J. Enzyme Inhibition, 2001, Vol. 16, pp. 89–94
Reprints available directly from the publisher
Photocopying permitted by license only

Slow Detection Reaction can Mimic Initial Inhibition of an Enzymic Reaction

JURE STOJAN*

Institute of Biochemistry, Medical faculty, University of Ljubljana, Vrazov trg 2, 1000 Ljubljana, Slovenia

(Received 15 January 2000)

An analysis of sigmoid-shaped progress curves in the reaction between *Electric Eel* acetylcholinesterase (acetylcholine acetylhydrolase, EC 3.1.1.7, AChE) and its substrate acetylthiocholine in low concentrations at pH 7 is presented. In order to be able to explain an initial apparent inhibition of the enzyme-substrate reaction, the rate of detection reaction had to be taken into account. The theoretical curves obtained by the fitting of differential equations for the reaction mechanism to the data of six progress curves simultaneously, exactly reproduce the course of the experimental curves. The measurements performed with various concentrations of detection reagent confirm the proposed cause of sigmoidity.

Keywords: Acetylcholinesterase, Kinetics, Progress curves, Numerical integration, Analytical solution

INTRODUCTION

In kinetic investigations of enzyme reaction mechanisms the time course of product formation or substrate turnover is usually followed photometrically after starting the reaction by mixing the enzyme with the substrate. The analysis of such progress curves may be difficult, especially if they are obtained under non-steady-state conditions.[1] Because the underlying system of differential equations in such cases cannot be solved analytically[2] a numerical method was proposed recently.[3] It is based on a semi-implicit mid-point rule extrapolation algorithm[4] implemented in a non-linear least square fitting program which is able to fit equations to data containing several independent variables.[5] The hydrolysis of acetylthiocholine by AChE in the presence of dithionitrobenzoic acid (DTNB, Ellman's reagent) at pH 8 was evaluated as a model reaction, and in that analysis it was correctly assumed, that the detection reaction is much faster than the formation of the product. However, if the reaction is monitored at pH 7 the rates of product formation and the detection reaction become comparable.[6] In this paper it is shown that experimentally obtained sigmoid-shaped progress curves are a consequence of these two reactions and an analysis is proposed for their kinetic separation.

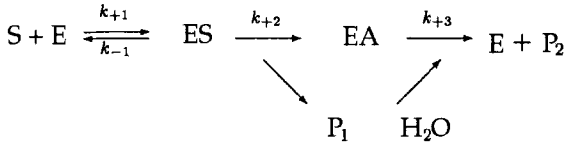
*Tel.: +386-1-5437649. Fax: +386-61-1320016. E-mail: stojan@ibmi.mf.uni-lj.si.

METHODS AND MATERIALS

Measurements at Various Enzyme Concentration

The hydrolysis of acetylthiocholine catalysed by AChE was recorded on a stopped flow apparatus. The experimental curves were obtained by following the product formation photometrically[7] after mixing together in the mixing chamber of the apparatus aliquots of two buffer solutions, one containing the enzyme and the other the substrate and the reagent. The absorbance was recorded until the rate of increase became constant. The time course of the product formation was followed at six different enzyme concentrations. The initial concentrations of the added substrate and reagent were always the same, 50 μM and 0.66 mM, respectively.

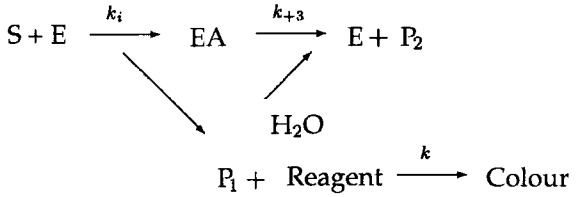
The kinetic model of AChE reaction at low substrate concentrations is shown in Scheme 1:[8]



SCHEME 1

where E is AChE, S is the substrate acetylthiocholine with an acetyl group A (P_2) and the leaving group thiocholine (P_1). ES is a reversible addition complex and the elementary rate constants are k_{+1}, k_{-1}, k_{+2} and k_{+3}.

It is well known that this enzyme converts the substrate with very high turnover ($k_{cat} = 8.5 \times 10^5$ min^{-1}).[8] Therefore, Antosiewicz *et al.*[9] suggested that, at low substrate concentrations, every substrate molecule that enters the active gorge is hydrolysed and, consequently, ES does not accumulate. In the presence of DTNB (Ellman's reagent), which upon reaction with thiocholine (P_1) gives a coloured product, Scheme 1 changes into Scheme 2.[3]



SCHEME 2

The reaction which proceeds in the surplus of the reagent can be followed on a stopped-flow apparatus from the very early stages until its completion. It was shown that the rate of detection reaction in Scheme 2 is pH dependent.[6] So, to evaluate the enzyme reaction at pH 7 the differential equations describing Scheme 2 are as follows:

$$\frac{d(E)}{dt} = -k_i \cdot (E) \cdot (S) + k_3 \cdot (EA) \quad (1)$$

$$\frac{d(S)}{dt} = -k_i \cdot (E) \cdot (S) \quad (2)$$

$$\frac{d(EA)}{dt} = k_i \cdot (E) \cdot (S) - k_3 \cdot (EA) \quad (3)$$

$$\frac{d(P_1)}{dt} = k_i \cdot (E) \cdot (S) - k \cdot (P_1) \cdot (R) \quad (4)$$

$$\frac{d(R)}{dt} = -k \cdot (P_1) \cdot (R) \quad (5)$$

$$\frac{d(Colour)}{dt} = k \cdot (P_1) \cdot (R) \quad (6)$$

$$\frac{d(P_2)}{dt} = k_3 \cdot (EA) \quad (7)$$

For the analysis of the experimental data there are two possibilities. (i) A direct evaluation of kinetic parameters from these equations is possible by fitting a numerically solved system simultaneously to all progress curves, using an appropriate computer program.[5] It should be stressed that the measured quantity is represented by Equation (6) and that the rate of colour production is not directly proportional to the

product formation Equation (4). (ii) An analytical solution of the system can be obtained by introducing the assumption that during the reaction substrate concentration remains constant. Of course, in our case this assumption is not realistic but we can omit late portions of progress curves in the analysis. To reach the analytical solution we can also simplify the derivation by assuming realistically, that presteady-state in the enzyme reaction is too fast to be detected with the stopped-flow apparatus. So, the explicit equation describing the time course of yellow colour production is:

$$(\text{Colour})_t = \frac{k_{+3}(\text{E})_0(\text{S})_0}{(\text{S})_0 + \frac{k_{+3}}{k_i}}\left[t - \frac{1 - e^{-(\text{R})_0 kt}}{k(\text{R})_0}\right] \quad (8)$$

where E_0, S_0 and R_0 are initial AChE, acetylthiocholine and reagent concentrations, respectively.

Measurements at Various Reagent Concentration

In order to prove, that the initial curvature of the progress curves is a consequence of a slow detection reaction we followed the hydrolysis of acetylthiocholine catalysed by very high AChE concentration (26 nM) at various concentrations of Ellman's reagent (55 μM, 0.33 mM, 0.66 mM and 1.33 mM).

Experiments were done at 25 °C in 10 mM phosphate buffer solution with a total ionic strength of 0.2 M, obtained by addition of NaCl (pH 7.0). The enzyme was *Electric Eel* AChE, purchased from Sigma Chemicals Ltd. (lot 128F8040, 1300 units/mg protein); the concentration of the enzyme active sites was estimated by pseudo-irreversible titration, according to the procedure described previously.[10] Acetylthiocholine iodide and 5,5′-dithio-bisnitrobenzoic acid (Ellman's reagent) were from BDH Biochemicals. All substances were reagent grade. The measurements were carried out on a stopped-flow apparatus PQ-SF 53 with theoretical dead time of 0.7 ms, manufactured by Hi-Tech Ltd., Salisbury, UK.

RESULTS AND DISCUSSION

The time course of product formation in the hydrolysis of acetylthiocholine by AChE in the presence of 0.66 mM Ellman's reagent is shown in Figure 1. In the inset it can be seen that in each individual curve a plateau is reached and that the level of the plateau corresponds to the initial concentration of the added substrate (50 μM). This proves that a complete conversion of the substrate takes place and that in the range at the plateau the concentrations of the enzyme and the substrate are comparable. Besides, it is obvious that the initial portions of the curves are concave upwards. There are several possible explanations for such sigmoid-shaped progress curves [cf. 1]. One explanation is a slow establishment of steady state in the enzyme reaction. However, the entrance of the substrate into the active gorge of the enzyme is known to be almost as fast as diffusion.[9] In this case the progress curves obtained by a stopped-flow apparatus with dead time around 1 ms would show an initial step [cf. 1] and the levels of the plateaus would appear lower than would be expected from the added substrate concentrations. Since this is not the case the most probable reason for initial sigmoidity is a slow rate of detection reaction.[6]

If the rate of product formation is faster than the rate of colour formation the sigmoid part of each progress curve must be a consequence of the transition state in the coupled reactions. The values of the rate constants in the enzyme reaction and the rate constant for colour formation obtained by the fitting of Equations (1–7) to all six progress curves simultaneously confirmed this hypothesis (Table I). Moreover, the rate of detection reaction, which runs under pseudo-first order conditions (the concentration of the reagent is at least 12 times higher than the concentration of the substrate) must depend on the concentration of the detection reagent. So, if the concentration of Ellman's reagent is increased one would expect less and less curvature at the

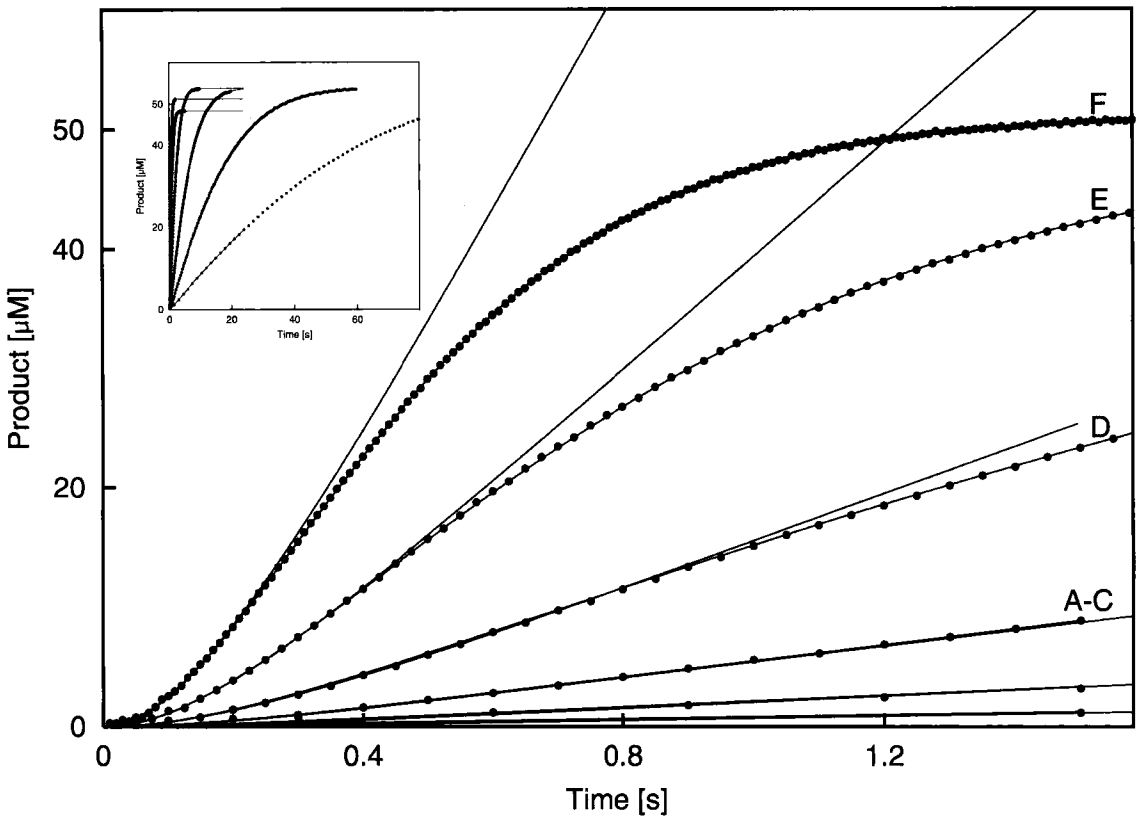


FIGURE 1 Time course of yellow colour formation in the reaction between acetylthiocholine and acetylcholinesterase in the presence of 0.66 mM Ellman's reagent. Final substrate concentrations were 50 μM. For enzyme concentrations see Table I. Theoretical curves matching experimental data were obtained by simultaneous fitting of differential Equations (1–7) to complete progress curves and those which deviate by fitting Equation (8) to the first fifteen points of each progress curve. Inset shows the same curves with a larger time scale.

TABLE I Rate constants and initial concentrations of the enzyme active sites and the substrate obtained by fitting Equations (1–7) to all experimental data in Figure 1

k_i [lmols^{-1}]	k_3 [s^{-1}]	k [lmols^{-1}]
$1.85 \pm 0.05 \times 10^8$	10206 ± 286	7002 ± 46
Added enzyme conc. [nM]	Calculated enzyme conc. [nM]	Calculated substrate conc. [μM]*
0.17	0.17 ± 0.04	57.3 ± 2.5
0.51	0.52 ± 0.15	54.2 ± 0.2
1.4	1.42 ± 0.04	53.9 ± 0.1
4.3	4.23 ± 0.12	$53.7 \pm < 0.1$
13	12.58 ± 0.08	$48.2 \pm < 0.1$
26	27.94 ± 0.08	$51.2 \pm < 0.1$

*Added substrate concentration was always 50 μM.

beginning of the curves. Figure 2 shows the progress curves obtained with the same enzyme and substrate concentrations, 26 nM and 50 μM, respectively, but with four different concentrations of the reagent (55 μM, 0.33 mM, 0.66 mM and 1.33 mM). It is obvious from the Figure that

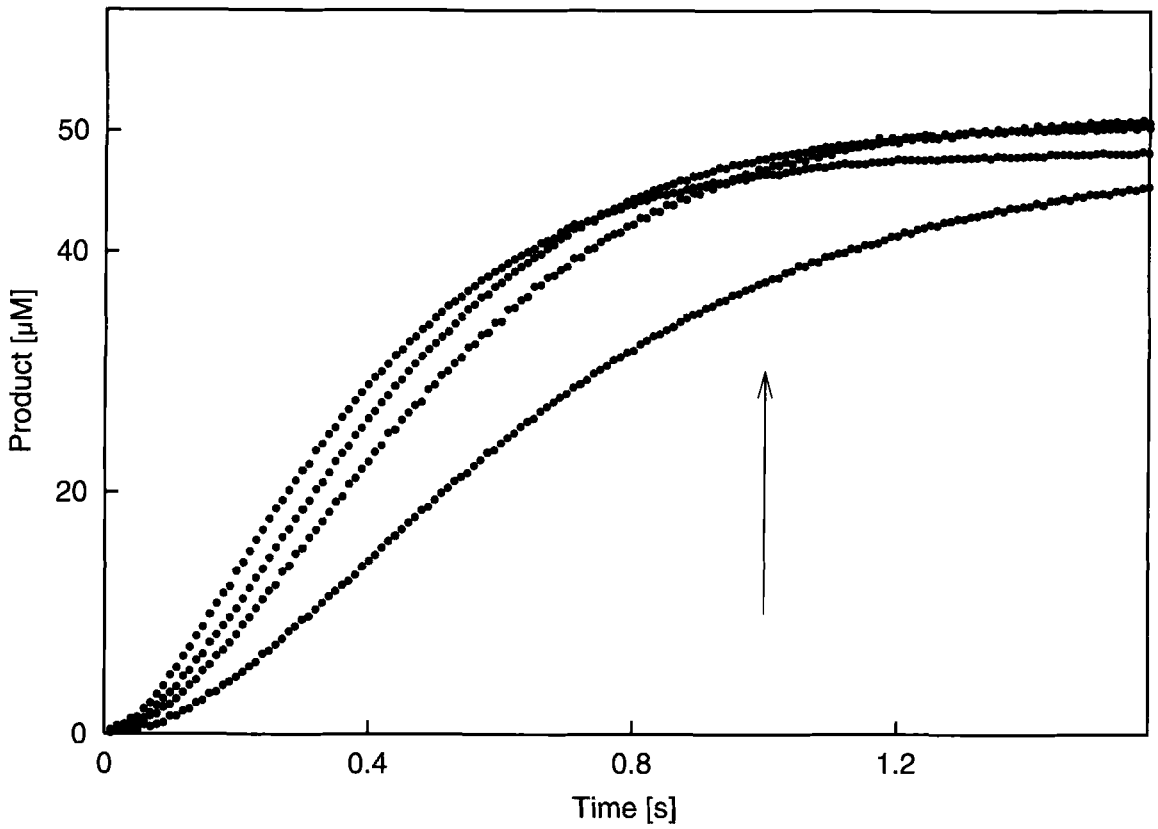


FIGURE 2 Time course of yellow colour formation in the reaction between acetylthiocholine and 26 nM acetylcholinesterase in the presence of various concentrations of Ellman's reagent (55 μM, 0.33 mM, 0.66 mM and 1.33 mM). Final substrate concentrations were always 50 μM. Arrow indicates increasing reagent concentrations.

with the increasing concentrations of Ellman's reagent the reaction goes faster. Unfortunately the concentration of the reagent cannot be further increased due to its limited solubility at pH 7, so, the complete disappearance of the sigmoidity at this very high enzyme concentration is not seen.

With our experiments we have shown that under certain conditions the slow rate of the detection reaction can distort the data of an enzyme reaction. Since the shapes of the curves representing initial product formation of enzymic reactions are well described in kinetic textbooks[1] such data might be misinterpreted as presteady state in the enzyme reaction. Similar deviations of the shapes from exponentiality are also expected when investigating the time course of the action of various irreversible or slow binding inhibitors. The analysis in such cases might be very complex *per se* and additional complication in the presence of the reagent can make it insoluble. We suggest two approaches in the analysis but it depends on the nature of the investigated reaction and technical possibilities which assumptions might be introduced in each particular case. It should also be recalled from Figure 1 that, the complication with the sigmoidity can sometimes be avoided by measuring the reaction with low enzyme concentrations and for longer time periods but under such conditions we may lose essential information pointing to the reaction mechanism.

On the other hand, it is possible to describe initial curvature of the curves analytically, but only under certain assumptions. In our case such assumptions are not realistic. It could be argued that by taking higher substrate concentrations its depletion during the reaction becomes negligible; this is true, but in cholinesterases for instance, negative cooperativity at higher substrate concentrations is the next complication.[1] Finally, it seems important to emphasize that complete conversion of the substrate in the reaction provides data at continuous substrate concentrations. Therefore, by evaluating even a small number of progress curves (six in our case) very reliable results are obtained.[11]

Finally it should be stressed that a number of powerful computer packages are commercially available that can simulate the course of such reactions by solving numerically large sets of differential equations. The presented kinetic analysis, however, requires evaluation of relevant reaction parameters and is not trivial: the computer program must be able to fit numerically solved system of stiff differential equations simultaneously to the data obtained for various independent variables. This is especially desirable when analysing time-dependent studies on the action of one or a combination of inhibitors.

Acknowledgments

The work was supported by the Ministry of Science and Technology of the Republic of Slovenia.

References

[1] K.J. Laidler and P.S. Bunting (1973) In *Enzyme Kinetics*, pp. 74, 187. Clarendon Press: Oxford.
[2] D. Brown and P. Rothery (1994) In *Models in Biology: Mathematics, Statistics and Computing*, pp. 331, 344. J. Wiley: New York.
[3] J. Stojan (1997) Analysis of progress curves in an acetylcholinesterase reaction: A numerical integration treatment. *J. Chem. Infor. Comp. Sci.*, **37**, 1025–1027.
[4] G. Bader and P.A. Deuflhard (1983) Semi-Implicit Mid-Point Rule for Stiff Systems of Ordinary Differential Equations. *Numer. Math.*, **41**, 373–398.
[5] R.G. Duggleby (1984) Regression Analysis of Nonlinear Arrhenius Plots: an Empirical Model and a Computer Program. *Comput. Biol. Med.*, **14** (4), 447–455.
[6] J. Stojan and M.R. Pavlič (1992) On the Velocity of Ellman's Reaction. *Neurochem. Res.*, **17**, 1207–1210.
[7] G.L. Ellman *et al.* (1961) A New and Rapid Colorimetric Determination of Acetylcholinesterase Activity. *Biochem. Pharmacol.*, **7**, 88–95.
[8] I.B. Wilson and M.A. Harrison (1961) Turnover number of acetylcholinesterase. *J. Biol. Chem.*, **236**, 2292–2295.
[9] J. Antosiewicz *et al.* (1995) Acetylcholinesterase: Diffusional Encounter Rate Constants for Dumbbell Models of Ligand. *Biophys. J.*, **68**, 62–68.
[10] J. Stojan and M. Zorko (1997) Kinetic Characterization of all Steps of the Interaction between Acetylcholinesterase and Eserine. *Biochim. Biophys. Acta.*, **1337**, 75–84.
[11] M. Markus and T. Pleser (1981) In: *Kinetic Data Analysis*, (Endrenyi, L. Ed.), pp. 317–339. Plenum Publishing Corporation. New York.