheme 3. *Possible reaction pathways for the interaction of amidase with chloroacetone*

Free enzyme combines with inhibitor to give a tetrahedral adduct, EI, which may react with a group Z in the active site to form EX or, if YH_2 is an amino group, may form a Schiff base, EI′, which in turn may react with the nucleophile Z to form the EX′ species. The k_{+1} step of Scheme 1 would correspond to the k_{+a} and k_{+b} steps.

hydroxyurea and by cyanate, each in a two-step reaction: the first involving a rapidly reversible adsorption with a K_I value of the order of millimolar and the second step a first-order process with a value of the rate constant of the order of $10^{-2}s^{-1}$ at pH 7.2 and 37°C. The overall apparent second-order rate constants are then of the order of $10 M^{-1}\cdot s^{-1}$, two orders of magnitude lower than that for EI formation from amidase and chloroacetone. Nevertheless it seems possible that the product of inhibition by urea corresponds in essence to the species EI in the inhibition by chloroacetones, as the urea inhibition product reverts slowly to active enzyme and unmodified urea after removal of excess inhibitor by gel filtration (Gregoriou & Brown, 1979).

The identities of these reversibly formed inactive species remain unknown. If they are not carbonyl addition compounds or Schiff-base analogues, it seems possible that they are complexes in which the inhibitor is held in the active site in such a way that a structural change in the enzyme is required before it is released.

It remains also to establish the nature of the species EX of Scheme 1. The most likely probability is that it results for reaction of a nucleophilic group in the active site of the enzyme (Z in Scheme 3) with the electrophilic $-CH_2$-Cl centre to give an essentially irreversibly formed $-CH_2-Z$ product. Candidates for the group Z would include the side chains of residues of aspartate, cysteine, glutamate, histidine, lysine, methionine or serine. Alternatively the second stage of inhibition by chloroacetone could involve a change of conformation in the enzymic protein. A change in aggregation state can be excluded as there was no observable change in the molecular weight of the amidase after modification with chloroacetone.

An observation that can be made from the data of Table 1 is that: as amidase is incubated with chloroacetone for longer periods of time both the 'rescue' reaction (k_{-1} step) and the 'irreversible' inhibition (k_{+2} step) become slower. That is, the inhibition is not monophasic. This behaviour could result from: a pre-existing asymmetry in the active sites; information transfer between active sites whereby a change in chemical reactivity at one site follows modification of another within the same molecule; or an effect on the reactivity at the active site after modification at a different set of loci.

The work described in the present paper demonstrates that chloroacetone is an effective active-site-directed inhibitor, but leaves a number of questions unanswered. In particular, the identity of reacting group(s) is unknown, as is the source of the apparent pK_a of about 7.8 in the pH profile for the rate of formation of the EI species. Answers to these questions should provide a deeper understanding of the catalytic mechanism of action of amidase.

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