A COMPETITION TIME-COURSE METHOD FOR FOLLOWING ENZYMIC REACTIONS APPLIED TO THE HYDROLYSIS OF ACETAMIDE CATALYSED BY AN ALIPHATIC AMIDASE

Michael R. HOLLAWAY and Tamara TICHO
Department of Biochemistry, University College London, Gower Street, London, WC1 6BT, England

Received 20 July 1979

1. Introduction

The inducible amidase (acylamide amidohydrolase EC 3.5.1.4) from *Pseudomonas aeruginosa* catalyses a number of reactions, including the hydrolysis of short-chain amides such as acetamide and propionamide [1]; the acyl transfer from acetamide to hydroxylamine and, at a far slower rate, the hydrolysis of ethyl acetate and other short-chain esters [2]. At pH 7 these reactions can be represented by eq. (1)–(3):

$$CH_3CONH_2 + H_2O = CH_3COO^- + NH_4^+ \quad (1)$$

$$CH_3CONH_2 + NH_2OH = CH_3CO.NHOH + NH_4^+ + OH^- \quad (2)$$

$$CH_3CO.OEt + H_2O = CH_3COO^- + EtOH + H^+ \quad (3)$$

The amide transferase reaction (2) has been used routinely as a convenient single time-point assay of amidase activity [3] and preparations of the enzyme from this laboratory give spec. act. ~1800 μmol .min^{-1} .mg^{-1} in this assay, comparable to the highest reported value of 2000 μmol .min^{-1} .mg^{-1} [4].

There are conflicting reports as to the catalytic activity of amidase in the acetamide hydrolase reaction (1). A k_{cat} for the reaction of 975 s^{-1} at pH 7.2 and 25°C was given [5] whereas the value that can be calculated from the data in [6] for closely similar conditions is 192 s^{-1}.

In the course of experiments directed to the determination of the number of functional active sites, n, in the amidase molecule, which comprises 6 identical subunits [7], we required a value for the number of acetamide molecules hydrolysed per amidase molecule per second, (corresponding to k_{cat} in [5] but defined here as k^A_{mol}). Division of k^A_{mol} by the turnover number of a single active site would give a value for n (cf. [8,9]). It was important to determine an accurate value for k^A_{mol}, not only for the latter study but also to establish a specific acetamide hydrolase activity for homogeneous preparations of amidase.

The conflicting values for k^A_{mol} [5,6] had been obtained by using discontinuous sampling methods involving the measurement of the concentration of ammonia in aliquots removed from the assay mixture. In preference we sought a method whereby a continuous time-course of acetamide hydrolysis could be recorded. A pH-stat method is not applicable at pH 7.2 as reaction (1) does not give any significant proton release or uptake at this pH value, so an alternative 'competition time-course' method was devized.

This approach involves the measurement of the extent of suppression of the rate of change of a signal associated with the transformation of one substrate on addition of a second substrate for which there is no signal change. In the present case the competition time-course method exploits the fact that the hydrolysis of ethyl acetate at neutral pH values gives a release of protons and so can be followed by using a pH-stat. Addition of ethyl acetate to an ethyl acetate assay medium caused a decrease in the rate of proton release as the enzyme switched over to the hydrolysis of the competing substrate (which gives no proton

release). The proton release returned to the original rate on exhaustion of the acetamide.

These observations indicate that the two substrates are probably hydrolysed at the same site but this communication is concerned mainly with how the steady-state kinetic parameters for the amidase-catalysed hydrolysis of acetamide can be determined from such competition time-courses and the precautions that must be taken in applying the method to this and other systems. The method is applicable in principle to many amide or peptide hydrolase reactions as well as other enzymic reactions.

2. Materials and methods

Preparations of amidase were carried out from 300 g batches of *Pseudomonas aeruginosa* PAC 142 [10], grown as in [3], by the procedure in [11]. The preparation employed for the present study gave spec. act. 1800 μmol .min^{-1} .mg^{-1} in the standard transferase assay [3]. The A_{280} of a 1 mg/ml solution of the enzyme was taken as 1.26 ± 0.02 (from dry wt measurements) and molarities of enzyme solutions were calculated on the basis of mol. wt 240 000 [12].

Acetamide was purified by crystallization from ethanol and ethyl acetate by fractional distillation and collection of liquid boiling at 76 ± 1°C.

The hydrolysis of ethyl acetate was followed by a pH-stat method employing a Metrohm (Herisau) Combititrator equipped with a 0.2 ml delivery syringe. The concentration of neutralizing NaOH was 0.03 M. Reaction mixtures (5 ml), contained 1 mM EDTA, 0.1 M NaCl and 0.05 M ethyl acetate, adjusted to pH 7.2 at 25°C and time-courses were initiated by addition of a small volume of a solution of amidase to give 0.0167 μM final conc. Competition time-courses were carried out by adding a small volume of a solution of acetamide to the pH-stat vessel while the ethyl acetate assay was in progress. Pen deflections on the chart recorder of the pH-stat were calibrated in units of molarity of protons released in the assay by addition of aliquots of standardized HCl to reaction mixtures.

3. Results and discussion

A typical competition time-course of acetamide hydrolysis catalysed amidase is shown in fig.1. A pH-stat assay with ethyl acetate as substrate was initiated, and after a linear time-course had been recorded a small volume of a solution of acetamide added to give 20 mM final conc. It can be seen that the evolution of protons from hydrolysis of the ethyl acetate ceased immediately and there was no further uptake of alkali for ~4 min. After this time the proton release resumed and the rate increased to reach eventually that obtained before addition of the acetamide. At this stage the process could be repeated by addition of a second aliquot of the acetamide solution, which gave rise to another time-course indistinguishable from the first.

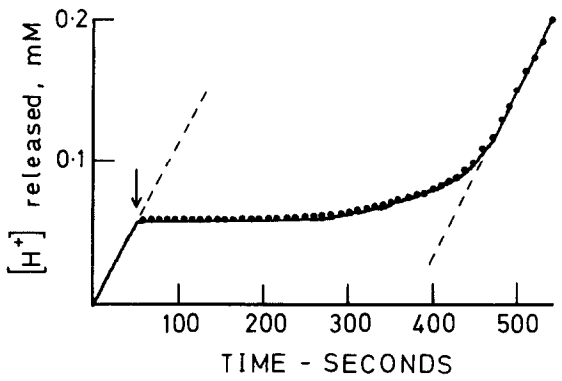

Fig.1. A competition time-course of the amidase-catalysed hydrolysis of acetamide with ethyl acetate as the indicator substrate. At zero time the pH-stat assay with 0.05 M ethyl acetate as substrate was initiated by addition of amidase to 0.0167 μM final conc. At the time indicated by the arrow, 0.1 ml of a 1 M solution of acetamide was added to give 20 mM final conc. The assay medium comprised 5 ml of a solution containing 1 mM EDTA, 0.1 M NaCl and the reaction components, at 25°C. The solid line is the pH-stat trace and the solid circles give the computed competition time-course for k^{A}_{mol} = 245 s^{-1} and K^{A}_{m} = 0.41 mM.

That the rate of the re-established reaction was the same as the original rate and the time-courses were reproducible with the same assay mixture shows first, that the enzyme does not lose activity during the time-course and second, that the products do not inhibit the reactions. Complications arising from these effects are therefore neglected in the following treatment.

As the concentration of ethyl acetate employed was <10% of the K_m value for this substrate (i.e., 0.05 M and 0.5 M, respectively [2]) the rate of hydrolysis of ethyl acetate, v, will be proportional to the concentration of free enzyme and eq. (4) will hold as a good approximation:

$$v = dB/dt \approx \frac{k^S_{mol}}{K^S_m} \cdot [S]_o.[E] \approx k_p.[E] \quad (4)$$

where k^S_{mol} is the number of ethyl acetate molecules hydrolysed per molecule of enzyme per second. The free ethyl acetate concentration can be written as constant at $[S]_o$ as the initial concentration in the time-course of fig.1 was 50 mM and only 0.2 mM of this was consumed by the completion of the observation. Thus the rate of proton release will be proportional to the concentration of enzyme not in the form of intermediates along the reaction pathway for acetamide hydrolysis, with the constant of proportionality:

$$k_P = k^S_{mol}.[S]_o/K^S_m$$

The hydrolysis of acetamide will make no contribution to the proton release under the conditions of the time-course of fig.1.

If it is assumed that the amidase-catalysed hydrolysis of acetamide follows Michaelis-Menten kinetics throughout the time-course, there being no product inhibition, then the expression for the concentration of acetamide $[A]_t$ remaining at any time, t, will be given by eq. (5):

$$([A]_o - [A]_t) + K^A_m.\ln([A]_o/[A]_t) = k^A_{mol}.[E]_o.t \quad (5)$$

where $[A]_o$ and $[E]_o$ are the initial, total concentrations of acetamide and enzyme, respectively, k^A_{mol} is the molecular activity of amidase with acetamide as substrate (i.e., the catalytic centre activity multiplied by the number of functional active sites per molecule) and K^A_m is the K_m for the acetamide reaction.

As $K^S_m >> [S]_o$ there will be no significant loss of the enzyme by binding of ethyl acetate, so the concentration of free enzyme at any time during the reaction is given by eq. (6) as a good approximation:

$$[E] = [E]_o/(1 + [A]_t/K^A_m) \quad (6)$$

so, from eq. (4), the observed rate of hydrolysis of ethyl acetate will be given by eq. (7):

$$dB/dt = v = k_P.[E]_o/1 + [A]_t/K^A_m \quad (7)$$

Equations (5) and (7) can be used in conjunction to determine values for k^A_{mol} and K^A_m from the competition time-course, given values for $[E]_o$, $[A]_o$ and k_P. We employed the computer program FACSIMILE [13] to compute best-fit values of these parameters by the following procedure. The program was given values for the concentration of alkali added at different times (B, corrected by calibration as in section 2) and values for $[E]_o$, $[A]_o$ and k_P. (The latter was obtained from the value of v in the absence of acetamide.) Least-squares, best-fit values of k^A_{mol}, and K^A_m were then computed by a cyclic, iterative routine involving numerical integration of eq. (7) and substitution of the $[A]_t$ value so obtained in eq. (5).

The values obtained from the time-course of fig.1 were: $k_{mol} = 245 \pm 2\ s^{-1}$ and $K^A_m = 0.41 \pm 0.05$ mM and these are the values used to calculate the theoretical curve of fig.1. The high precision of the k^A_{mol} value arises from the consideration that the duration of the flat part of the competition time-course gives information mainly about the k_{mol} value, whereas the curvature as the rate approaches that in the absence of substrate is determined essentially by the value of K_m. The greater the curvature, the less the value of K_m. In the case of the time-course of fig.1 the time of zero rate is relatively long and the curvature is rather sharp.

Other experiments were carried with different concentrations of substrate. For example, the k^A_{mol} values obtained with 4 mM, 8 mM and 20 mM acetamide were $195 \pm 5\ s^{-1}$, $204 \pm 2\ s^{-1}$ and $245 \pm 2\ s^{-1}$, respectively; i.e., the value of k^A_{mol} is $215 \pm 20\ s^{-1}$. The small, apparently systematic variation in the values at different concentration of substrate is not significant as the response time of the pH-stat renders the values at low substrate concentration less accurate, although the computer program gives the same, high precision to the parameter. A number of other time-courses gave similar results, which were independent of the concentration of enzyme.

It is not clear why the k^A_{mol} value of $215 \pm 20\ s^{-1}$ obtained in the present study should differ so widely from the value of $975\ s^{-1}$ reported [5]. The enzyme preparation used here had spec. act. 1800 units/mg in the hydroxamate transfer assay, close to the value of 2000 units/mg quoted for a preparation of amidase shown to be homogeneous by a number of criteria [4,7]. The difference cannot be accounted for by a

difference in the two strains of bacterium employed as the structural genes in PAC 111 and PAC 142 are identical (Professor P. H. Clarke, personal communication). On the other hand the value of k^{A}_{mol} = $215 \pm 20\ s^{-1}$ is in good agreement with the value of the same parameter that can be calculated from the data in [6] obtained at the same temperature and pH value.

The competition time-course of fig.1 provides evidence for acetamide and ethyl acetate being hydrolysed at the same site in the amidase molecule. However it does not constitute a final proof as it could be argued that there were separate sites for the two substrates and occupation of one site precluded catalysis at the other. In this respect it is noteworthy that a common use of competition between substrates has been to establish that the substrates bind to the same site [14], although measurement of an initial velocity of an equimolar mixture of two substrates has been used to determine the value of K_m for one substrate when that of the other is known [15].

The competition time-course approach provides a method for following enzymic reactions for which there is no suitable signal change on product formation. It does require that there is some other substrate that gives a signal change and is transformed at the same site, although a relatively poor and not necessarily specific 'indicator' substrate would be adequate. If such a substrate is available the method provides an alternative to the use of coupled enzyme assays. For example, it would be applicable to the study of peptide hydroytic reactions at neutral pH values using a *p*-nitroanilide as a spectrophotometric reporter or an ester substrate as a proton-releasing reporter.

In the present case, the competing, indicator substrate (ethyl acetate), was at a concentration much less than its K_m value. This allows the simplifying assumption to be made that the indicator reaction did not perturb significantly the reaction under study. However it would be possible, by using an analogous procedure to that described here, to elucidate kinetic parameters for a system of substrates where this assumption did not hold.

Acknowledgements

The authors thank the SRC for financial support. Batches of *Pseudomonas aeruginosa* were kindly given by Professor P. H. Clarke.

References

[1] Kelly, M. and Clarke, P. H. (1962) J. Gen. Microbiol. 27, 305–316.
[2] McFarlane, N. D., Brammar, W. J. and Clarke, P. H. (1965) Biochem. J. 95, 24c–25c.
[3] Brammar, W. J. and Clarke, P. H. (1964) J. Gen. Microbiol. 37, 307–309.
[4] Lilly, M. D., Clarke, P. H., Houldsworth, M., Currie, J. A. and Dunnill, P. (1969) Biotechnol. Eng. 11, 283–292.
[5] Findlater, J. D. and Orsi, B. A. (1973) FEBS Lett. 35, 109–111.
[6] Brown, P. R., Gregoriou, M. and Tata, R. (1978) Mol. Gen. Genet. 165, 213–219.
[7] Brown, P. R., Smyth, M. J., Clarke, P. H. and Rosemeyer, M. A. (1973) Eur. J. Biochem. 34, 177–187.
[8] Hollaway, M. R., White, H. A., Makin, R. A., Joblin, K. N., Johnson, A. W., Lappert, M. F. and Wallis, O. C. (1978) Hoppe-Seyler's Z. Pysiol. Chem. 359, 1043.
[9] Gutfreund, H. (1975) in: Enzymes: Physical Principles, p. 201, Wiley, New York.
[10] Brown, P. R. and Clarke, P. H. (1972) J. Gen. Microbiol. 70, 287–298.
[11] Brown, J. E., Brown, P. R. and Clarke, P. H. (1969) J. Gen. Microbiol. 57, 275–285.
[12] Gregoriou, M. and Brown, P. R. (1979) Eur. J. Biochem. 96, 101–108.
[13] Chance, E. M., Curtis, A. R., Jones, I. P. and Kirby, C. R. (1977) FACSIMILE: a computer program for flow and chemistry simulation and general initial value problems. Comput. Sci. Systems Div. AERE, R.8775, Harwell, Oxon.
[14] Webb, E. C. and Morrow, P. F. W. (1959) Biochem. J. 73, 7–15.
[15] Adams, D. H. and Whittaker, V. P. (1950) Biochim. Biophys. Acta 4, 543–558.