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# KINETIC MECHANISM OF THE ALIPHATIC AMIDASE FROM *PSEUDOMONAS AERUGINOSA*

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## Summary

The kinetic constants for hydrolysis and transfer (with hydroxylamine as the alternate acceptor) of the aliphatic amidase (acylamide amidohydrolase, EC 3.5.1.4) from *Pseudomonas aeruginosa* were determined for a variety of acetyl and propionyl derivatives. The results obtained were consistent with a ping-pong or substitution mechanism.

Product inhibition, which was pH dependent, implicated an acyl-enzyme compound as a compulsory intermediate and indicated that ammonia combined additionally with the free enzyme in a dead-end manner.

The uncompetitive activation of acetamide hydrolysis by hydroxylamine and the observation that the partitioning of products between acetic acid and acetohydroxamate was linearly dependent on the hydroxylamine concentration substantiated these conclusions and indicated that deacylation was at least partially rate limiting.

With propionamide as the acyl donor apparently anomalous results, which included inequalities in certain kinetic constants and a hyperbolic dependence of the partition ratio on the hydroxylamine concentration, could be explained by postulating a compulsory isomerisation of the acyl-enzyme intermediate prior to the transfer reaction.

## Introduction

The inducible aliphatic amidase (acylamide amidohydrolase, EC 3.5.1.4) from *Pseudomonas aeruginosa* which catalyses the hydrolysis of simple aliphatic amides (acetamide and propionamide) was discovered by Kelly and Clarke [1]; later Kelly and Kornberg [2,3] partially purified it and demonstrated that the enzyme was also capable of catalysing the transfer of the acyl group of an amide to hydroxylamine to form the corresponding acylhydroxa-

mate (acyltransferase activity). Since that early work much effort has been devoted to studying the regulation of the synthesis of the enzyme [4] and to the mutants which produce the amidase with an altered specificity [5—7]. Although the enzyme has been purified to homogeneity and some of its physicochemical properties studied [8] little was known about its mechanism of action, the general assumption being that it behaved like an acyltransferase of the serine or thiol protease type. However, the observation that acetaldehyde-ammonia was an extremely potent inhibitor, possibly acting as a transition-state analogue, suggested that the reaction may proceed through a direct elimination rather than a substitution reaction [9]; in contrast enzyme, partially inactivated by iodoacetamide, gave kinetics characteristic of a substitution mechanism [10]. This paper is concerned with the kinetics of the amidase in an attempt to resolve its kinetic mechanism.

## Materials and Methods

*Materials*

DL-Lactamide was supplied by Sigma Chemical Company, Missouri, U.S.A.; acetamide and urea were obtained from May and Baker Ltd., Dagenham, England; propionamide and hydroxylamine hydrochloride were obtained from B.D.H. Chemicals Ltd., Poole, England; acetohydroxamate, acetohydrazide, *N*-methylacetamide and *N*-methylpropionamide were purchased from the Aldrich Chemical Company, Wembley, England. Propionohydroxamate was prepared by the method of Fishbein et al. [11]. [1-$^{14}$C]Acetamide (1.83 Ci/mol) was obtained from ICN Pharmaceuticals Inc., California, U.S.A. and [1-$^{14}$C]propionic acid (53 Ci/mol) was obtained from the Radiochemical Centre, Amersham, England.

[1-$^{14}$C]Propionamide was prepared by treating 1.18 $\mu$mol of [1-$^{14}$C]-propionic acid with trimethylacetyl chloride at 25°C to form a mixed anhydride; excess $NH_3$ was added to form the mixed amides and the resulting white precipitate extracted with water in which trimethylacetamide is only very sparingly soluble. The [1-$^{14}$C]propionamide was purified by paper chromatography with acetone/$NH_4OH$ (density 0.88 g/ml) (9 : 1, v/v) as the solvent; in this solvent propionamide, propionohydroxamate and propionic acid have $R_F$ values of 0.75, 0.42 and 0.17, respectively. [1-$^{14}$C]Acetohydrazide was prepared by refluxing for 1 h 75 $\mu$mol of [1-$^{14}$C]acetamide and 148 $\mu$mol of hydrazine. The solution was reduced to dryness and the residue taken up in a small quantity of water. The [1-$^{14}$C]acetohydrazide was purified by paper chromatography in the acetone/$NH_4OH$ solvent in which the $R_F$ values for acetohydrazide, acetamide, acetohydroxamate and acetic acid are 0.73, 0.56, 0.17 and 0.03, respectively.

The amidase was obtained by the procedure of Brown et al. [5]. A partially purified preparation was very kindly supplied by Professor P.H. Clarke of University College, London and the final steps involving separation on a DEAE-Sephadex column carried out in this laboratory. The enzyme obtained by this method is essentially pure in that only a single protein/activity band is seen after polyacrylamide gel electrophoresis at pH 8.5. Prior to being used the enzyme was dialysed against 0.1 M sodium phosphate buffer (pH 7.2) containing 2 mM EDTA and 1 mM dithiothreitol.

*Methods*

All assays were done in duplicate. Acylamide and *N*-methylacylamide hydrolysis was measured by incubating the appropriate amides at 25°C with 0.1 M sodium phosphate buffer (pH 7.2) in a total volume of 980 μl; the reaction was started by the addition of 20 μl of suitably diluted amidase solution and the reaction followed by the removal of 100-μl samples at zero time and suitable time intervals thereafter. The products of the reaction, ammonia or methylamine, were estimated colorimetrically at 630 nm by a modification [12] of the Berthelot procedure of Fawcett and Scott [13]; in this method the first of the colour-producing reagents (sodium phenate) also serves to stop the enzyme reaction. Hydroxylamine, hydrazine and urea have been found to interfere with this colour reaction. For a standard assay an initial acetamide concentration of 100 mM was used. Acylhydroxamate hydrolysis was assayed by measuring the decrease in acylhydroxamate concentration. The incubation conditions were the same as previously described but the 100-μl samples were added to 1 ml of ferric chloride solution (0.11 M in 0.33 N HCl) and the absorbance measured at 540 nm [14]. Acylhydrazide hydrolysis was measured by the indicator method of Findlater and Orsi [15] using bromothymol blue and 1 mM sodium phosphate buffer (pH 7.2) at 25°C. Acyltransferase activity was assayed by including hydroxylamine in the basic incubation mixture and measuring the increase in acylhydroxamate concentration with time using the ferric chloride procedure. A standard acyltransferase assay contained 100 mM acetamide and 750 mM hydroxylamine.

In certain experiments it was necessary (a) to measure hydrolysis ($v_{acylacid}$) in the presence of substances that interfered with the Berthelot procedure and (b) to measure the transfer reaction ($v_{acylhydroxamate}$) concomitantly with hydrolysis ($v_{acylacid} + v_{acylhydroxamate} = v_{NH_3}$), in these instances the substrates used were the [1-$^{14}$C] derivatives. Samples (50 μl) removed at various times were added to 10 μl of 2 N HCl followed by 10 μl of 2 N NaOH; a 25 μl sample was then applied to Whatman No. 1 paper and the reactants separated using the acetone/$NH_4OH$ solvent. The labelled compounds were located, cut out and suspended in 0.5% (w/v) PPO in toluene and counted in a Packard Tricarb scintillation counter. In the case of [1-$^{14}$C]propionamide it was found that the use of HCl to stop the reaction was unsatisfactory and led to low recoveries of total radioactivity; for these experiments the reaction was stopped by the addition of 10 mM acetaldehydeammonia ($K_i = 1.6 \cdot 10^{-5}$ M) a potent competitive inhibitor [9].

The initial velocities were determined by plotting absorbance or cpm as a function of time; in most instances a straight line was obtained the slope of which was taken as the initial velocity; in the few experiments where definite curvature was observed the data was fitted to a cubic equation in time and the initial velocity is given by the coefficient of $t$. The kinetic constants were obtained by fitting the initial velocity-substrate concentration data directly to the Michaelis-Menten hyperbola [16] and, if necessary, fitting the reciprocals of these kinetic constants as a linear, parabolic or hyperbolic function of inhibitor concentration or the reciprocal of a second substrate concentration [17]; the standard errors of the constants are shown in parentheses. The terminology is that advocated by Cleland [18] and the kinetic constants that

can be determined from the hydrolase reaction alone are indicated with h as a superscript; all other constants refer to the transfer reaction.

## Results

*Initial rate patterns and kinetic constants*

With propionamide as the acyl donor linear reciprocal plots were obtained in both hydrolysis and transfer reactions and in the latter case increasing the concentration of the alternate acceptor hydroxylamine produced the intersecting pattern expected from a transferase carrying out a simultaneous hydrolytic reaction (Appendix, Eqn. 2). Essentially similar results were obtained with acetamide, acetohydrazide and *N*-methylacetamide as the acyl donors. The kinetic constants for both hydrolysis and transfer for all these substrates are shown in Table I. This table also shows that although *N*-methylpropionamide was a substrate for hydrolysis and transfer the rate of the transfer reaction was so low it was not possible to obtain accurate estimates of the kinetic constants. The other point worth noting from Table I is the apparent absence of hydrolysis of propionohydrazide even though the transfer reaction proceeded at a low but reasonable rate. Estimates of the hydrolytic kinetic constants from the transfer kinetic constants gave values of 35 $s^{-1}$ for $k_{cat}^{h}$ and 1595 mM for $K_a^h$ and no difficulty should have been encountered detecting hydrolase activity in the very sensitive indicator method [15]. Propionohydrazide is the only substrate found so far that undergoes the transfer reaction without being subject to hydrolysis.

Except for DL-lactamide no substrate inhibition has ever been observed in the hydrolase reaction even at substrate concentrations as high as 2.5 M. In contrast, in the transferase reaction both acetamide and hydroxylamine are

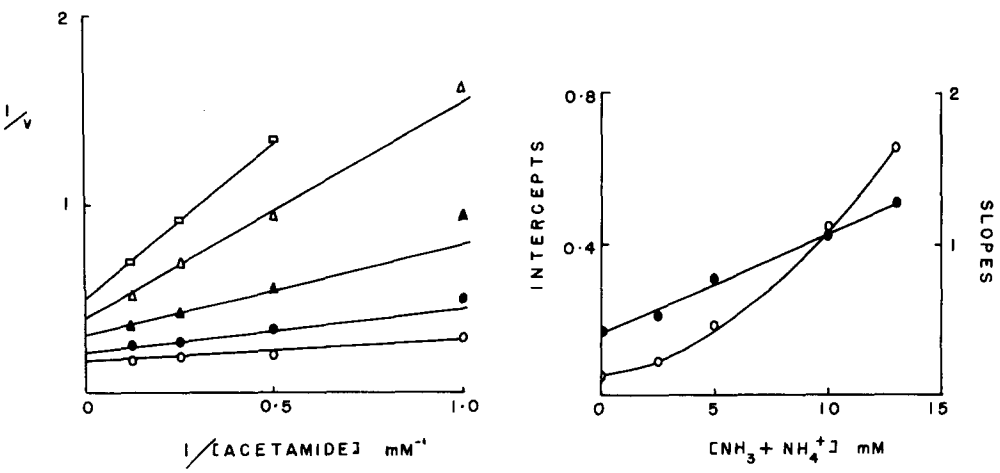



Fig. 1. Inhibition by $(NH_4)_2SO_4$ of acetamide hydrolysis at 25°C in 0.1 M sodium borate buffer, pH 9.0; $v$ = μmol [1-$^{14}$C]acetate/min. $(NH_4)_2SO_4$ concentrations were 0 mM (○), 1.25 mM (●), 2.5 mM (▲), 5 mM (△) and 6.5 mM (□).

Fig. 2. Replot of the slopes (○) and intercepts (●) from Fig. 1 as a function of the concentration of $(NH_3 + NH_4^+)$.

TABLE I

KINETIC CONSTANTS

The subscripts a and b refer to the acyl donor and acylacceptor ($NH_2OH$), respectively; the superscript h refers to the hydrolase reaction only, the other constants refer to the transferase reaction. The values for $k^h_{cat}$ and $k_{cat}$ were obtained from the corresponding maximum velocities assuming that the enzyme has a molecular weight of $2 \cdot 10^5$ with six independent active sites [8].

| Substrate | $K^h_a$ (mM) | $k^h_{cat}$ ($s^{-1}$) | $K_a$ (mM) | $K_b$ (mM) | $k_{cat}$ ($s^{-1}$) | $k^h_{cat}/K_a$ ($s^{-1} \cdot mM^{-1}$) | $k_{cat}/K^h_a$ ($s^{-1} \cdot mM^{-1}$) | $k_{cat}/K_b$ ($s^{-1} \cdot mM^{-1}$) | $K^h_a K_b/K_a = K'_{ib}$ (mM) |
|---|---|---|---|---|---|---|---|---|---|
| Acetamide | 0.83 | 162 | 4.0 | 91 | 790 | 195 | 198 | 8.7 | 19 |
| | (0.07) | (17) | (0.12) | (15) | (106) | (26) | (27) | (1.8) | (3.5) |
| Acetohydroxamate | 6.7 | 14 | — | — | — | 2.1 | — | — | — |
| | (2) | (1) | | | | (0.64) | | | |
| Acetohydrazide | 81 | 10 | 215 | 35 | 20 | 0.12 | 0.093 | 0.57 | 14 |
| | (16.5) | (1) | (23) | (4) | (2.5) | (0.025) | (0.015) | (0.096) | (3.4) |
| *N*-Methylacetamide | 233 | 1080 | 239 | 26 | 890 | 4.6 | 3.7 | 34 | 25 |
| | (30) | (71) | (16) | (7.6) | (35) | (0.67) | (0.3) | (10) | (8) |
| Propionamide | 7.8 | 374 | 34 | 310 | 465 | 48 | 13 | 1.5 | 71 |
| | (1.2) | (22) | (4) | (70) | (55) | (8) | (2.1) | (0.38) | (21) |
| Propionohydroxamate | 75 | 22 | — | — | — | 0.29 | — | — | — |
| | (9) | (3.5) | | | | (0.058) | | | |
| Propionohydrazide | * | * | 1640 | 73 | 9.5 | * | 0.006 | 0.13 | * |
| | | | (405) | (14) | (0.6) | | (0.0015) | (0.026) | |
| *N*-Methylpropionamide | 248 | 0.3 | ** | ** | ** | 0.0012 | ** | ** | ** |
| | (64) | (0.024) | | | | (0.003) | | | |

* No detectable activity.

** Activity detectable but too small to estimate the constants.

substrate inhibitors, non-competitive and competitive, respectively [10], of each other.

*Product inhibition*

The inhibition of acetamide hydrolysis by either product was dependent on pH and showed that $NH_3$, the base form, and acetic acid, the acid form, are the true inhibitors. At pH 5.7 acetic acid was found to be a linear competitive inhibitor with an inhibition constant of 6.7 mM. With ammonia as the product inhibitor a non-competitive pattern was observed (Fig. 1) but although the intercepts were a linear function of the ammonia concentration the slopes showed an upward curving line (Fig. 2) these data were fitted to a parabolic equation. These product inhibition patterns are summarised in Table II.

*Alternate product effects*

During amide hydrolysis hydroxylamine acts as an alternate product and it produces a linear non-competitive effect on acetamide hydrolysis when $v_{acetate}$ is used as a measure of the reaction rate (Appendix, Eqn. 3). In contrast to this when $v_{NH_3}$ ($v_{acetate} + v_{acetohydroxamate}$) was used hydroxylamine produced an uncompetitive activation (Fig. 3) and a replot of the intercepts as

TABLE II

INHIBITION PATTERNS AND CONSTANTS

The variable substrate in all cases was acetamide. All inhibition constants (mM) have been corrected for the concentration of inhibitor in the correct ionic form assuming p*K*a of 4.75 and 9.27 for acetic acid and ammonia respectively. For slope inhibition patterns the general equation used was:

$$\text{slope} = \frac{K_a^h}{V_m^h}\left(1 + \frac{[I]}{K_{is}} + \frac{[I]^2}{K'_{is}}\right),$$

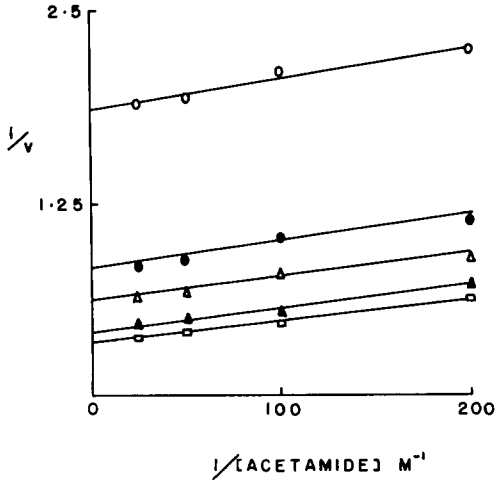
in the case of linear effects $K'_{is}$ was considered to be infinite. For intercept inhibition patterns the general equation used was:

$$\text{intercept} = \frac{1}{V_m^h}\,\frac{(1 + [I]/K_{ii})}{(1 + [I]/K_{in})}$$

and in the case of linear effects $K_{in}$ was considered to be infinite.
All inhibitors were fitted to the linear form unless otherwise indicated.

| Inhibitor | pH | Reaction mixture measured | Type of inhibition | Slope | | Intercept | |
|---|---|---|---|---|---|---|---|
| | | | | $K_{is}$ | $K'_{is}$ | $K_{ii}$ | $K_{in}$ |
| Urea | 7.2 | [$CH_3COOH$] | C | 3.1 (0.67) | — | — | — |
| Acetic acid | 5.7 | [$NH_3$] | C | 6.7 (1.2) | — | — | — |
| Hydroxylamine | 7.2 | [$CH_3COOH$] | NC | 16.3 (2.2) | — | 150 (54) | — |
| Hydroxylamine | 7.2 | [$CH_3COOH$] + [$CH_3CONHOH$] | UC * | — | — | 155 (40) | 16.8 (1.7) |
| Ammonia | 9.0 | [$CH_3COOH$] | NC ** | 1.0 (0.26) | 6.6 (0.74) | 2.1 (0.24) | — |

* Intercepts fitted to hyperbolic equation.
** Slopes fitted to parabolic equation.



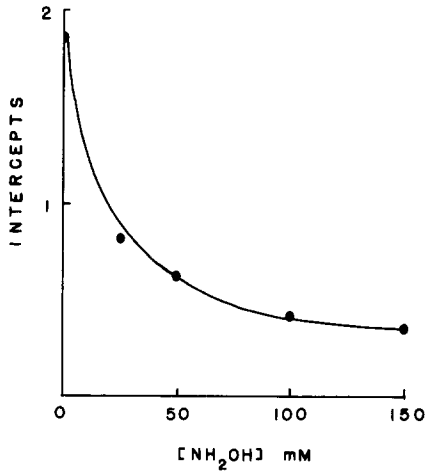



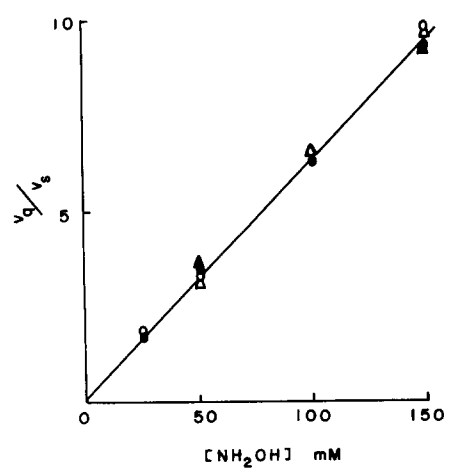

Fig. 3. Activation of acetamide disappearance by hydroxylamine at 25°C and pH 7.2; $v$ = μmol ([1-$^{14}$C]-acetate + [1-$^{14}$C]acetohydroxamate)/min. Hydroxylamine concentrations were : 0 mM (○); 25 mM (●), 50 mM (△), 100 mM (▲) and 150 mM (□).

Fig. 4. Replot of the intercepts from Fig. 3 as a function of the hydroxylamine concentration.

a function of the hydroxylamine concentration showed that there was a hyperbolic relationship (Fig. 4) (Appendix, Eqn. 4). The inhibition constants obtained are shown in Table II.

*Dead-end inhibition*

Urea was found to be an effective linear competitive inhibitor of hydrolysis



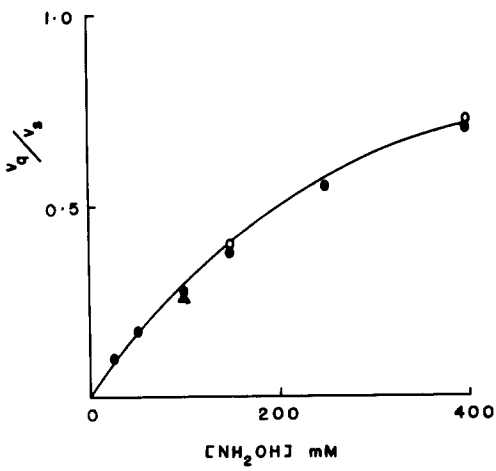




Fig. 5. Effect of the hydroxylamine concentration on the partition ratio ($v_Q/v_S$ = $v_{acetohydroxamate}/v_{acetate}$) for acetamide (5 mM (○), 20 mM (●)) and acetohydrazide (100 mM (△), 150 mM (▲)) as acyl donors.

Fig. 6. Effect of the hydroxylamine concentration on the partition ratio ($v_Q/v_S$ = $v_{propionohydroxamate}/v_{propionate}$) for propionamide (50 mM (○), 100 mM (●), 150 mM (△)) as the acyl donor.

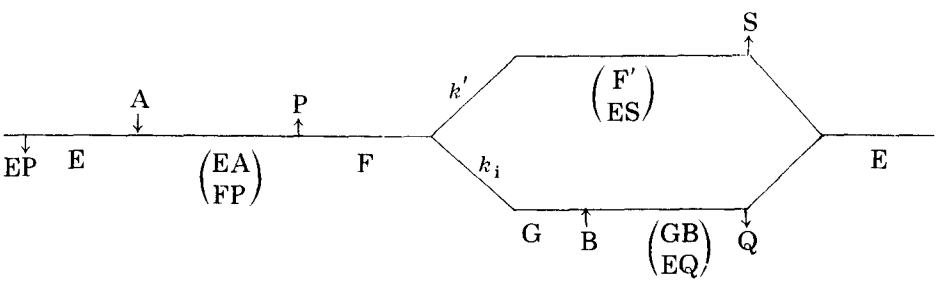
and a similar effect was seen on the transfer reaction but in this case the inhibition constant was a linear function of the hydroxylamine concentration.

*Partitioning of products*

With either acetamide or acetohydrazide as the acyl donor the ratio of $v_{acetohydroxamate}/v_{acetate}$ was found to be independent of the acyl donor or its concentration but to have a linear dependence on the hydroxylamine concentration (Fig. 5) the slope of which yielded a value of 0.062 $mM^{-1}$ (±0.0022). In contast, with propionamide as the acyl donor the relationship between $v_{propionohydroxamate}/v_{propionate}$ and the hydroxylamine concentration was hyperbolic (Fig. 6) although it was still independent of the propionamide concentration. Unfortunately no other suitable propionyl derivative was available as a substrate to test the independence of the ratio to the leaving group.

## Discussion

Except for two observations the results presented can be readily interpreted in terms of a CPP mechanism (see Appendix). The kinetic constants of the various substrates (Table I) show that for acetamide, acetohydrazide and *N*-methylacetamide the first order rate constants for hydrolysis ($k^h_{cat}/K^h_a$) and transfer ($k_{cat}/K_a$) are essentially equal although the values differ for the various substrates as would be expected as the leaving group changes. A CO mechanism (see Appendix), although not inconsistent with these results, is less likely in view of the widely different values of $k^h_{cat}$ and $K^h_a$. In contrast to the acetyl derivatives the $k^h_{cat}/K^h_a$ for propionamide was about four times greater than $k_{cat}/K_a$ an observation not in keeping with a CO mechanism and the most likely explanation is that a CPP mechanism is operating but in addition there is a compulsory isomerisation of F to G (Scheme III) prior to the transfer reaction taking place. In Scheme III A is the acyl donor, P is ammonia, S is the acyl acid, Q is the acylhydroxamate, and B is hydroxylamine the alternate acceptor. The relative constancy of the $K'_{ib}$ values for the acetyl-derivatives supports the general conclusion of a CPP mechanism and the fact that the mean value (19.3 ± 3.2 mM) differs appreciably from that obtained for propionamide (71 mM) tends to eliminate a rapid equilibrium random mechanism from consideration. Unfortunately no other suitable propionyl derivatives were available




Scheme III

to test the constancy of this $K'_{ib}$ as the leaving group is changed. A third factor in favour of this view is that as $k^h_{cat}$ increases from acetohydroazide to acetamide and *N*-methylacetamide, suggesting that the rate of acylation is increasing, so the first order rate constant for the reaction of the alternate acceptor in the transfer reaction ($k_{cat}/K_b$) also increases, an observation inconsistent with a CO mechanism.

The linear competitive inhibition by acetic acid (pH 5.7) of acetamide hydrolysis suggests that the acyl acid is the last product to leave the active site and implies that an acyl-enzyme compound is a compulsory intermediate in the reaction sequence. The inhibition produced by ammonia at pH 9 was more complex and whilst the intercept effect was linear, and the inhibition constant of 2.1 mM can be equated with $K^h_{ip}$ (Appendix, Eqn. 1) the slope effect appeared to be of a degree greater than one suggesting that ammonia (P) was forming a dead-end complex with the free enzyme in addition to its role as a product inhibitor [19,21], this situation would produce a slope term in the reciprocal rate equation as shown in Eqn. 6 where $K_I$ represents the binding constant for ammonia acting as a dead-end inhibitor.

$$\frac{K^h_a}{V^h_m}\left(1+\left(\frac{1}{K_I}+\frac{K^h_{ia}}{K^h_a K^h_{ip}}\right)[P]+\frac{K^h_{ia}[P]^2}{K^h_a K^h_{ip} K_I}\right) \qquad (6)$$

Fitting the data from Fig. 2 to a parabolic equation, and using the values for $K^h_a$ and $K^h_{ip}$ already obtained, yielded estimates of 3.4 mM (±0.34) and 5.4 mM (±0.71) for $K^h_{ia}$ and $K_I$, respectively. If this interpretation is correct then an unusual situation is occurring in that the inhibition constant for dead-end binding ($K_I$) is uncommonly low and it is possible that this is a control mechanism enabling ammonia to be an effective product inhibitor even around neutrality where the concentration of free base is very small.

If the reasonable assumption is made that $K^h_{ia}$ behaves like $K^h_a$ as a function of pH then the predicted value of $K^h_{ia}$ at pH 7.2 is 1.7 mM [22] and assuming $K^h_{ip}$ and $K^h_{is}$ (the inhibition constant for acetic acid, 6.7 mM) do not change appreciably with pH then it is possible to obtain a value of 0.008 M (±0.0018) for the composite constant $K^h_{ip}K^h_{is}/K^h_{ia}$. Hsu et al. [21] have shown for a simple ordered uni-bi enzyme that this composite constant should be equal to or greater than the overall equilibrium constant of the chemical reaction catalysed. A value of 34.7 M for $K_{eq}$ for propionamide hydrolysis can be calculated from the data given by Morawetz and Otaki [23] and assuming the value for acetamide would not be too different then it is obvious that the simple ordered uni-bi mechanism is inadequate. The simplest way in overcoming this objection is to postulate the occurrence of an additional enzyme containing species after the release of the first product P and prior to the relase of the second product S, this presumably being the modified enzyme essential to a CPP mechanism. Similar results have been obtained for a phosphomonoesterase [21], adenosine aminohydrolase [19] and an ATP phosphohydrolase [24].

Urea was a linear competitive inhibitor of acetamide in both the hydrolytic and transfer reactions indicating that it acts as a substrate analogue. The proportional increase of the inhibition constant in the transfer reaction as the hydroxylamine concentration increases shows that urea and hydroxylamine are competing for the same binding site and supports the previous conclusion that

hydroxylamine is a competitive substrate inhibitor of acetamide in the transfer reaction [10]. Preliminary experiments with hydroxylamine as a product inhibitor of acetohydroxamate hydrolysis have shown that an S-parabolic I-linear non-competitive pattern is obtained and this again is consistent with hydroxylamine binding to the free enzyme in a dead-end manner similar to that for ammonia.

Hydroxylamine was a linear non-competitive inhibitor of acetamide hydrolysis and the slope inhibition constant, 16.3 mM, should give a value for $K'_{ib}$ (19.3 mM) and the intercept inhibition constant 150 mM, should give a value for $K_b$ (91 mM, Table I); these reasonable agreements are, however, consistent with either a CPP or CO mechanism. In contrast to this when the total reaction was measured ($v_{NH_3}$) hydroxylamine was an uncompetitive activator (Fig. 3) a result much more readily accommodated by a CPP mechanism. Evaluation of the constants show that $K_{in}$ (16.8 mM) and $K_{ii}$ (155 mM) reflect reasonably well the constants $K'_{ib}$ and $K_b$, respectively. The strong uncompetitive activation observed indicates that acylation is not rate limiting and that the acylated enzyme is accumulating in the steady state [25].

If a common intermediate is formed from a series of different substrates an identical ratio of products will be observed at fixed concentrations of acceptors regardless of whether acylation or deacylation is the rate-determining step [26]. The results from the partitioning experiments show that this is the case for acetamide and acetohydrazide (Fig. 5). The ratio obtained was independent of the acyl donor concentration, which Frere [27] has shown eliminates the possibility that the acyl acceptor is binding before the acyl donor, but linearly dependent on the hydroxylamine concentration; the reciprocal of the slope of this line, 16.1 mM (±0.56) gives a good estimate of $K'_{ib}$. The hyperbolic dependence of this ratio for propionamide (Fig. 6) indicates that Scheme III is probably operating and in this case the ratio is equal to $a[B]/(K'_{ib} + (1-a)[B])$ where $a$ equals $V_m K^h_a / V^h_m K_a$. A value of 77 mM for $K'_{ib}$ can be extracted from this data and compares favourably with the value of 71 mM obtained from initial rate studies (Table I).

From these results it seems most likely that the kinetic mechanism for this amidase is of a ping-pong type with the compulsory isomerisation of the acylated enzyme (F) prior to the transfer reaction only (Scheme III). In the case of the acetyl substrates $k' \ll k_i$ and the observed kinetics are consistent with a simple CPP mechanism; in the case of propionamide this relationship does not hold and thus the apparently anomalous results can be easily explained.

The two observations which are not readily explained by Scheme III are the lack of hydrolysis of propionohydrazide and the lack of substrate inhibition by acetamide in hydrolysis even though it causes non-competitive substrate inhibition in the transfer reaction. One possible explanation for the latter observation is that the enzyme, a hexamer, behaves as a triplet of dimers. These dimers act independently of one another until the compulsory isomerisation of F to G has occurred when the binding of a second acyl donor to the adjacent empty site is completely suppressed. At the present time the only, and rather unsatisfactory, explanation that can be offered for the propionohydrazide results is that after binding and the subsequent reaction to form the acyl-enzyme an isomerization

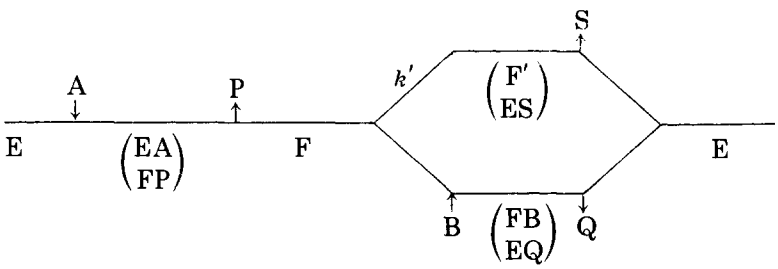
occurs which is capable of reacting with alternate acceptors but incapable of reacting with water.

Two important problems remain outstanding with this enzyme. The first concerns the nature of the amino acid residue that becomes acylated during the reaction. Although thiols are necessary for stabilisation of the active enzyme they do not appear to be involved directly at the catalytic site [8,10], in addition various phosphofluoridates have no inhibitory action even at high concentrations [28]. The second problem concerns the metabolic control of the enzyme as distinct from its genetic control [4]. At the present time no cellular metabolite has been found that will effectively modulate the enzyme's activity [4,28] in spite of its polymeric nature and that it is the first enzyme in a metabolic pathway that is capable of providing a source of nitrogen, carbon and energy for the growth of the organism.

## Appendix

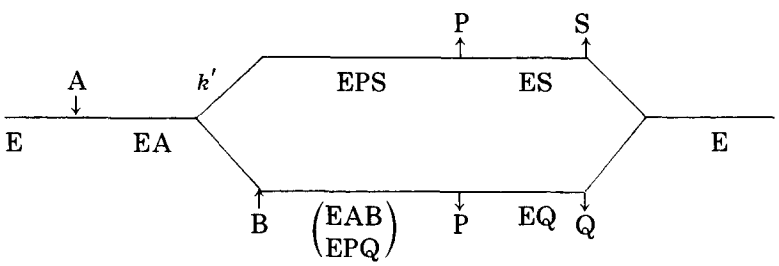
*Theory*

The distinction between a substitution mechanism (Scheme I) and a sequential mechanism (Scheme II) is greatly facilitated by the presence of an alternate acceptor




Scheme I

to water. These two main possibilities have been termed crypto ping pong (CPP) and crypto ordered (CO), respectively [19].




Scheme II

In these schemes S is the hydrolytic product, B the alternate acceptor giving rise to the alternate product Q, the common product of both reactions is P and $k'$ indicates the water reaction.

For either of these mechanisms the initial rate equation for hydrolysis in the absence of B is given by Eqn. 1:

$$v_s = \frac{V_m^h[A]}{K_a^h + [A] + \frac{[A][P]}{K_{ip}^h} + \frac{K_{ia}^h[P]}{K_{ip}^h} + \frac{K_a^h[S]}{K_{is}^h}} \quad (1)$$

and for the transfer reaction by Eqn. 2:

$$v_q = \frac{V_m[A][B]}{K_a^h K_b + K_a[B] + K_b[A] + [A][B]} \quad (2)$$

and thus they appear to be indistinguishable. Analysis of the kinetic constants shows that for Scheme I $V_m^h/K_a^h = V_m/K_a$ [20] but that for Scheme II $V_m^h/K_a^h \leqslant V_m/K_a$. For a CPP mechanism the composite constant $K_a^h K_b/K_a = K'_{ib}$ is expected to remain constant as the substrate A changes if the change is only in the nature of the leaving group P [20]; no such constancy for $K'_{ib}$ is expected for a CO mechanism. A third diagnostic constant is $V_m/K_b$ the magnitude of which depends to a large extent on the relative rates of release of the two products in the hydrolytic reaction, for a CPP mechanism this constant gets larger in a hyperbolic manner as the release of S becomes more and more rate limiting; a CO mechanism shows the converse change.

The effect of the alternate acceptor B on the hydrolytic reaction is identical for both mechanisms and Eqn. 3 shows the reciprocal rate equation.

$$\frac{1}{v_s} = \frac{1}{[A]} \frac{K_a^h}{V_m^h} \left(1 + \frac{[B]}{K'_{ib}}\right) + \frac{1}{V_m^h} \left(1 + \frac{[B]}{K_b}\right) \quad (3)$$

In contrast the effect of B on the total reaction is different in that a hyperbolic uncompetitive effect is seen with Scheme I (Eqn. 4):

$$\frac{1}{v_p} = \frac{1}{[A]} \frac{K_a^h}{V_m^h} + \frac{1}{V_m^h} \frac{(1 + [B]/K_b)}{(1 + [B]/K'_{ib})} \quad (4)$$

and a hyperbolic noncompetitive effect is found with Scheme II (Eqn. 5):

$$\frac{1}{v_p} = \frac{1}{[A]} \frac{K_a^h}{V_m^h} \frac{(1 + [B]/K'_{ib})}{(1 + [B]/K_{bb})} + \frac{1}{V_m^h} \frac{(1 + [B]/K_b)}{(1 + [B]/K_{bb})} \quad (5)$$

Finally the partitioning of products between hydrolysis and transfer ($v_q/v_s$) for both mechanisms is linearly dependent on the concentration of B but for the CPP mechanism this ratio is equal to $[B]/K'_{ib}$ whereas for a CO mechanism it is equal to $[B]/K_{bb}$.

The importance of these rate equations is that the same kinetic constants appear in different guises depending on the experiment involved.

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## References


1 Kelly, M. and Clarke, P.H. (1962) J. Gen. Microbiol. 27, 305—316
2 Kelly, M. and Kornberg, H.L. (1962) Biochim. Biophys. Acta 64, 190—191
3 Kelly, M. and Kornberg, H.L. (1964) Biochem. J. 93, 557—566
4 Clarke, P.H. (1970) Adv. Microbiol. Physiol. 4, 179—222
5 Brown, J.B., Brown, P.R. and Clarke, P.H. (1969) J. Gen. Microbiol. 57, 273—285
6 Brown, P.R. and Clarke, P.H. (1972) J. Gen. Microbiol. 70, 287—298
7 Betz, J.L. and Clarke, P.H. (1972) J. Gen. Microbiol. 73, 161—174
8 Brown, P.R., Smyth, M.J., Clarke, P.H. and Rosemeyer, M.A. (1973) Eur. J. Biochem. 34, 177—187
9 Findlater, J.D. and Orsi, B.A. (1973) FEBS Lett. 35, 109—111
10 Woods, M.J. and Orsi, B.A. (1974) Biochem. Soc. Trans. 2, 1344—1346
11 Fishbein, W.N., Daly, J. and Streeter, C.L. (1969) Anal. Biochem. 28, 13—24
12 Wisdom, G.B. and Orsi, B.A. (1969) Eur. J. Biochem. 7, 223—230
13 Fawcett, J.K. and Scott, J.E. (1960) J. Clin. Pathol. 13, 156—159
14 Fishbein, W.N., Winter, T.S. and Davidson, J.D. (1965) J. Biol. Chem. 240, 2402—2406
15 Findlater, J.D. and Orsi, B.A. (1974) Anal. Biochem. 58, 294—300
16 Cleland, W.W. (1967) Adv. Enzymol. 29, 1—32
17 Cleland, W.W. (1963) Nature 198, 463—465
18 Cleland, W.W. (1963) Biochim. Biophys. Acta 67, 104—137
19 Orsi, B.A., McFerran, N., Hill, A. and Bingham, A. (1972) Biochemistry 11, 3386—3392
20 Wallin, B.K. and Arion, W.J. (1973) J. Biol. Chem. 248, 2380—2386
21 Hsu, R.Y., Cleland, W.W. and Anderson, L. (1966) Biochemistry 5, 799—807
22 Woods, M.J., Edgeworth, M.A. and Orsi, B.A. (1975) Biochem. Soc. Trans. 3, 1216—1219
23 Morawetz, H. and Otaki, P.S. (1963) J. Am. Chem. Soc. 85, 463—468
24 Robinson, J.D. (1970) Biochim. Biophys. Acta 212, 509—511
25 Greenzaid, P. and Jencks, W.P. (1971) Biochemistry 10, 1210—1221
26 Epand, R.M. and Wilson, I.B. (1963) J. Biol. Chem. 238, 1718—1723
27 Frere, J.M. (1973) Biochem. J. 135, 469—481
28 Woods, M.J. (1976) Ph.D. Thesis, University of Dublin, Ireland