Analysis of Progress Curves in an Acetylcholinesterase Reaction: A Numerical Integration Treatment

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During the course of enzyme reactions in which a complete conversion of the reactants into the products occurs, the assumptions for the derivation of steady-state rate equations become violated. Therefore, the integrated form of these equations is not appropriate for the analysis of progress curves which describe such reactions. Since the solution of the system of ordinary differential equations without steady-state assumptions is impossible, the analysis is proposed which is based on a numerical integration method. The parameters of numerically solved differential rate equations for the reaction mechanism of acetylcholinesterase (acetylcholine acetylhydrolase, EC 3.1.1.7, AChE) at low substrate concentrations are fitted to the experimental data by a nonlinear regression computer program. For the numerical solutions of stiff systems of ordinary differential equations a semi-implicit midpoint rule extrapolation method is used.

INTRODUCTION

Usually an enzyme reaction is monitored by measuring the amount of substrate remaining or product formed at several intervals after starting the reaction by mixing the enzyme with the substrate. Most kinetic models are formulated in terms of rate equations obtained only by the analysis of initial rates determined from the tangents through their origins although the complete progress curves provide considerably more information.[1] There are several traditional and practical reasons for such analysis,[2] but it is also true that even the simplest rate equation cannot be integrated to express the dependent variable as an explicit function of all independent variables.[3,4] On the other hand, the first implicit form of an integrated Michaelis−Menten equation was given by Victor Henry[5] in 1903. A practical method for solving implicit equations, the Newton−Raphson procedure described by Nimmo and Atkins,[6] is now used as a general approach in the analysis of progress curves in enzyme kinetics.[2,7]

There is, however, a fundamental objection to such an analysis. The underlying rate equations are derived under a steady-state assumption, which can only be satisfied if the substrate−enzyme ratio is 1000 or greater.[4] This can almost always be achieved in initial rate experiments by making the concentration of the substrate sufficiently large. But, if the reaction is followed over an extended period of time, the steady-state conditions can easily be violated, especially if the enzyme reaction proceeds with a high turnover. Additionally, in biological experiments the conditions are usually such that the reaction goes far to the right, resulting in almost complete conversion of the reactants into the products.

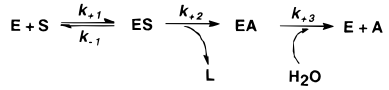
New preparative and rapid kinetic techniques make it possible to obtain great amounts of pure enzymes which can be used to observe enzyme reactions from early stages until their completion. The catalytic hydrolysis of acetylcholine by AChE is an extremely fast reaction.[8,9] If relatively high concentrations of the enzyme and low concentrations of the substrate are used, it can be followed on a stopped-flow apparatus, in the presence of an appropriate reagent, until its completion. In such experiments, however, the concentrations of the enzyme and the substrate become comparable at a certain point during the course of the reaction. Thus, the kinetic analysis of progress curves obtained under such conditions cannot be based on the steady-state assumptions. Without these assumptions, however, the exact derivation of progress curve equations is impossible.[10]

In the present paper the differential rate equations for the reaction mechanism of AChE at low substrate concentrations are numerically integrated and the parameters fitted to the experimental data by a nonlinear regression computer program which was originally written by R. G. Duggleby.[11] Since this program was developed for the analysis of kinetic models with explicit solutions, in this paper we introduce a modification in which a semi-implicit midpoint rule extrapolation method,[12] designed for the numerical solutions of stiff systems of ordinary differential equations, is used.

METHODS AND MATERIALS

Theoretical. The reaction mechanism of many hydrolytic enzymes involves two stages: the formation of an addition complex followed by the formation of a covalent intermediate in which a hydroxyl group on the enzyme becomes acylated. In the case of an AChE reaction, the enzyme hydroxyl group of serine[200] which is buried at the bottom of a 20 Å deep gorge[13] is acetylated and the choline part of the substrate is released. The reaction mechanism of the hydrolysis of the substrate by AChE in the presence of the substrate in concentrations low enough to avoid the inhibition can be represented by Scheme 1.[8]

Scheme 1



In this scheme E is AChE, S is the substrate acetylcholine with an acetyl group A, and the leaving group choline L.

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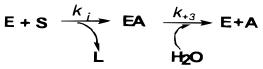
ES is a reversible addition complex, and the elementary rate constants are k_{+1}, k_{-1}, k_{+2}, and k_{+3}.

AChE has a remarkably high catalytic power ($k_{cat} = 8.3 \times 10^5 min^{-1}$).[8] Therefore, it was suggested that, at low substrate concentrations, every substrate molecule that enters the gorge is converted into its hydrolytic products,[14] and, consequently, ES does not accumulate. So, Scheme 1 may be reduced to Scheme 2.

Scheme 2



Usually, the formation of product L is followed photometrically according to Ellman et al.[15] with acetylthiocholine as the substrate. In this case, the leaving group thiocholine yields a yellow product upon reaction with 5,5′-dithiobis-dinitrobenzoic acid (Ellman's reagent). Under appropriate conditions the rate of Ellman's reaction is much faster than the rate of thiocholine release, which can thus be assumed to be proportional to the yellow color production.[16] The differential equations for this scheme are

$$\frac{d[E]}{dt} = -k_i[E][S] + k_{+3}[EA] \quad (1)$$

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

$$\frac{d[EA]}{dt} = -k_i[E][S] - k_{+3}[EA] \quad (3)$$

$$\frac{d[L]}{dt} = k_i[E][S] \quad (4)$$

$$\frac{d[A]}{dt} = k_{+3}[EA] \quad (5)$$

Although Scheme 2 represents the simplest one-intermediate enzyme reaction mechanism, no exact solution of the underlying nonlinear differential equations can be given[10] (see the product "[E][S]" on the right hand sides of the eqs 1−4). The equations, however, can be solved numerically. Still, our attempt to apply a Runge−Kutta algorithm[17] to this case remained unsuccessful. Very low concentrations of the enzyme and the substrate, on the one hand, and very high values of the reaction rate constants, on the other hand, make the system extremely stiff.[10,12] However, a semi-implicit midpoint rule extrapolation method[12] makes it possible to integrate numerically eqs 1−5. The method is based on a type of discretization which permits a quadratic asymptotic expansion in terms of the stepsize, thus backing the expectations that it is useful for solving stiff problems. This integration alone, however, does not solve the whole problem. It remains to evaluate the kinetic parameters k_i and k_{+3} in eqs 1−5. We carried this out by the implementation of the semi-implicit midpoint rule extrapolation method[12] in a nonlinear regression computer program which is able to fit simultaneously theoretical curves to the experimental data containing several independent variables.[11] In addition to the two rate constants (k_i, k_{+3}), there were also the initial concentrations of the enzyme active sites and the initial substrate concentrations which were set as fitting parameters. The initial concentrations of the added substrate were set as the first estimates in the fitting, while the initial concentrations of the enzyme active sites were estimated by the so-called pseudoirreversible titration, according to the procedure described previously.[18] The other boundary conditions at $t = 0$ were always set to (EA) = 0, (L) = 0, (A) = 0.

Experimental. The hydrolysis of acetylthiocholine catalyzed by AChE was recorded spectrophotometrically[15] on a stopped-flow apparatus. Aliquots of two buffer solutions, one containing the enzyme and the other the substrate and the reagent, were mixed together in the mixing chamber of the apparatus. The absorbance of the reaction mixture was recorded until the rate of increase in absorbance became constant. The time course of the product formation was followed at six different enzyme concentrations (see Table 1). The initial concentrations of the added substrate were 10 μM and, in the experiment with the highest enzyme concentration, 11.5 μM.

Experiments were done at 25 °C in a buffer solution prepared according to Britten and Robinson[19] with a pH of 8.0 and a total ionic strength of 0.2 M, obtained by the addition of NaCl. The enzyme used was Electric Eel AChE, purchased from Sigma Chemical LTD (lot 128F8040, 1580 units/mg protein). Acetylthiocholine iodide and 5,5′-dithiobisnitrobenzoic acid (Ellman's reagent) were from BDH Biochemicals. All substances were of reagent grade. The measurements were performed on a stopped-flow apparatus PQ-SF 53 with a theoretical dead time of 0.7 ms, manufactured by High-Tech Ltd., Salisbury, UK.

RESULTS AND DISCUSSION

The experimental data are presented in Figure 1. A plateau is reached in each individual progress curve, and the level of the plateau in each measurement corresponds to the initial concentration of the substrate used. Additionally, the level also exactly corresponds to the level of the plateau obtained in a control experiment (not shown, but see ref 16) in which thiocholine in the same concentration reacted with Ellman's reagent. It can therefore be concluded that complete hydrolysis of acetylthiocholine takes place in the presence of AChE. It should be recalled from the previous publication[16] that under specified experimental conditions, the reaction between thiocholine and Ellman's reagent is completed in approximately 200 ms. Thus, Ellman's reaction does not need to be included in the analysis of data obtained in much longer time intervals.

The kinetic parameters of ordinary nonlinear differential equations (eqs 1−5) were fitted simultaneously[20] to all data from six progress curves (200 points per curve) by means of a nonlinear regression program using the semi-implicit midpoint rule extrapolation method.[12] The values of these parameters, the two rate constants (k_i, k_{+3}) together with the initial substrate, and the enzyme active sites concentrations are given in Table 1.

It can be seen from Table 1 that the estimated concentrations of enzyme active sites obtained by the pseudoirreversible titration practically do not differ from the fitted values. It is obvious from Figure 1 that the fitted curves agree very well with the experimental data. The correctness of the determined kinetic parameters is also supported by the very good agreement between the determined deacylation rate constant k_{+3} with the value found in literature (19 000 s^{-1}).[8,9]

Table 1. Rate Constants and Initial Concentrations of the Substrate and the Enzyme Active Sites Obtained by Simultaneously Fitting Numerically Integrated Differential Eqs 1−5 to All Experimental Data in Figure 1

rate constant	k_i L mol^{-1} s^{-1}	k_{+3} s^{-1}
	$308.1 \pm 3.3 \times 10^6$	12121 ± 541
added initial concn of enzyme active sites (nM)[a]	concns of enzyme active sites fitted values (nM)	concns of acetylthiocholine[b] fitted values (μM)
0.22	0.21 ± 0.02	10.05 ± 0.002
0.45	0.46 ± 0.05	9.75 ± 0.001
0.9	0.80 ± 0.09	9.70 ± 0.013
1.8	1.80 ± 0.19	9.98 ± 0.008
5.4	5.20 ± 0.55	9.93 ± 0.009
16.2	17.04 ± 1.92	11.43 ± 0.007

[a] Estimated according to the pseudoirreversible titration. (See Methods and Materials and ref 18.) [b] Initial concentrations of the added acetylthiocholine were 10 μM and in the experiment with the highest enzyme concentration, 11.5 μM.

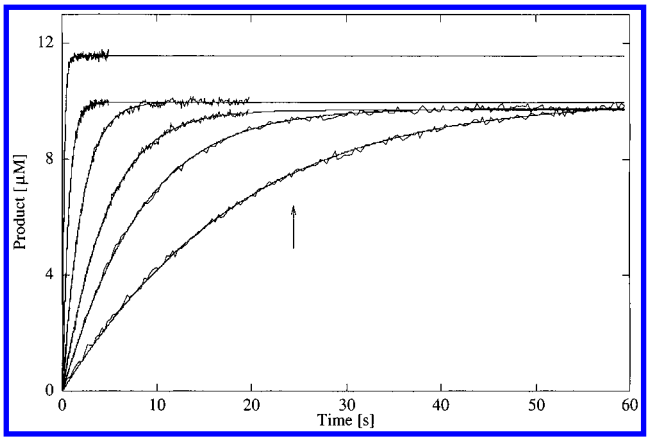


Figure 1. Progress curves for the hydrolysis of acetylthiocholine catalyzed by acetylcholinesterase. Initial concentrations of the substrate and the enzyme active sites are given in Table 1. The arrow indicates the rising enzyme active sites concentrations. The zigzag curves are experimental, and the smooth curves are the fits of differential eqs 1−5 (see text), using the corresponding parameters from Table 1.

All these facts prove that the reaction between AChE and low acetylthiocholine concentrations can indeed be described by the proposed kinetic model (Scheme 2). Additionally, it can be concluded that at some point in the time course of the reaction the concentration of the substrate becomes comparable to the concentration of the enzyme active sites. In this part of the curves the assumptions under which Michaelian type of steady-state rate equations are derived are no more valid. Consequently, the analysis of the progress curves, according to the corresponding integrated equation (for instance Henry's equation), in their entire course is not acceptable. Omitting the parts of the curves where these conditions occur, however, means to leave off the most informative portion of the experimental data calling for much more measurements to perform, in order to obtain the same statistical significance.[1] Therefore, the numerical integration treatment in the analysis seems to be well justified.

At the end, it should be stated that a number of very powerful computer packages are available for solving numerically the systems of stiff differential equations utilizing Gear's method.[21] But with these packages the implementation of the simultaneous[20] analysis could be much more difficult than with the proposed simple computer program which can also be very easily adopted for the use in similar kinetic investigations.

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APPENDIX

The computer program used in this study can be downloaded from the author's homepage at 'http//:www2.mf.uni-lj.si/∼stojan/stojan.html'. A test version of the program in BASIC in which the example of Yamaoka and Nakagawa[17] is solved and also added in order to enable the comparison of the flow of both programs. Attention should be paid to the stepsize in the integration which must be approximately ten times smaller than in the original program due to the nature of the integration method (semi-implicit vs explicit).

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