Ribozymes: Analytical Solution of the One-substrate, Two-intermediate Reversible Scheme for Enzyme Reactions

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Abstract The paper presents a kinetic analysis of a reversible enzymatic reaction $S \rightleftharpoons P$ involving two intermediate compounds under the condition $[E]_0 \gg [S]_0 + [P]_0$. For the case of mono-exponential behavior, we derive an equation for k_{obs} as a function of $[E]_0$, which emphasizes the pitfalls of oversimplifying kinetic schemes (such as the Michaelis-Menten model) for ribozyme studies. This novel apparent rate constant, which has been arrived at through mechanistic considerations, is analyzed, and the characteristic parameters obtained. The equation, which seems to fit experimental data better than conventional approximations, is used to analyze a single turnover study on an ADC1 ribozyme drawn from hepatitis delta virus RNA. The microscopic kinetic constants for such enzyme are evaluated and its mono-exponential behavior verified.

Key words ribozyme kinetics · Michaelis-Menten · low catalytic enzyme · kinetic analysis · single turnover

1 Introduction

The irreversible Michaelis-Menten scheme is widely used in biochemistry to describe enzymatic processes in solution. Normally, three microscopic constants appear in the scheme: k_1 , k_{-1} and k_2 . The progress curve for the irreversible accumulation of the products of enzymatic reactions is described by the integrated Michaelis-Menten equation [1], in which the dependence of $[S]$ on time is implicit. If the substrate is suicidal, the enzyme loses activity during incubation. Waley proposed implicit integrated equations to describe

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both the consumption of substrate and the loss of enzyme activity [2, 3]. From the mechanism discussed by Waley, explicit time-course equations for the accumulation of the product were successively derived [4] under the assumption that the substrate's concentration remains nearly constant over the considered time interval. This condition can be achieved experimentally by using a suitable value for the enzyme concentration or by reducing enzyme catalytic activity. Attempts to formulate explicit solutions to non-approximate equations were first made by Swoboda [5], then by Ignatik [6], though a general solution for differential equations describing the kinetics of all species has yet to be found. However, by applying particular initial conditions to the Michaelis-Menten scheme (see below), it is easy to simplify the set of differential equations. Good results in solving the Michaelis-Menten scheme have been obtained via the quasi-steady state approximation (QSSA) method, also referred to as the pseudo-steady state hypothesis, which has proven very fruitful in the analysis of differential equations, as it enables approximate analytical solutions and simple schemes for parameter estimation [7].

The simplest reversible enzymatic reaction kinetic is represented by Scheme 1, which includes the microscopic rate constant of the enzyme-product reaction, (k_2). In such a scheme the enzyme-substrate complex concentration is the same as that of the enzyme-product complex (equilibrium is rapidly established between ES and EP). A recent study on enzyme kinetics has used the limiting case of this Michaelis-Menten reversible scheme – where the enzyme-product binding rate approaches zero – to study irreversible, consumptive, enzymatic reactions [8]. A slightly more complicated scheme (Scheme 2) is necessary to describe the reversible process when a second intermediate (EP), different from the enzyme-substrate complex, is partitioned between turnover ($E + P$) and enzyme-substrate complex production; this situation applies when the intermediate reversible reaction ($ES \rightleftharpoons EP$) is limiting with respect to both association steps of S and P with the enzyme. Partial solution of this reaction scheme was achieved by Varon et al. [9], who reduced the set of differential equations to a linear system (by assuming $[S] \cong [S]_0$). Taking their work as the point of departure, we reduce the differential equation set for Scheme 1 to a linear system by instead assuming $[E] \cong [E]_0$, and thereby obtain analytical solutions, as described below [10]. The ability to arrive at such solutions is important for the study of enzymes such as ribozymes, which must be present in high concentrations.

Many years have passed since the discovery of catalytic RNA. Although the earliest discoveries of ribozymes (RNA enzymes) involved phosphoryl transfer reactions on RNA substrates, the biological repertoire of these enzymes was recently expanded as a result of new evidence suggesting that the RNA component of the ribosome catalyzes peptide bond formation [11]. Ribozymes have posed novel challenges for mechanistic studies, but recent investigations have yielded increasing support for chemical mechanisms involving precisely positioned nucleic acid bases with environmentally perturbed pK_a values and metal ions. Mathematical kinetic studies of ribozymes have been, for the most part, very simple (adhering closely to the Michaelis-Menten scheme), but in some cases, such as the hammerhead ribozyme and other particular ribozyme types [12–14], the reaction scheme had two or more intermediates, and in numerous cases Scheme 2 seems appropriate. If there are rate-limiting conformational changes, however, or even conformational changes with

Scheme 1 Michaelis-Menten scheme

Scheme 2 Two-intermediate scheme

rates approaching those of the chemical step of the reaction, an expanded scheme is instead more appropriate (for example, Scheme 3). In this case, the observed rate may be dominated by conformational changes (k_3) or by chemistry ($k_{4\text{chem}}$), or even a combination of both. Alternatively, if a rapid equilibrium is established between [ES] and [ES*], the resulting overall rate will reflect any changes affecting the equilibrium population of [ES*] [11].

The aims of the present paper are: (1) to find analytical solutions for all species taking part in enzyme reactions evolving according to Scheme 2, under the assumption that all the enzyme is substantially in the free form; such solutions turn out to require three exponents; (2) to reduce such tri-exponential solutions to single-exponent behavior; (3) to determine the dependence of the first order rate constant of this reaction on the total enzyme concentration (useful for only slightly reversible catalytic enzyme reactions); (4) to suggest analytical procedures for evaluating the kinetic parameters based on experimental data.

2 Ribozyme: Single Turnover Method

Most ribozymes are inefficient catalysts, so, in studying them, the enzyme concentration must be quite high in order to obtain a sufficient concentration of product in operative time periods. This problem is resolved by applying the single turnover method under conditions of enzyme excess ($[\text{E}]/[\text{S}] > 5$) [15]. Time traces of product formation are fit to the single-exponent, first-order rate equation [9]. Different ribozyme concentrations ($[\text{Rz}]$) are used to determine the catalytic rate constant k_{cleav} under standard conditions by linear fitting of the dependence of k_{obs} on ribozyme concentration. K_M describes the apparent ribozyme Michaelis constant for the substrate (1).

$$k_{\text{obs}} = k_{\text{cleav}} \cdot \frac{[\text{Rz}]}{[\text{Rz}] + K_M}. \quad (1)$$

Although most ribozyme studies have revealed single turnover behavior [14, 16, 17], sometimes the data are better fit by bi-exponential kinetics [18].

3 Kinetic Analysis

The reversible Michaelis-Menten scheme, that is, a single-substrate, reversible reacting system in which only one intermediate is formed, is described by Scheme 1 [8], where E, S, and P represent the enzyme, substrate and product, respectively, and ES is the enzyme-substrate complex. It should be noted, however, that this scheme can also be adopted if a ribozyme reaction is characterized by the rapid attainment of equilibrium between all the intermediates. On the other hand, if a rapid intermediate equilibrium does not occur, it is

Scheme 3 Three-intermediate scheme with explicit conformational change and chemistry step

necessary to use a single-substrate, reversible reaction scheme, in which two intermediates take part (Scheme 2), and where EP is the extra intermediate enzyme complex [19].

The equations describing the kinetics of the species in Scheme 2 are:

$$d[E]/dt = -k_1 \cdot [S] \cdot [E] + k_{-1} \cdot [ES] + k_2 \cdot [EP] - k_2 \cdot [P] \cdot [E] \quad (2)$$

$$d[ES]/dt = k_1 \cdot [E] \cdot [S] - k_{-1} \cdot [ES] + k_L \cdot [EP] - k_R \cdot [ES] \quad (3)$$

$$d[EP]/dt = k_2 \cdot [E] \cdot [P] - k_{-2} \cdot [EP] + k_R \cdot [ES] - k_L \cdot [EP] \quad (4)$$

$$d[S]/dt = -k_1 \cdot [E] \cdot [S] + k_{-1} \cdot [ES] \quad (5)$$

$$d[P]/dt = -k_2 \cdot [E] \cdot [P] + k_{-2} \cdot [EP] \quad (6)$$

$$[E]_0 = [E] + [ES] + [EP] \quad (7)$$

$$[S]_0 + [P]_0 = [S] + [ES] + [EP] + [P]. \quad (8)$$

The set of differential Eqs. 2, 3, 4, 5 and 6, is non-linear and therefore has no analytical solution. However, under the following assumption (9):

$$[S]_0 + [P]_0 \ll [E]_0 \quad (9)$$

the mass balance (conservation equation) for the enzyme (7) simplifies to:

$$[E]_0 \cong [E]. \quad (10)$$

By adopting assumption (9), we convert the set of differential Eqs. 2, 3, 4, 5 and 6 into a linear one (see Appendix A). By then inserting Eq. 10 into the differential equation set, we obtain the matrix form, Eq. 45. By applying the Galvez–Varon method [20], it is possible to obtain the general differential equation of both the substrate and product. Starting with the same coefficient matrix (46), we show that the differential equation for the substrate is equal to that for the product. Its solution is arrived at via textbook methods for linear differential equation systems with constant coefficients based on successive derivations and substitutions (see also [20]). By solving the fourth-order differential Eq. 11

$$\frac{d^4x}{dt^4} + G_1 \cdot \frac{d^3x}{dt^3} + G_2 \cdot \frac{d^2x}{dt^2} + G_3 \cdot \frac{dx}{dt} = 0 \quad (11)$$

in which $x=[S]$ or $x=[P]$, and

$$G_1 = k_1 \cdot [E]_0 + k_{-1} + k_R + k_L + k_2 \cdot [E]_0 + k_{-2} \quad (12)$$

$$G_2 = k_R \cdot k_{-2} + k_L \cdot k_{-1} + (k_R + k_L) \cdot (k_1 \cdot [E]_0 + k_2 \cdot [E]_0) \\ + (k_2 \cdot [E]_0 + k_{-2}) \cdot (k_1 \cdot [E]_0 + k_{-1}) \quad (13)$$

$$G_3 = k_1 \cdot [E]_0 \cdot k_R \cdot (k_2 \cdot [E]_0 + k_{-2}) + k_2 \cdot [E]_0 \cdot k_L \cdot (k_1 \cdot [E]_0 + k_{-1}) \quad (14)$$

we reach solutions for all the species involved (see Appendix A). δ_h ($h=1,2,3$) are the eigenvalues of the coefficient matrix of (45), i.e., the real roots of the polynomial $\delta^3 + G_1 \cdot \delta^2 + G_2 \cdot \delta + G_3 = 0$ (47).

Note that the roots are all negative and the following relations exist between δ_1 , δ_2 and δ_3 :

$$\delta_1 + \delta_2 + \delta_3 = -G_1 \quad (15)$$

$$\delta_1 \cdot \delta_2 \cdot \delta_3 \cdot \delta_1 \cdot \delta_3 = G_2 \quad (16)$$

$$\delta_1 \cdot \delta_2 \cdot \delta_3 = -G_3. \quad (17)$$

Recalling that the reaction scheme in question is a reversible one, if both substrate and product were present at time zero, the direction of the reaction would be different, depending on the values of the kinetic constants. Under the assumption that $[P]_0=0$, instead, the reversible reaction mechanism brings about consumption of the substrate, S, and the production of the P species, so the analytical solutions for the species EP and P simplify to:

$$[EP] = \frac{k_1 \cdot k_R \cdot k_2 \cdot [S]_0 \cdot [E]_0^2}{G_3} + \sum_{h=1}^3 \frac{k_1 \cdot k_R \cdot [S]_0 \cdot [E]_0 \cdot (k_2 \cdot [E]_0 - \delta_h)}{\delta_h \cdot \prod_{\substack{p=1 \\ p \neq h}}^3 (\delta_h - \delta_p)} \cdot e^{\delta_h \cdot t} \quad (18)$$

$$[P] = \frac{k_1 \cdot k_R \cdot k_{-2} \cdot [S]_0 \cdot [E]_0}{G_3} + \sum_{h=1}^3 \frac{k_1 \cdot k_R \cdot k_{-2} \cdot [S]_0 \cdot [E]_0}{\delta_h \cdot \prod_{\substack{p=1 \\ p \neq h}}^3 (\delta_h - \delta_p)} \cdot e^{\delta_h \cdot t}. \quad (19)$$

4 Results and Discussion

4.1 Mono-exponential Behavior

By definition, mono-exponential decay implies that one of the rate constants is lower than the others; so relation (20) will hold:

$$-\delta_2, -\delta_3 \gg -\delta_1 \quad (20)$$

By denoting this rate constant as δ_1 and accounting for Eqs. 15, 16, 17, and 20, we obtain:

$$\delta_1 \cong -G_3/G_2 \quad (21)$$

$$\delta_2 \cong -\frac{1}{2} \cdot \left(G_1 + \sqrt{G_1^2 - 4 \cdot G_2} \right) \quad (22)$$

$$\delta_3 \cong -\frac{1}{2} \cdot \left(G_1 - \sqrt{G_1^2 - 4 \cdot G_2} \right). \quad (23)$$

The condition of mono-exponential decay is always guaranteed at sufficiently high enzyme concentrations, as we can see by evaluating the leading order estimates of the limits:

$$\lim_{[E]_0 \rightarrow \infty} G_1 = (k_1 + k_2) \cdot [E]_0 \quad (24)$$

$$\lim_{[E]_0 \rightarrow \infty} G_2 = k_1 \cdot k_2 \cdot [E]_0^2 \quad (25)$$

$$\lim_{[E]_0 \rightarrow \infty} G_3 = k_1 \cdot k_2 \cdot (k_R + k_L) \cdot [E]_0^2 \quad (26)$$

and correspondingly

$$\lim_{[E]_0 \rightarrow \infty} \delta_1 = -\lim_{[E]_0 \rightarrow \infty} G_3/G_2 = -(k_R + k_L) \quad (27)$$

$$\lim_{[E]_0 \rightarrow \infty} \delta_2 = -k_1 \cdot [E]_0 \quad (28)$$

$$\lim_{[E]_0 \rightarrow \infty} \delta_3 = -k_2 \cdot [E]_0. \quad (29)$$

4.2 Product Formation Rate Equation

In the case of mono-exponential decay, the terms with δ_2 or δ_3 in the exponent can be neglected in Eqs. 18 and 19; so from Eq. 19 we obtain:

$$[P] = \frac{k_1 \cdot k_R \cdot k_{-2} \cdot [S]_0 \cdot [E]_0}{G_3} \left(1 - e^{-\left(G_3/G_2\right) \cdot t} \right). \quad (30)$$

Equation 30 is consistent with the linear dependence of the initial rate of free product formation at low enzyme concentration [21], as in the Michaelis-Menten analysis. When the enzyme concentration is increased, in the case of reversibility, the initial rate (or the free product first derivative at time zero) reaches a maximum, and then falls hyperbolically to

zero. The decrease in the initial rate is due to the value of the microscopic association constant of the product, k_2 . In the event that k_2 is zero (irreversibility of the dissociation process), the hyperbolic decrease is lacking.

By deriving Eq. 30 and setting time to zero, we obtain the initial rate, called v_0 :

$$v_0 = \frac{k_1 \cdot k_R \cdot k_{-2} \cdot [S]_0 \cdot [E]_0}{[E]_0^2 \cdot (k_1 \cdot k_2) + [E]_0 \cdot (k_1 \cdot (k_{-2} + k_R + k_L) + k_2 \cdot (k_{-1} + k_R + k_L)) + k_{-1} \cdot k_{-2} + k_{-1} \cdot k_L + k_{-2} \cdot k_R} \quad (31)$$

In the case that k_2 is close to zero (irreversibility of the process), the quadratic term in the denominator disappears, and Eq. 31 becomes:

$$v_0 = \frac{k_1 \cdot k_R \cdot k_{-2} \cdot [S]_0 \cdot [E]_0}{[E]_0 \cdot (k_1 \cdot (k_{-2} + k_R + k_L)) + k_{-1} \cdot k_{-2} + k_{-1} \cdot k_L + k_{-2} \cdot k_R} \quad (32)$$

which expresses a simple dependence of the initial velocity on the total enzyme concentration (rectangular hyperbola).

To simplify the analysis of (31), we divide both numerator and denominator by $k_1 \cdot k_2$, and Eq. 31 becomes:

$$v_0 = \frac{k_R \cdot K_{d(P)} \cdot [S]_0 \cdot [E]_0}{[E]_0^2 + \left((k_R + k_L) \cdot \left(\frac{1}{k_1} + \frac{1}{k_2} \right) + K_{d(S)} + K_{d(P)} \right) \cdot [E]_0 + K_{d(S)} \cdot K_{d(P)} + \frac{k_R}{k_1} \cdot K_{d(P)} + \frac{k_L}{k_2} \cdot K_{d(S)}} \quad (33)$$

Although this precludes imposing a value of zero on the microscopic association constants, Eq. 33 is easier to understand and study: indeed, $K_{d(S)}$ and $K_{d(P)}$ are the dissociation constants of the substrate and product, respectively.

Generally, in Michaelis-Menten studies, $[E]_0 \ll [S]_0$, and the initial rate is a function of the total substrate concentration, and is moreover linear with respect to $[E]_0$. In the current case, instead, the substrate is the limited component, so the initial reaction rate is a function of the total enzyme concentration and is linear with respect to $[S]_0$. The characteristic values of equation (33) are summarized in Fig. 1.

Note that the term c (42) has dimensions $[M]^2$, and that rate v_0 reaches its maximum value at a total enzyme concentration of $\sqrt{c}$, after which it decreases very slowly.

The existence of a second intermediate depends on the values of k_R and k_L being very near the other microscopic constants in Scheme 2 (if a rapid equilibrium is established between [ES] and [EP], the system can be simply described with a single intermediate). On the other hand, low enzyme catalytic activity is one of various conditions for mono-exponential behavior of the reaction over time.

4.3 Ribozyme: Useful Rate Equation

In the case of a ribozyme reaction mechanism involving two intermediates (Scheme 2), when excess enzyme is present, a non-negligible amount of product is bound to the ribozyme. In experimental measurements, the reaction is stopped and the product concentration is determined. Thus, Eq. 34, which yields the sum of the EP and P

concentrations, represents a better fit to the experimental data:

$$\begin{aligned}
 [\text{EP}] + [\text{P}] = & \frac{k_1 \cdot k_R \cdot [\text{S}]_0 \cdot [\text{E}]_0 (k_2 \cdot [\text{E}]_0 + k_{-2})}{G_3} \\
 & + \sum_{h=1}^3 \frac{k_1 \cdot k_R \cdot [\text{S}]_0 \cdot [\text{E}]_0 \cdot (k_2 \cdot [\text{E}]_0 - \delta_h + k_{-2})}{\delta_h \cdot \prod_{\substack{p=1 \\ p \neq h}}^3 (\delta_h - \delta_p)} \cdot e^{\delta_h \cdot t}. \quad (34)
 \end{aligned}$$

Otherwise, in the case of mono-exponential behavior, from Eq. 34, we obtain:

$$\begin{aligned}
 [\text{EP}] + [\text{P}] = & \frac{k_1 \cdot k_R \cdot [\text{S}]_0 \cdot [\text{E}]_0 (k_2 \cdot [\text{E}]_0 + k_{-2})}{G_3} \\
 & - \frac{k_1 \cdot k_R \cdot [\text{S}]_0 \cdot [\text{E}]_0 \cdot \left(k_2 \cdot [\text{E}]_0 + \frac{G_3}{G_2} + k_{-2} \right)}{G_3} \cdot e^{-\left(G_3/G_2 \right) \cdot t}. \quad (35)
 \end{aligned}$$

Derivation of equation (35) furnishes a rate equation for the production of product in both free and bound form.

$$\frac{d([\text{EP}] + [\text{P}])}{dt} = \frac{k_1 \cdot k_R \cdot k_{-2} \cdot [\text{S}]_0 \cdot [\text{E}]_0}{G_2} + \frac{k_1 \cdot k_R \cdot k_2 \cdot [\text{S}]_0 \cdot [\text{E}]_0^2}{G_2} + \frac{k_1 \cdot k_R \cdot [\text{S}]_0 \cdot [\text{E}]_0 \cdot G_3}{G_2^2}. \quad (36)$$

Equation 36 is given in explicit form in the Appendix (Eq. 60).

4.4 Ribozymes: k_{obs} for a Two-intermediate Scheme

Starting with equation (30), which represents a simple exponential function, we can express the exponential term (i.e., k_{obs}) as the ratio of G_3 vs G_2 :

$$k_{\text{obs}} = \frac{((k_R + k_L) \cdot k_1 \cdot k_2) \cdot [\text{E}]_0^2 + (k_1 \cdot k_R \cdot k_{-2} + k_2 \cdot k_L \cdot k_{-1}) \cdot [\text{E}]_0}{k_2 \cdot k_1 \cdot [\text{E}]_0^2 + ((k_R + k_L) \cdot (k_1 + k_2) + k_2 \cdot k_{-1} + k_1 \cdot k_{-2}) \cdot [\text{E}]_0 + k_{-2} \cdot k_{-1} + k_R \cdot k_{-2} + k_L \cdot k_{-1}}. \quad (37)$$

Dividing both the numerator and denominator of Eq. 37 by $k_1 \cdot k_2$ yields:

$$k_{\text{obs}} = \frac{((k_R + k_L)) \cdot [\text{E}]_0^2 + (k_R \cdot K_{d(\text{P})} + k_L \cdot K_{d(\text{S})}) \cdot [\text{E}]_0}{[\text{E}]_0^2 + \left((k_R + k_L) \cdot \left(\frac{1}{k_1} + \frac{1}{k_2} \right) + K_{d(\text{S})} + K_{d(\text{P})} \right) \cdot [\text{E}]_0 + K_{d(\text{S})} \cdot K_{d(\text{P})} + \frac{k_R}{k_1} \cdot K_{d(\text{P})} + \frac{k_L}{k_2} \cdot K_{d(\text{S})}}. \quad (38)$$

Now, using the values of f , g , b and c defined in Eqs. 39, 40, 41 and 42, respectively, we can write (43) as formulated in Fig. 2:

$$f = k_R + k_L \quad (39)$$

$$g = k_R \cdot K_{d(\text{P})} + k_L \cdot K_{d(\text{S})} \quad (40)$$

Fig. 1 Characteristic values of Eq. 33

$$b = (k_R + k_L) \cdot \left(\frac{1}{k_1} + \frac{1}{k_2} \right) + K_{d(S)} + K_{d(P)} \quad (41)$$

$$c = K_{d(S)} \cdot K_{d(P)} + \frac{k_R}{k_1} \cdot K_{d(P)} + \frac{k_L}{k_2} \cdot K_{d(S)} \quad (42)$$

$$k_{\text{obs}} = \frac{f \cdot [E]_0^2 + g \cdot [E]_0}{[E]_0^2 + b \cdot [E]_0 + c} \quad (43)$$

This expression for k_{obs} is consistent with the other polynomial expressions, indicating that the simple rectangular hyperbola is only an approximation of function [11].

It is thus possible to express Eq. 38 in a simpler form, in accordance with most classical ribozyme studies. Via simple algebraic manipulation, we obtain:

$$k_{\text{obs}} = \frac{(k_R + k_L) \cdot [E]_0}{[E]_0 + \frac{1}{2} \cdot \left((k_R + k_L) \cdot \left(\frac{1}{k_1} + \frac{1}{k_2} \right) + K_{d(S)} + K_{d(P)} \right) \cdot \left(1 + \sqrt{1 - \frac{4 \left(K_{d(S)} \cdot K_{d(P)} + \frac{k_R}{k_1} \cdot K_{d(P)} + \frac{k_L}{k_2} \cdot K_{d(S)} \right)}{\left((k_R + k_L) \cdot \left(\frac{1}{k_1} + \frac{1}{k_2} \right) + K_{d(S)} + K_{d(P)} \right)^2}} \right)} \quad (44)$$

where, by using the values given in Fig. 2, the two fundamental enzymatic constants in Eq. 1, k_{cleav} and K_M , are expressed as f and $\frac{1}{2} \cdot \left(b + \sqrt{b^2 - 4 \cdot c} \right)$, respectively (the minus

sign was discarded on the basis of asymptotic considerations); k_{cleav} is the k_{obs} asymptote, while K_M is the $[E]_0$ value at which k_{obs} is at its half-maximum value ($k_{\text{cleav}}/2$). However, this function fits experimental data only when the total ribozyme concentration is equal to or greater than K_M ; thus, in a linear plot of the approximate Eq. 44, a high ribozyme concentration must be used. Note that Eq. 44 is not useful for analysis of microscopic kinetic constants.

By using data drawn from the literature [10] and Eq. 38, the values of all microscopic constants involved in Scheme 2 can be determined.

Shih and Been have formulated a kinetic scheme (Scheme 4) for cleavage reactions catalyzed by a *trans*-acting HDV ribozyme (ADC1) [10]. The scheme is based on measurements of individual elemental rate constants, including cleavage of the phosphodiester bond, as well as the association and dissociation of substrate and product [10]. In their single-turnover experiments for cleavage reactions, the ribozyme and substrate were pre-incubated at 37°C for 10 min in a “buffer 1” solution, containing 40 mM Tris-HCl (pH 8.0) and 1 mM EDTA. The reaction was initiated by adding MgCl_2 to yield “standard conditions” [40 mM Tris-HCl (pH 8.0), 1 mM EDTA, and 11 mM MgCl_2 at 37°C; “buffer 2”]. Aliquots were taken at various times, quenched with an equal volume of 0.1 M EDTA, and fractionated on PEI plates in 1M LiCl. The fraction cleaved was visualized and quantified using a PhosphorImager (Molecular Dynamics). First-order rate constants (k_{obs}) were determined. In multiple-turnover reactions, the substrate binds to the ribozyme to form the ES complex; the portion that goes on to the chemistry step EP5'P3' (EP intermediate) generates two cleavage products (P5' and P3' products). These two products dissociate from the ribozyme along a kinetically ordered pathway because the dissociation rate constant of the P5' product is at least $2 \cdot 10^3$ fold faster than that of the P3' product. Dissociation of the P3' product, which is 140-fold slower than the chemistry step, is the rate limiting step for multiple turnover. Thus, in Scheme 4, we can ignore the faster step of P5' dissociation.

The numerical data extrapolated from single-turnover experiments seem to fit a simple rectangular hyperbola [Eq. 44], as illustrated in Fig. 3. We fitted the data to a double reciprocal plot, obtaining values of 0.979, $(7.33 \pm 1.80) \cdot 10^{-1} \text{ min}^{-1}$ and $(8.92 \pm 2.17) \cdot 10^{-8} \text{ M}$, respectively, for r^2 , k_{cleav} and K_M (Fig. 3a). Better results would be obtained by using data points with enzyme concentration values higher than K_M . It should be emphasized that our

Fig. 2 Characteristic values of Eq. 38

Scheme 4 Shih and Been model [10] for cleavage reaction catalyzed by ADC1

novel approximation for the apparent mono-exponential rate constant fits experimental data better than the conventional plot ($r^2=0.9958$ vs $r^2=0.979$, respectively).

Although the experimental points were rather limited in number for a non-linear fitting, this method enabled determination of the sum of k_R and k_L values as $(9.33 \pm 0.54) \cdot 10^{-1} \text{ min}^{-1}$, similar to the value obtained in the original study. The four parameter values obtained via the non-linear fitting of b , c , f and g were $(1.51 \pm 0.33) \cdot 10^{-7} \text{ M}$, $(8.28 \pm 2.72) \cdot 10^{-16} \text{ M}^2$, $(9.33 \pm 0.54) \cdot 10^{-1} \text{ min}^{-1}$, $(8.73 \pm 1.22) \cdot 10^{-9} \text{ M} \cdot \text{min}^{-1}$, respectively. The f asymptote and the linear dependence of the first-order rate constants on low concentration of the ribozyme, or a g/c of $(1.05 \pm 0.37) \cdot 10^7 \text{ M}^{-1} \cdot \text{min}^{-1}$, were also in agreement with the results of the original work $((9.1 \pm 0.2) \cdot 10^{-1} \text{ min}^{-1}$ and $(7.5 \pm 0.5) \cdot 10^6 \text{ M}^{-1} \cdot \text{min}^{-1}$, respectively).

Starting with the four parameters b , c , f and g , as expressed in Eqs. 39, 40, 41 and 42, we can write the four-equation linear system:

$$\begin{cases} f = k_R + k_L \\ g = k_R \cdot K_{d(P)} + k_L \cdot K_{d(S)} \\ b = (k_R + k_L) \cdot \left(\frac{1}{k_1} + \frac{1}{k_2} \right) + K_{d(S)} + K_{d(P)} \\ c = K_{d(S)} \cdot K_{d(P)} + \frac{k_R}{k_1} \cdot K_{d(P)} + \frac{k_L}{k_2} \cdot K_{d(S)} \end{cases}$$

Solving this system by using the values of the dissociation constants for both substrate and product (P_3'), we have obtained the values of the kinetic rate constants presented in Table 1.

Fig. 3 (a) Double reciprocal data plot with results using k_{cleav} and K_M values of $(7.33 \pm 1.80) \cdot 10^{-1} \text{ min}^{-1}$ and $(8.92 \pm 2.17) \cdot 10^{-8} \text{ M}$, respectively, with $r^2=0.979$; (b) rectangular hyperbola obtained using k_{cleav} and K_M values of $(7.33 \pm 1.80) \cdot 10^{-1} \text{ min}^{-1}$ and $(8.92 \pm 2.17) \cdot 10^{-8} \text{ M}$, respectively, versus experimental data (indicated by circles)

Table 1 Microscopic kinetic constants values obtained via non-linear fitting

Constant	k_1 ($\text{min}^{-1} \cdot \text{M}^{-1}$)	k_{-1} (min^{-1})	k_R (min^{-1})	k_L (min^{-1})	k_2 ($\text{min}^{-1} \cdot \text{M}^{-1}$)	k_{-2} (min^{-1})
Value	$(1.07 \pm 0.28) \cdot 10^7$	$(6.19 \pm 1.62) \cdot 10^{-1}$	$(8.17 \pm 1.64) \cdot 10^{-1}$	$(1.15 \pm 0.35) \cdot 10^{-1}$	$(2.99 \pm 0.66) \cdot 10^8$	$(7.49 \pm 1.66) \cdot 10^{-1}$

Fig. 4 shows the plots of two graphs of k_{obs} versus $[E]_0$ made using Eq. 37 and the microscopic kinetic constant values from both Table 1 and those of Shih and Been [10]. Note that using the microscopic kinetic constants obtained by Shih and Been and Eq. 37, the simulated curve does not fit the experimental data (Fig. 4b).

By using Eq. 47 and the kinetic rate constants presented in Table 1, we have obtained the values of δ_1, δ_2 and δ_3 as a function of $[E]_0$. As shown in Fig. 5, for both ratios, δ_1/δ_2 and δ_1/δ_3 , the value of d_1 must be much smaller (by an absolute value of about one order of magnitude) than the others in order for the mono-exponential single turnover to fit the kinetics of the particular ribozyme under study.

5 Conclusions

This paper presents a kinetic analysis of reversible enzymatic reactions $S \rightleftharpoons P$ involving one and two intermediate compounds, as illustrated in Schemes 1 and 2, under the condition that $[E]_0 \gg [S]_0 + [P]_0$. All species in the kinetic analysis present bi- or tri-exponential terms in the one- and two-intermediate schemes, respectively.

For the case of mono-exponential behavior, equations have been derived by which the initial velocity, v_0 , of product formation is a function of $[E]_0$; such equations have been analyzed and their characteristic parameters obtained.

Fig. 4 Simulated plot of k_{obs} versus $[ADC1]$ using Eq. 37 and (a) the microscopic kinetic values obtained via a non-linear data fit using Eq. 38 and the MatLab 5.1 program, with $r^2=0.9958$ and the values in Table 1 ($k_1 (1.07 \pm 0.28) \cdot 10^7 \text{ min}^{-1} \cdot \text{M}^{-1}$, $k_{-1} (6.19 \pm 1.62) \cdot 10^{-1} \text{ min}^{-1}$, $k_R (8.17 \pm 1.64) \cdot 10^{-1} \text{ min}^{-1}$, $k_L (1.15 \pm 0.35) \cdot 10^{-1} \text{ min}^{-1}$, $k_2 (2.99 \pm 0.66) \cdot 10^8 \text{ min}^{-1} \cdot \text{M}^{-1}$, $k_{-2} (7.49 \pm 1.66) \cdot 10^{-1} \text{ min}^{-1}$); (b) the microscopic kinetic values from Shih and Been (Scheme 4; $k_1 2.1 \cdot 10^7 \text{ min}^{-1} \cdot \text{M}^{-1}$, $k_{-1} 1.4 \text{ min}^{-1}$, $k_R 9.1 \cdot 10^{-1} \text{ min}^{-1}$, $k_2 2.3 \cdot 10^6 \text{ min}^{-1} \cdot \text{M}^{-1}$, $k_{-2} 5.8 \cdot 10^{-3} \text{ min}^{-1}$)

Fig. 5 Simulated ratio of δ_1/δ_2 (solid line) and δ_1/δ_3 (grey line) versus $[E]_0$ using Eq. 47 and the microscopic rate constants presented in Table 1 (k_1 $(1.07 \pm 0.28) \cdot 10^7 \text{ min}^{-1} \cdot \text{M}^{-1}$, k_{-1} $(6.19 \pm 1.62) \cdot 10^{-1} \text{ min}^{-1}$, k_R $(8.17 \pm 1.64) \cdot 10^{-1} \text{ min}^{-1}$, k_L $(1.15 \pm 0.35) \cdot 10^{-1} \text{ min}^{-1}$, k_2 $(2.99 \pm 0.66) \cdot 10^8 \text{ min}^{-1} \cdot \text{M}^{-1}$, k_{-2} $(7.49 \pm 1.66) \cdot 10^{-1} \text{ min}^{-1}$)

We furthermore derive an alternative, apparently mono-exponential rate constant (k_{obs} equation as a function of $[E]_0$), which underscores the pitfalls of oversimplifying kinetic schemes (the use of the Michaelis-Menten equation for ribozyme studies). This novel apparent rate constant, whose formulation was mechanistically motivated, has also been analyzed, and its characteristic parameters determined. The rate equation has moreover been manipulated algebraically to obtain the classical equation for ribozyme analysis. However, the classical equation proves to be simpler and mathematically less robust. The proposed equation, which seems to fit the data better than the conventional approximation, has been applied to analyze a single-turnover study on a ADC1 ribozyme from hepatitis delta virus RNA. The microscopic kinetic constants have been evaluated, and the mono-exponential behavior verified.

Appendix A

By inserting Eq. 10 into the differential equation set, we obtain the matrix form:

$$\begin{bmatrix} d[S]/dt \\ d[ES]/dt \\ d[EP]/dt \\ d[P]/dt \end{bmatrix} = \begin{bmatrix} -k_1 \cdot [E]_0 & k_{-1} & 0 & 0 \\ k_1 \cdot [E]_0 & -k_{-1} - k_R & k_L & 0 \\ 0 & k_R & -k_{-2} - k_L & k_2 \cdot [E]_0 \\ 0 & 0 & k_{-2} & -k_2 \cdot [E]_0 \end{bmatrix} \begin{bmatrix} [S] \\ [ES] \\ [EP] \\ [P] \end{bmatrix} \quad (45)$$

the determinant $D(x)$ is:

$$D(x) = \begin{bmatrix} -k_1 \cdot [E]_0 - x & k_{-1} & 0 & 0 \\ k_1 \cdot [E]_0 & -k_{-1} - k_R - x & k_L & 0 \\ 0 & k_R & -k_{-2} - k_L - x & k_2 \cdot [E]_0 \\ 0 & 0 & k_{-2} & -k_2 \cdot [E]_0 - x \end{bmatrix} \quad (46)$$

where the roots δ_1 , δ_2 and δ_3 are non-zero and different from $\delta_4=0$, and their explicit values are given by:

$$\delta_{n+1} = 2 \cdot \sqrt{-\frac{3 \cdot G_2 - G_1^2}{9}} \cdot \cos \left(\frac{1}{3} \cdot \arccos \left(\frac{1}{6} \cdot \frac{9 \cdot G_1 \cdot G_2 - 27 \cdot G_3 - 2G_1^3}{\sqrt{-3 \cdot G_2 - G_1^2}} \right) + \frac{2}{3} \cdot \pi \cdot n \right) - \frac{1}{3} \cdot G_1 \quad (47)$$

$(n = 0, 1, 2)$

Defining

$$\alpha_P = [P]_0 \quad (48)$$

$$\beta_P = -k_2 \cdot [P]_0 \quad (49)$$

$$\chi_P = (k_2 + k_{-2} + k_L) \cdot k_2 \cdot [P]_0 - k_L \cdot k_2 \cdot [P]_0 \quad (50)$$

and

$$\begin{aligned} \varepsilon_P = & k_{-2} \cdot k_R \cdot k_1 \cdot [S]_0 - (k_2 + k_{-2} + k_L + k_R + k_{-1}) \cdot ((k_2 + k_{-2} + k_L) \cdot k_2 \cdot [P]_0 - k_L \cdot k_2 \cdot [P]_0) \\ & + (k_{-1} \cdot k_2 + k_{-1} \cdot k_{-2} + k_{-1} \cdot k_L + k_R \cdot k_2 + k_R \cdot k_{-2} + k_L \cdot k_2) \cdot (k_2 \cdot [P]_0) - k_{-1} \cdot k_L \cdot k_2 \cdot [P]_0 \end{aligned} \quad (51)$$

we have

$$[P] = \alpha_P + \sum_{h=1}^3 \left(\frac{\beta_P \cdot \prod_{\substack{p=1 \\ p \neq h}}^3 \delta_p - \chi_P \cdot \sum_{\substack{p=1 \\ p \neq h}}^3 \delta_p + \varepsilon_P}{\delta_h \cdot \prod_{\substack{p=1 \\ p \neq h}}^3 (\delta_h - \delta_p)} \cdot (e^{\delta_h t} - 1) \right) \quad (52)$$

$$\begin{aligned} [EP] = & \frac{k_2 \cdot [E]_0}{k_{-1}} \cdot \alpha_P \\ & + \sum_{h=1}^3 \left(\frac{\left(\beta_P \cdot \prod_{\substack{p=1 \\ p \neq h}}^3 \delta_p - \chi_P \cdot \sum_{\substack{p=1 \\ p \neq h}}^3 \delta_p + \varepsilon_P \right)}{\delta_h \cdot \prod_{\substack{p=1 \\ p \neq h}}^3 (\delta_h - \delta_p)} \cdot \left(\frac{k_2 \cdot [E]_0 \cdot (e^{\delta_h t} - 1) - \delta_h \cdot e^{\delta_h t}}{k_{-2}} \right) \right). \end{aligned} \quad (53)$$

Defining

$$\alpha_S = [S]_0 \quad (54)$$

$$\beta_S = -k_1 \cdot [S]_0 \quad (55)$$

$$\chi_S = (k_1 + k_{-1} + k_R) \cdot k_1 \cdot [S]_0 - k_R \cdot k_1 \cdot [S]_0 \quad (56)$$

and

$$\begin{aligned} \varepsilon_S = & k_{-1} \cdot k_L \cdot k_2 \cdot [P]_0 - (k_1 + k_{-1} + k_R + k_L + k_{-2}) \cdot ((k_1 + k_{-1} + k_R) \cdot k_1 \cdot [S]_0 - k_R \cdot k_1 \cdot [S]_0) \\ & + (k_{-2} \cdot k_1 + k_{-2} \cdot k_{-1} + k_{-2} \cdot k_R + k_L \cdot k_1 + k_L \cdot k_{-1} + k_R \cdot k_1) \cdot (k_1 \cdot [S]_0) - k_{-2} \cdot k_R \cdot k_1 \cdot [S]_0 \end{aligned} \quad (57)$$

we have

$$[S] = \alpha_S + \sum_{h=1}^3 \left(\frac{\beta_S \cdot \prod_{\substack{p=1 \\ p \neq h}}^3 \delta_p - \chi_S \cdot \sum_{\substack{p=1 \\ p \neq h}}^3 \delta_p + \varepsilon_S}{\delta_h \cdot \prod_{\substack{p=1 \\ p \neq h}}^3 (\delta_h - \delta_p)} \cdot (e^{\delta_h \cdot t} - 1) \right) \quad (58)$$

$$[ES] = \frac{k_1 \cdot [E]_0}{k_{-1}} \cdot \alpha_S + \sum_{h=1}^3 \left(\frac{\left(\beta_S \cdot \prod_{\substack{p=1 \\ p \neq h}}^3 \delta_p - \chi_S \cdot \sum_{\substack{p=1 \\ p \neq h}}^3 \delta_p + \varepsilon_S \right)}{\delta_h \cdot \prod_{\substack{p=1 \\ p \neq h}}^3 (\delta_h - \delta_p)} \cdot \left(\frac{k_1 \cdot [E]_0 \cdot (e^{\delta_h \cdot t} - 1) - \delta_h \cdot e^{\delta_h \cdot t}}{k_{-1}} \right) \right). \quad (59)$$

Appendix B

$$\frac{d([EP] + [P])}{dt} = \frac{\left(\begin{array}{l} k_1 \cdot k_R \cdot [S]_0 \cdot ((k_{-2} \cdot k_2 \cdot k_1 + k_2 \cdot ((k_R + k_L) \cdot (k_1 + k_2) + k_2 \cdot k_{-1} + k_1 \cdot k_{-2}) + ((k_R + k_L) \cdot k_1 \cdot k_2))) \cdot [E]_0^4 + \\ k_1 \cdot k_R \cdot [S]_0 \cdot ((k_{-2} \cdot ((k_R + k_L) \cdot (k_1 + k_2) + k_2 \cdot k_{-1} + k_1 \cdot k_{-2}) + k_2 \cdot (k_{-2} \cdot k_{-1} + k_R \cdot k_{-2} + k_L \cdot k_{-1}) + (k_1 \cdot k_R \cdot k_{-2} + k_2 \cdot k_L \cdot k_{-1}))) \cdot [E]_0^3 + \\ k_1 \cdot k_R \cdot [S]_0 \cdot k_{-2} \cdot (k_{-2} \cdot k_{-1} + k_R \cdot k_{-2} + k_L \cdot k_{-1}) \cdot [E]_0 \end{array} \right)}{\left(\begin{array}{l} (k_2 \cdot k_1)^2 \cdot [E]_0^4 + \\ 2 \cdot k_2 \cdot k_1 \cdot ((k_R + k_L) \cdot (k_1 + k_2) + k_2 \cdot k_{-1} + k_1 \cdot k_{-2}) \cdot [E]_0^3 + \\ ((k_R + k_L) \cdot (k_1 + k_2) + k_2 \cdot k_{-1} + k_1 \cdot k_{-2})^2 + 2 \cdot k_2 \cdot k_1 \cdot (k_{-2} \cdot k_{-1} + k_R \cdot k_{-2} + k_L \cdot k_{-1}) \cdot [E]_0^2 + \\ 2 \cdot (k_{-2} \cdot k_{-1} + k_R \cdot k_{-2} + k_L \cdot k_{-1}) \cdot ((k_R + k_L) \cdot (k_1 + k_2) + k_2 \cdot k_{-1} + k_1 \cdot k_{-2}) \cdot [E]_0 + \\ (k_{-2} \cdot k_{-1} + k_R \cdot k_{-2} + k_L \cdot k_{-1})^2 \end{array} \right)} \quad (60)$$

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