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ORIGINAL PAPER

Analysis of the fractional modification of the monocyclic enzyme cascades, defined in an alternative way involving the two forms of the modified protein

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Abstract This paper presents the discussion of a more complete definition of the fractional modification of an monocyclic enzyme cascade suggested, but not discussed, by Varon et al. (Bull Math Biol 68(7):1461-1493, 2006) as the quotient of the sum of all forms of the modified protein, i.e. the free one and the intermediate complex converter enzyme of the original protein-modified protein, between the initial concentration of the target protein. From this general equation, obtained under three assumptions necessary to linearize the set of differential equations describing the kinetic of the system, we derive, as particular cases, other simpler expressions, by applying additional sim-

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plifying assumptions, which are, therefore, of a smaller range of validity. We discuss the relationships between the kinetic parameters and concentrations needed for the fulfillment of both the necessary and unnecessary assumptions. The goodness of the analysis was tested by using the shape in the steady state of the simulated time progress curves obtained by numerical integration. Seven arbitrarily chosen examples differing in one or more of the values of the concentrations and/or kinetic parameters have been used to support the results.

Keywords Fractional modification · Monocyclic enzyme cascades · Modified protein · Target protein

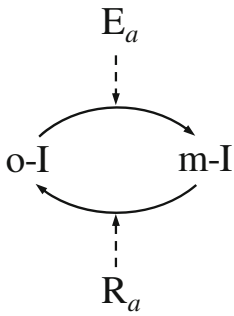
1 Introduction

There are many processes of cell regulation where a protein is reversibly and covalently modified by the enzyme catalyzed transfer of a group from a donor to a specific amino acid residue located at the active site of the acceptor [1]. These covalent modification and demodification reactions are catalyzed by a specific converter enzyme, often protein kinases and phosphoprotein phosphatases [2]. The modification of the protein by a converter enzyme and the opposite reaction, in which the modified protein or peptide is "demodified" by another converter enzyme, form a monocyclic enzyme cascade. The converter enzymes for both modification and demodification reactions also undergo a modification process (activation or inactivation) induced by an allosteric effector. Monocyclic enzyme cascades are ubiquitous in biological systems.

A very important steady-state parameter related with monocyclic enzyme cascades is the fractional modification of the interconvertible protein, once it has reached this state, i.e. the ratio between the concentration of the modified interconvertible enzyme at the steady state and the total interconvertible enzyme concentration. This parameter is often the basis for defining important steady state regulatory properties of monocyclic cascades, such as signal amplification, amplitude and sensitivity [3–8] or the transient time of the interconvertible protein and the mean regulation rate of the cascade [9].

The steady state fractional modification of the monocyclic enzyme cascades has been extensively studied, more or less under assumptions allowing the derivation of symbolic equations which are simplest when more assumptions are made [3,9–14]. In all these contributions the fractional modification refers to the fraction of the molecules of the free modified protein. Varon et al. [12] suggested a wider and more complete definition of the fractional modification including all forms of the modified protein, the free one and the intermediate complex converter enzyme of the original protein-modified protein. They gave the general expression of fractional modification hereby defined, denoted as f_∞, for the monocyclic cascades, but no discussion was made concerning the usual definition which was extensively studied by these authors. Obviously, both definitions coincide in those cases in which it is assumed that the intermediate complex mentioned above is negligible, but this assumption, although frequently used by some authors [15], as we will see, is unnecessary to reach an analytical expression of the fractional modification and limits the applicability of this expression.

Scheme 1 Scheme used for the study of the four different monocyclic cascades



This paper has, as a starting point, the general expression of f_∞ given by Varon et al. [12] and from this expression discusses: (1) the validity of this analytical expression and the fulfillment of the assumptions under which the expression was derived; (2) The possible additional assumptions that simplify the general expression for f_∞ yielding simplified expressions and (3) the applicability of the general equation for the complete fractional modification and its simplified expressions.

2 Material and methods

Steady state analytical solutions for the cyclic enzyme cascade in Scheme 1 under the linearizing *Assumptions 1–3* were found using the specific software wREFERASS [16] for the acquisition of the steady state equations of enzyme reactions in which the different interconversions between the enzyme forms are of first or pseudofirst order. Numerical integration and the corresponding simulated progress curves were obtained from the set of differential Eqs. (8)–(11), using arbitrary sets of rate constants and initial concentration values. This numerical solution was found by the Runge-Kutta-Fehlberg algoritm [17,18] using the software WES implemented in Visual C++ 6.0 [19]. Progress simulated curves in Fig. 1 are directly given by WES. Plots in Fig. 2 have been obtained using the SigmaPlot Scientific Computing System for Windows version 8.02 (SPSS Inc.). Both wREFERASS and WES software are disposable in the link http://oretano.iele-ab.uclm.es/~BioChem-mg/software.php.

3 The monocyclic cascades model

We will study the four different monocyclic cascades in the Scheme 1, where E_a and R_a are the active forms of the converter enzymes E and R, respectively and $o-I$ and $m-I$ are the original and modified forms, respectively, of the interconvertible protein, I. Scheme 1 yields four different Schemes [3–15], which we will denote as 1(a), 1(b), 1(c) and 1(d), according to how the active enzyme forms E_a and R_a are related with the corresponding inactive forms of E and R, E_i and R_i, through the modifying action (activating or deactivating) of the allosteric effectors e_1 and e_2 of E and R, respectively. In Table 1 we show the possible interactions between both the active and inactive forms of the enzymes E and R and their corresponding effectors

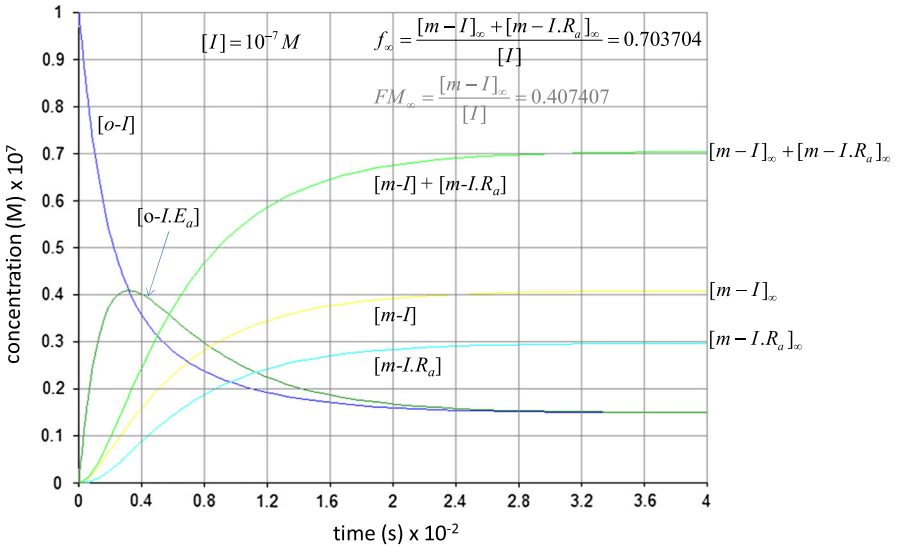


Fig. 1 Simulated time course curves of the different species involved in reactions [I] and [II] of Scheme 2, corresponding to case 7 in Table 3, obtained by numerical integration of the corresponding set of differential Eqs. (8)–(11) assuming constant $[E_a]$ and $[R_a]$, i.e. by adding to the above set of differential equations the following two ones: $d[E_a]/dt = 0$ and $d[R_a]/dt = 0$. Note that *Assumption a* is not observed. At the steady state, in this case, $[o - I]_\infty = [o - I.E_a]_\infty$ as expected from Eqs. (12) and (13) and the values of the rate constants and initial concentrations involved in them

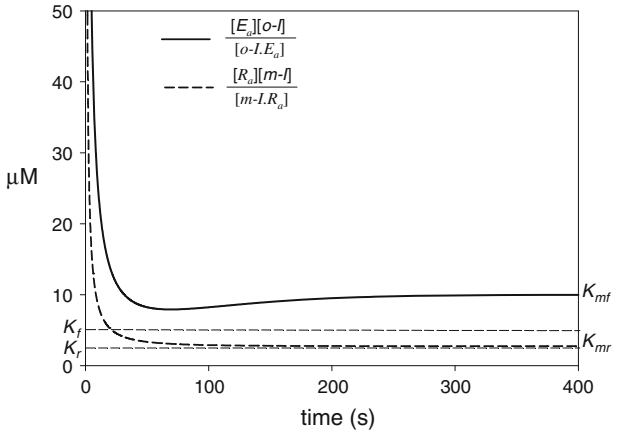


Fig. 2 Time course of the quotients $[E_a][o - I]/[o - I.E_a]$ and $[R_a][m - I]/[m - I.R_a]$ obtained from the results in Fig. 1. Note that in the dissociation of the complex $o - I.E_a$ the rapid equilibrium approach does not prevail (there is no coincidence of $[E_a][o - I]/[o - I.E_a]$ with K_f) neither at the transient phase nor at the steady state in which the quotient above is the value of K_{mf}. Nevertheless, in the dissociation of the complex $m - I.R_a$ the equilibrium is reached from approximately 100 s, being the quotient $[R_a][m - I]/[m - I.R_a]$ slightly higher than the equilibrium constant K_r that, therefore, is also approximately equal to K_{mr}. The values of K_{mf}, K_{mr} K_f and K_r obtained both from the values of the rate constants used for this example and the definitions given in Eqs. (4)–(7) are, respectively, 10, 2.75, 5 and 2.5 μM

Table 1 Possible interactions between both the active and inactive forms of the enzymes E and R in Schemes 1 and their corresponding effectors

Scheme	Step in which the active converter enzyme E_a is involved previously to its reaction with $o-I$	Step in which the active converter enzyme E_a is involved previously to its reaction with $m-I$
1(a)	$E_i + e_1 \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} E_a$	$R_i + e_2 \underset{k'_{-1}}{\overset{k'_1}{\rightleftarrows}} R_a$
1(b)	$E_i + e_1 \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} E_a$	$R_i \underset{k'_{-1}}{\overset{k'_1}{\rightleftarrows}} R_a + e_2$
1(c)	$E_i \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} E_a + e_1$	$R_i + e_2 \underset{k'_{-1}}{\overset{k'_1}{\rightleftarrows}} R_a$
1(d)	$E_i \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} E_a + e_1$	$R_i \underset{k'_{-1}}{\overset{k'_1}{\rightleftarrows}} R_a + e_2$

Scheme 2 Set of reaction steps in Scheme 1 where the interconvertible protein is involved

$$o-I+E_a \underset{k_{-2}}{\overset{k_2}{\rightleftarrows}} o-I.E_a \xrightarrow{k_3} E_a+m-I \qquad \text{[I]}$$

$$m-I+R_a \underset{k'_{-2}}{\overset{k'_2}{\rightleftarrows}} m-I.R_a \xrightarrow{k'_3} R_a+o-I \qquad \text{[II]}$$

for each of the Schemes 1(a)–1(b). From here, we will write Schemes 1 when referring to the common features of the four Schemes 1(a)–1(d).

The detailed set of reaction steps in Scheme 1 where the interconvertible protein is involved is shown in Scheme 2.

3.1 Notation

The notation in this work is chosen to coincide in part with that used in previous contributions [3,9–12,20] in order to facilitate the comparison that is made in the Results and Discussion section. Part of the notation has been introduced in the text above; further notations follow:

[I]: Concentration of the target interconvertible enzyme I

$[o-I]$, $[o-I.E_a]$, $[m-I]$, $[m-I.R_a]$: Concentrations at any time, t, of the enzyme species $o-I$, $o-I.E_a$, $m-I$ and $m-I.R_a$, respectively. From the mass conservation law it is observed that:

$$[I] = [o-I] + [o-I.E_a] + [m-I] + [m-I.R_a] \quad \text{(at any reaction time)} \qquad (1)$$

$[o-I]_0$, $[o-I.E_a]_0$, $[m-I]_0$, $[m-I.R_a]_0$: Initial concentrations, i.e. at the reaction time t = 0, of enzyme species $o-I$, $o-I.E_a$, $m-I$ and $m-I.R_a$, respectively.

Note that $[I]$ coincides with $[o-I]_0$ if, as assumed in the present analysis, it is observed that $[o-I.E_a]_0 = [m-I]_0 = [m-I.R_a]_0 = 0$.

$[o-I]_\infty$, $[o-I.E_a]_\infty$, $[m-I]_\infty$, $[m-I.R_a]_\infty$: Constant concentrations of enzyme species $o-I$, $o-I.E_a$, $m-I$ and $m-I.R_a$, respectively, at the steady state, i.e.: $[o-I]_\infty = \lim_{t\to\infty}[o-I]$; $[o-I.E_a]_\infty = \lim_{t\to\infty}[o-I.E_a]$; $[m-I]_\infty = \lim_{t\to\infty}[m-I]$; $[m-I.R_a]_\infty = \lim_{t\to\infty}[m-I.R_a]$.

f_∞ : Fraction of the interconvertible protein, I, that, in the steady state, was transformed into the enzyme forms containing $m-I$. In the cases of Schemes 1 it is given by:

$$f_\infty = \frac{[m-I]_\infty + [m-I.R_a]_\infty}{[I]} \tag{2}$$

In the following we will refer to f_∞ merely as *the complete fractional modification*. This complete fractional modification was introduced by Varon et al. [12] and its expressions were given, but no discussion of it has yet been carried out. In the literature about cyclic cascades, the fractional modification, which will denote here as FM_∞, is defined as:

$$FM_\infty = \frac{[m-I]_\infty}{[I]} \tag{3}$$

Obviously, the fractional modification thus defined approximately coincides with that in Eq. (2) in those cases, and only in those cases, in which $[m-I.R_a]_\infty \ll [m-I]_\infty$.

K_1: Equilibrium constant corresponding to the reversible reaction step where E_i, e_1 and E_a are involved, defined as: $K_1 = \frac{k_{-1}}{k_1}$

$K_1^{'}$: Equilibrium constant corresponding to the reversible reaction step where R_i, e_2 and R_a are involved, defined as: $K_1^{'} = \frac{k_{-1}^{'}}{k_1^{'}}$

K_{mf} : Michaelis constant of the forward reaction in step [I], i.e.:

$$K_{mf} = \frac{k_{-2}+k_3}{k_2} \tag{4}$$

K_{mr} : Michaelis constant of the reverse reaction in step [II], i.e.:

$$K_{mr} = \frac{k_{-2}^{'}+k_3^{'}}{k_2^{'}} \tag{5}$$

K_f : Equilibrium constant corresponding to the dissociation of the complex $o-I.E_a$, i.e.:

$$K_f = \frac{k_{-2}}{k_2} \tag{6}$$

K_r : Equilibrium constant corresponding to the dissociation of the complex $m-I.R_a$, i.e.:

$$K_r = \frac{k_{-2}^{'}}{k_2^{'}} \tag{7}$$

Table 2 *Assumptions 1, 2* and *3* (in the following *Assumptions 1–3*) are the minimal of those usually accepted in analysis of monocyclic enzyme cascades to their corresponding set of differential equations describing their kinetic behavior becoming linear. In the last column the global result of the *Assumptions 1–3* is shown

Scheme	*Assumption 1*	*Assumption 2*	*Assumption 3*	Global result of *Assumptions 1–3*
	Rapid equilibrium of the activation or inactivation reaction of the converter enzymes	$[o-I.E_a] \ll [E_a]$, $[E_i]$ and $[m-I.R_a] \ll [R_a]$, $[R_i]$	The concentrations of the allosteric effectors are maintained at constant levels	Constance of $[E_a]$ and $[R_a]$ and their expressions
(a)	$K_1 = \frac{[E_i][e_1]}{[E_a]}$ $K_1^{'} = \frac{[R_i][e_2]}{[R_a]}$	$[E] \simeq [E_i] + [E_a]$ $[R] \simeq [R_i] + [R_a]$	$[e_1]$ constant $[e_2]$ constant	$[E_a] = \frac{[E][e_1]}{K_1+[e_1]}$ $[R_a] = \frac{[R][e_2]}{K_1^{'}+[e_2]}$
(b)	$K_1 = \frac{[E_i][e_1]}{[E_a]}$ $K_1^{'} = \frac{[R_i]}{[R_a][e_2]}$	The same as in Scheme (a)	The same as in Scheme (a)	$[E_a] = \frac{[E][e_1]}{K_1+[e_1]}$ $[R_a] = \frac{K_1^{'}[R]}{K_1^{'}+[e_2]}$
(c)	$K_1 = \frac{[E_i]}{[E_a][e_1]}$ $K_1^{'} = \frac{[R_i][e_2]}{[R_a]}$	The same as in Scheme (a)	The same as in Scheme (a)	$[E_a] = \frac{K_1[E]}{K_1+[e_2]}$ $[R_a] = \frac{[R][e_2]}{K_1^{'}+[e_2]}$
(d)	$K_1 = \frac{[E_i]}{[E_a][e_1]}$ $K_1^{'} = \frac{[R_i]}{[R_a][e_2]}$	The same as in Scheme (a)	The same as in Scheme (a)	$[E_a] = \frac{K_1[E]}{K_1+[e_2]}$ $[R_a] = \frac{K_1^{'}[R]}{K_1^{'}+[e_2]}$

α_{mf} : Ratio between the rate constants $k_3$and the Michaelis constant K_{mf}, i.e.: $\alpha_{mf} = \frac{k_3}{K_{mf}}$

α_{mr} : Ratio between the rate constants $k_3^{'}$ and the Michaelis constant K_{mr}, i.e.: $\alpha_{mr} = \frac{k_3^{'}}{K_{mr}}$

α_f : Ratio between the rate constants k_3 and the equilibrium constant K_f, i.e.: $\alpha_f = \frac{k_3}{K_f}$

α_r : Ratio between the rate constants $k_3^{'}$ and the Michaelis constant K_r, i.e.: $\alpha_r = \frac{k_3^{'}}{K_r}$

3.2 Assumptions

It is very useful to make some reasonable assumptions which permit us to obtain approximate analytical solutions for $[o-I]_\infty$, $[o-I.E_a]_\infty$, $[m-I]_\infty$, $[m-I.R_a]_\infty$ in Schemes 1. We have performed part, but not all, of the assumptions made by other authors to obtain their steady state equations [3,10]. The three assumptions made are indicated in Table 2.

Assumptions 1–3 predict that $[E_a]$ and $[R_a]$ remain approximately constant from the onset of the reaction being their expressions for Schemes 1 given on the last column in Table 2.

4 Theory

The constancy of $[E_a]$ and $[R_a]$ allows the enzyme cascade be described only by the reactions in Scheme 2 whose corresponding set of differential equations are:

$$\frac{d[o-I]}{dt} = -k_2[E_a][o-I] + k_{-2}[o-I.E_a] + k_3^{'}[m-I.R_a] \tag{8}$$

$$\frac{d[o-I.E_a]}{dt} = -(k_{-2}+k_3)[o-I.E_a] + k_2[o-I][E_a] \tag{9}$$

$$\frac{d[m-I]}{dt} = -k_2^{'}[R_a][m-I] + k_{-2}^{'}[m-I.R_a] + k_3[o-I.E_a] \tag{10}$$

$$\frac{d[m-I.R_a]}{dt} = -(k_{-2}^{'}+k_3^{'})[m-I.R_a] + k_2^{'}[m-I][R_a] \tag{11}$$

At the steady state, $[o-I]$, $[o-I.E_a]$, $[m-I]$, $[m-I.R_a]$ reach the constant values $[o-I]_\infty$, $[o-I.E_a]_\infty$, $[m-I]_\infty$ and $[m-I.R_a]_\infty$. To find the complete fractional modification, f_∞, of Schemes 1 at the steady state we need to know $[m-I]_\infty$ and $[m-I.R_a]_\infty$ and then to divide their sum by [I], according to Eq. (2). It will also be interesting below to know also the expressions of $[o-I]_\infty$ and $[o-I.E_a]$.

The derivation of equations which gives us the enzyme species concentrations and/or the rate of any ligand species at the steady state for any reaction mechanism is a task which necessarily has to be carried out in most of the kinetic analysis of all enzyme reactions. Done by hand using the usual well-known procedures, this becomes tedious, time-consuming and prone to human errors even for reaction mechanisms which are not too complex, as the one here analyzed. This is the reason of the considerable number of contributions dealing with the implementation of adequate software which allows obtaining either the steady state equations [21–32] or both the transient and steady state equations [21–23,33–36]. We have used the software wREFERASS [16] with the following results:

$$[o-I]_\infty = \frac{k_2^{'}k_3^{'}(k_{-2}+k_3)[R_a][I]}{Den} \tag{12}$$

$$[o-I.E_a]_\infty = \frac{k_2k_2^{'}k_3^{'}[E_a][R_a][I]}{Den} \tag{13}$$

$$[m-I]_\infty = \frac{k_2k_3(k_{-2}^{'}+k_3^{'})[E_a][I]}{Den} \tag{14}$$

$$[m-I.R_a]_\infty = \frac{k_2k_2^{'}k_3[E_a][R_a][I]}{Den} \tag{15}$$

where:

$$Den = k_2k_3(k_{-2}^{'}+k_3^{'})[E_a] + k_2^{'}k_3^{'}(k_{-2}+k_3)[R_a] + k_2k_2^{'}(k_3+k_3^{'})[E_a][R_a] \tag{16}$$

If in Eq. (2) we take into account Eqs. (12)–(16), we have, after some convenient rearrangement:

Table 3 Set of concentrations and initial concentrations, arbitrarily chosen, for different cases we will use to support our results

Case	[I] (M)	$[E_a]$ (M)	$[R_a]$ (M)	k_2 ($M^{-1}s^{-1}$)	k_{-2} (s^{-1})	k_3 (s^{-1})	k'_2 ($M^{-1}s^{-1}$)	k'_{-2} (s^{-1})	k'_3 (s^{-1})
1	10^{-7}	10^{-5}	5×10^{-6}	10^7	100	1	2×10^7	200	2
2	10^{-7}	10^{-8}	5×10^{-8}	10^8	100	1	4×10^8	800	2
3	10^{-7}	10^{-8}	2×10^{-8}	10^8	100	100	4×10^8	800	200
4	10^{-7}	10^{-5}	2×10^{-6}	10^8	100	100	4×10^8	800	200
5	10^{-7}	10^{-5}	5×10^{-8}	10^8	100	1	4×10^8	800	2
6	10^{-6}	10^{-5}	5×10^{-6}	10^7	100	1	2×10^7	200	2
7	10^{-7}	10^{-5}	2×10^{-6}	4×10^3	0.02	0.02	4×10^4	0.1	0.01

$$f_\infty = \left[1 + \frac{\alpha_{mr}[R_a]}{\alpha_{mf}[E_a]} + \frac{[R_a]}{K_{mr}} + \frac{\alpha_{mr}[R_a]}{k_3}\right]^{-1}\left(1 + \frac{[R_a]}{K_{mr}}\right) \tag{17}$$

5 Results and discussion

In this paper we discuss the fractional modification, given by Eq. (17), for the important monocyclic reversible enzyme cascades shown in Scheme 1. To the derivation of Eq. (17) one needs *Assumptions 1–3*, therefore, it is valid whenever these assumptions are observed. It is to be noted that, according to Eq. (17) f_∞ is $[I]$-independent.

Equation (17) is valid for the four different specific Schemes 1(a)–1(d) arising from Scheme 1 and Table 1. These schemes differ in the way the active enzymes E_a and R_a are related with the corresponding effectors. Thus, in Schemes 1(a) and 1(b) E_a proceeds from the activation by e_1 of E_i, whereas in Schemes 1(c) and 1(d) E_a is inactivated by e_1 to give E_i. Likewise, in Schemes 1(a) and 1(c) R_a proceeds from the activation by e_2 of R_i, whereas in Schemes 1(b) and 1(d) R_a is inactivated by e_2 to give R_i.

The dependence of Eq. (17) on $[E_a]$ and $[R_a]$ is the same for the four Schemes 1 [Schemes 1(a)–1(b)]. Nevertheless they formally differ when the expressions for $[E_a]$ and $[R_a]$ are replaced by those indicated in Table 2 for each of the different Schemes 1 so that now the corresponding equations for each of the Schemes 1(a)–1(d) show a different dependence of f_∞ on $[E]$, $[e_1]$, $[R]$ and $[e_2]$. The obtaining of these specific equations for each of the four enzyme cascades mentioned, using the procedure commented above, is obvious. Thus, as an example, for Scheme 1(a) the resulting equation is, after some ease rearrangement:

$$f_\infty = \left[1 + \frac{\alpha_{mr}[R][e_2]}{K_1' + [e_2]}\left(\frac{K_1 + [e_1]}{\alpha_{mf}[E][e_1]} + \frac{1}{k_3'} + \frac{1}{k_3}\right)\right]^{-1}\left(1 + \frac{[R][e_2]}{K_{mr}(K_1' + [e_2])}\right) \tag{18}$$

So as not to unnecessarily increase the length of this paper, we do not give the corresponding expressions for the other three enzyme cascades.

Table 4 Values of the parameters α_{mf}, α_{mr} and K_{mr} involved in Eq. (17) and the corresponding f_∞-value obtained from this equation, for each of the cases 1–7 in Table 1 [the k_3-value involved in Eq. (17) was already given in Table 3 for each case]

Case	α_{mf}	α_{mr}	K_{mr}	f_∞ Eq. (17)	FM_∞ Eq. (20)
1	9.90×10^4	1.98×10^4	1.01×10^{-5}	0.429	0.287
2	9.90×10^5	1.00×10^6	2.01×10^{-6}	0.168	0.164
3	5.00×10^7	8.00×10^7	2.50×10^{-6}	0.239	0.237
4	5.00×10^7	8.00×10^7	2.50×10^{-6}	0.484	0.269
5	9.90×10^5	1.00×10^7	2.01×10^{-6}	0.949	0.926
6	9.90×10^4	2.00×10^5	1.01×10^{-5}	0.429	0.287
7	2.00×10^3	3.64×10^3	2.75×10^{-6}	0.704	0.407

In the last column the value of a fractional modification, denoted as FM_∞, for cases 1–7, defined in an alternative, simpler way explained below, is also given. Note that for cases 1 and 6, differing only in their $[I]$-values we have the same result. This is due to the fact that expressions for f_∞ and FM_∞ are $[I]$-independent

In Table 4 we show the values of the kinetic parameters corresponding to cases 1–7 of Table 3 involved in Eq. (17).

5.1 Another more simple definition for the steady state fractional modification of monocyclic enzyme cascades

The different regulatory properties of the monocyclic enzyme cascade, such as the signal amplification, amplitude and sensitivity [5–7,37–40] and other regulatory properties suggested by Varon et al. [9] are based on a simpler definition of the steady state fractional modification of monocyclic cascades, which we denote as FM_∞ to distinguish it from the fractional modification, f_∞, studied here, as:

$$FM_\infty = \frac{[m - I]}{[I]} \tag{19}$$

which, taking into account Eqs. (14), (16) and (19), becomes:

$$FM_\infty = \left[1 + \frac{\alpha_{mr}[R_a]}{\alpha_{mf}[E_a]} + \frac{[R_a]}{K_{mr}} + \frac{\alpha_{mr}[R_a]}{k_3}\right]^{-1} \tag{20}$$

Note that, from Eqs. (17) and (20), the following relationships between f_∞ and FM_∞ is:

$$f_\infty = FM_\infty \left(1 + \frac{[R_a]}{K_{mr}}\right) \tag{21}$$

i.e., it is always observed that $f_\infty > FM_\infty$, as expected from the respective definitions. Definition in Eq. (19) only refers to the fraction of molecular species $m - I$. The definition used in this paper is more general because it refers to all forms of the

modified protein, i.e. to $m - I$ and $m - I.R_a$ species. Varon et al. [12] carried out an extensive analysis of the fractional modification of the same monocyclic enzyme cascades studied here, but based exclusively on the definition in Eq. (20). These same authors suggested the definition of the fractional modification given in Eq. (2), but then they omitted the corresponding analysis, which we have developed in the present contribution.

For the same Schemes 1 the results of defining the fractional modification as in Eq. (17) or as Eq. (20) may be very different. In Table 4 we have included the values obtained for the fractional modification of cases 1–7 using both equations. This differences makes it advisable to revisit the regulatory properties of the cascades on the basis of the expression for the complete fractional modification, f_∞, and thus to obtain new expressions for these properties.

5.2 Particular cases of general Eq. (17)

General Eq. (17) for the fractional modification, f_∞, has been derived under *Assumptions 1–3*. Nevertheless, the steady state kinetics of monocyclic enzyme cascades has been extensively studied under *Assumptions 1–3* and other additional ones which are not strictly necessary for the derivation of analytical equations and which are, in most cases, unwarranted [3,11,12]. The unnecessary additional assumptions have, apparently, three advantages: (1) if they are used previously to attempt the derivation of analytical solutions, they facilitate this derivation; (2) the final form of these analytical solutions are simpler and more manageable; (3) if these additional assumptions are made after the obtaining of an analytical solution, then this may become considerably reduced and, therefore, as said above, more manageable. The most frequent additional assumptions used are the following *Assumptions a* and *b*.

Assumption a: Both reversible steps in reactions [I] and [II] from Scheme 2 are in equilibrium, i.e.:

$$\frac{k_3}{k_{-2}} \ll 1 \text{ and } \frac{k_3^{'}}{k_{-2}^{'}} \ll 1 \tag{22}$$

Relationship (22) must be fulfilled simultaneously and if they are keeping in mind in Eqs. (4)–(7), we have that this *Assumption a* yields:

$$K_{mf} \simeq K_f;\ K_{mf} \simeq K_f;\ \alpha_{mf} \simeq \alpha_f;\ \alpha_{mr} \simeq \alpha_r \tag{23}$$

Assumption b: The concentrations of the enzyme-converter protein complexes are negligible in comparison with the concentration of the active and inactive enzymes, i.e.:

$$[o - I]_\infty + [o - I.E_a]_\infty + [m - I]_\infty + [m - I.R_a]_\infty \simeq [o - I]_\infty + [m - I]_\infty \tag{24}$$

For the fulfillment of *Assumption b*, it is necessary and sufficient that the four following relationships are observed:

$$[o - I.E_a]_\infty \ll [o - I]_\infty;\ [m - I.R_a]_\infty \ll [o - I]_\infty;$$
$$[o - I.E_a]_\infty \ll [m - I]_\infty;\ [m - I.R_a]_\infty \ll [m - I]_\infty \tag{25}$$

If in relationship (25), arising from *Assumption b,* Eqs. (12)–(15) are taken into account, the following four conditions (26) must be simultaneously fulfilled:

$$\frac{[E_a]}{K_{mf}} \ll 1;\ \frac{\alpha_{mf}[E_a]}{k_3^{'}} \ll 1;\ \frac{\alpha_{mr}[R_a]}{k_3} \ll 1;\ \frac{[R_a]}{K_{mr}} \ll 1 \tag{26}$$

If, previously to *Assumption b, Assumption a* is made, then the relationship (26) corresponding to *Assumption b* must be expressed as:

$$\frac{[E_a]}{K_f} \ll 1;\ \frac{\alpha_f[E_a]}{k_3^{'}} \ll 1;\ \frac{\alpha_r[R_a]}{k_3} \ll 1;\ \frac{[R_a]}{K_r} \ll 1 \tag{27}$$

In Eq. (17) we may introduce either only *Assumption a*, only *Assumption b* or both *Assumptions a* and *b*.

Effect of introducing *Assumption a* in Eq. (17).

If in Eq. (17) we insert relationship (23) corresponding to *Assumption a*, it becomes:

$$f_\infty = \left[1 + \frac{\alpha_r[R_a]}{\alpha_f[E_a]} + \frac{[R_a]}{K_r} + \frac{\alpha_r[R_a]}{k_3}\right]^{-1}\left(1 + \frac{[R_a]}{K_r}\right) \tag{28}$$

Effect of introducing *Assumption b* in Eq. (17).

If in Eq. (17) we insert relationship (27) corresponding to *Assumption b*, it becomes:

$$f_\infty = \left[1 + \frac{\alpha_{mr}[R_a]}{\alpha_{mf}[E_a]}\right]^{-1} \tag{29}$$

Effect of introducing in Eq. (17) **simultaneously both *Assumptions a* and *b***

Finally, if both *Assumptions a* and *b* are inserted in Eq. (17), then we directly obtain:

$$f_\infty = \left[1 + \frac{\alpha_r[R_a]}{\alpha_f[E_a]}\right]^{-1} \tag{30}$$

Comparison of the expressions of the fractional modification, f_∞, given by the general and the particular ones emanating from it.

In Table 5 we give the values of k_3/k_{-2}, $k_3^{'}/k_{-2}^{'}$, $[E_a]/K_{mf}$, $\alpha_{mf}[E_a]/k_3^{'}$, $\alpha_{mr}[R_a]/k_3$, $[R_a]/K_{mr}$ for each of the cases 1–7 in Table 3 pointing out if *Assumptions a* and/or *b* are observed and in Table 6 we give, for each of these same cases, the values obtained for f_∞ using general Eq. (17) and its particular Eqs. (28)–(30). Note that when only *Assumption a*, only *Assumption b* or both of them are observed, Eqs. (28)–(30) furnish good results, but no in the contrary case.

As an example, in Fig. 1 we show the simulated progress curves, from t=0, for all of the enzyme species involved in the cascade for the case 7 in Table 3. These

Table 5 Values of the relationship involving rate and/or equilibrium constants and/or concentrations given in Eqs. (22) and (26) determining whether *Assumptions a* and *b* are observed or not

Case	k_3/k_{-2}	$k_3^{'}/k_{-2}^{'}$	$[E_a]/K_{mf}$	$\alpha_{mf}[E_a]/k_3^{'}$	$\alpha_{mr}[R_a]/k_3$	$[R_a]/K_{mr}$	Is *Assumption a* observed?	Is *Assumption b* observed?
1	0.010	0.010	0.990	0.495	0.990	0.495	Yes	No
2	0.010	0.025	0.099	0.005	0.050	0.025	Yes	Yes
3	1.000	0.250	0.005	0.003	0.016	0.008	No	Yes
4	1.000	0.250	9.901	4.950	1.995	0.998	No	No
5	0.010	0.025	0.099	0.005	0.050	0.025	Yes	Yes
6	0.010	0.010	0.990	0.495	0.990	0.495	Yes	No
7	1.000	0.100	1.000	2.000	0.364	0.727	No	No

Table 6 Values of f_∞ given by simplified Eqs. (28)–(30). To allow comparison with the corresponding true results for f_∞, these ones are indicated, on the 3rd column, extracted from Table 4 and repeated here for ease

Case	*Assumptions a* or *b* observed, if any, according to Table 5	f_∞ from Eq. (17)	f_∞ from Eq. (28)	f_∞ from Eq. (29)	f_∞ from Eq. (30)
1	Only *Assumption a* is observed	0.429	0.429	0.500	0.500
2	Both *Assumptions a* and *b* are observed	0.168	0.169	0.166	0.167
3	Only *Assumption b* is observed	0.239	0.333	0.238	0.333
4	Neither *Assumption a* nor *Assumption b* are observed	0.484	0.476	0.758	0.833
5	Both *Assumption a* and *b* are observed	0.949	0.949	0.995	0.995
6	Only *Assumption a* is observed	0.429	0.429	0.500	0.500
7	Neither *Assumption a* nor *Assumption b* are observed	0.704	0.750	0.733	0.833

progress curves have been obtained from the set of differential Eqs. (8) and (11) to which must be added the following two: $d[E_a]/dt = 0$ and $d[R_a]/dt = 0$ because the consequence of *Assumptions 1–3* is that both $[E_a]$ and $[R_a]$ remain constant during the reactions progress.

From Fig. 1, it is obvious that neither $[o - I.E_a]_\infty$ nor $[m - I.R_a]_\infty$ is negligible in comparison with both $[o - I]_\infty$ and $[m - I]_\infty$, i.e. that *Assumption b* is not fulfilled. To see the lack of fulfillment of *Assumption a* we have plotted, from the simulated progress curves in Fig. 1, curves in Fig. 2. Note that although K_{mr} is near to K_r, K_{mf} it is very different from K_f so that *Assumption a* is not observed.

In Table 6 we compare the results given by general Eq. (17) and the simplified Eqs. (28)–(30). Note the high level of concordance between the true result given by Eq. (17) and that of the corresponding simplified equation when *Assumption a*, *b* or both are observed.

5.3 Some additional remarks about general Eq. (17) and simplified Eqs. (28)–(30)

In this paper we have discussed the steady state fractional modification defined as in Eq. (17), which is a more complete definition than the previous one. The most important contribution of this paper is that in it one analyses Eq. (17), arising from Eq. (2) and Eqs. (12)–(15), in a triple sense: (1) by checking its validity using numerical integration, (2) by comparing it with another previous, more limited equation for the fractional modification [12] and (3) showing the way to obtain, from Eq. (17), different equations when *Assumption a, Assumption b* or both *Assumptions a* and *b* are simultaneously used, Eqs. (28)–(30) respectively. One of these particular equations, Eq. (28), is obtained here the first time. One of them, Eq. (29), was also obtained by Varon et al. [12] as a particular case of Eq. (20) when only *Assumption b* was used. Finally, Eq. (29) was already obtained in an individualized analysis of the monocyclic cascades when Assumption a and b, together with *Assumptions 1–3*, were used from the beginning of the analysis [3] and also by Varon et al. [12] as a particular cases of Eq. (20) when in it both *Assumptions a* and *b* were inserted.

Both sets of assumptions, necessary *Assumptions 1–3* required to derive Eq. (17), and additional unnecessary *Assumptions a* and *b* used to obtain Eqs. (28)–(30) either as particular cases of Eq. (17) or in an individualized analysis, require certain restrictive relationships involving rate and/or equilibrium constants and/or concentrations as those above given for each of these assumptions. Obviously, the necessary *Assumptions 1–3* are indispensable, but the use of the additional, unnecessary *Assumptions a* and *b* has great inconveniences which outweigh the above mentioned advantages of their use. One of these inconveniences is that the simpler equations Eqs. (28)–(30) resulting are only applicable to the enzyme system under study, if the mentioned relationships are fulfilled. The greater the number of additional assumptions used to derive the kinetic equations or to simplify them once obtained, the more they move away from the real system for which such equations are intended, i.e. the range of applicability of the equations diminishes and they become less accurate.

Simplified Eqs. (28)–(30) are only applicable if the assumptions under which they have been obtained are observed. Thus, Eq. (28) is only applicable only if *Assumptions 1–3* and *a* are observed, Eq. (29) is applicable only if *Assumptions 1–3* and *b* are observed and Eq. (30) is applicable only if *Assumptions 1–3* and *a* and *b* are observed. The problem is that it is impossible to know *a priori* what assumptions are observed in a particular enzyme cascade under study. Obviously, the probability of the results for the kinetic behavior of the system being correct will be greater if we use directly Eq. (17), which is less demanding and more general than Eqs. (28)–(30) which are very demanding and more limited. Obviously, simplified Eqs. (28)–(30) could be applied to cases to which they should not be applied but then, the results would be inaccurate.

As above commented for general Eq. (17), particular Eqs. (28)–(30), are valid for any of the four different Schemes 1(a)–1(d) shown in Table 1. If one wants to express the fractional modification as a function of $[E]$, $[R]$, $[e_1]$ and $[e_2]$ then both $[E_a]$ and $[R_a]$ must be merely replaced by the corresponding expressions in Table 2 for each of the four cascades 1(a)–1(d), resulting now in different expressions of the fractional modification for each of the four Schemes. As an example, Eq. (29) for Scheme 1(c) becomes:

$$f_\infty = \left[1 + \frac{\alpha_{mr}[R][e_2](K_1 + [e_1])}{\alpha_{mf} K_1 [E](K_1^{'} + [e_2])}\right]^{-1} \tag{31}$$

Let us finally point out, as a summary of this section, that the relevance of the steady state fractional modification is largely justified in the previous contributions for monocyclic enzyme cascades ([9–12]). As explained here, in these enzyme systems, part of the original enzyme is modified yielding a free modified enzyme and the intermediate complexes modified enzyme-activating enzyme of the modified enzyme and this fact is indicated in the corresponding reaction mechanisms. Nevertheless, this intermediate was not yet taken into account in the obtaining of the expression of the steady state fractional modification, i.e. of the fraction of the original enzyme which has been transformed into a modified enzyme. The neglection of this intermediate is generally based on the biologically unjustified assumption that its concentration at the steady state is much lower than that of the free modified enzyme. This assumption has, as immediate results, a more easily derivation of the expression for the fractional modification and a simpler final formula. But this assumption has the disadvantage that it doesn't correspond to the system under study, as is obvious from Figs. 1 and 2. Here we circumvent this limiting situation giving a general expression, Eq. (17), for the fractional modification which includes, as some of its different particular cases those situations in which the concentration of the intermediate mentioned can be reasonably neglected Eqs.(28) and (30).

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