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Kinetics of thermal deactivation of enzymes: a simple three parameters phenomenological model can describe the decay of enzyme activity, irrespectively of the mechanism.

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Abstract

Heat induced enzyme inactivation or protein denaturation is now well documented, due to progresses in methods, instruments and computation resources. Complex mechanisms, rather than the classic simple "one step - two states" model (still in use) are recognized in many cases, leading investigators to manipulate more or less complicated kinetic expressions describing the heat induced decay of enzyme activity.

We show that the different kinetic expressions related to different mechanisms among the most frequently encountered can be arranged in a common simple three-parameters biexponential equation.

This unifying simplification is of interest for people focusing attention to phenomenological rather than mechanistic description of the kinetics of heat induced enzyme deactivation. Moreover, the reduction in the number of parameters reduces the risk of cross-correlation and allows a better estimation of the apparent rate constants (which are in many cases the pertinent required information). It also illustrates the difficulty to make inference of mechanism from kinetics, since the same equation applies for a variety of mechanisms ("kinetic homeomorphism") - in particular, it stresses out the need of caution when reporting on existence of isoenzymes from deactivation kinetics.

Application of this simple 3-parameters biexponential kinetic expression has been validated with a number of results in the Literature and current investigations in our laboratory. Two examples are given.

Keywords: Thermal denaturation; Enzyme deactivation; Kinetics

1. Introduction

Endogenous enzymes in raw food ingredients may have beneficial or detrimental effects on the nutritional, functional - sensorial properties of the derived feeds. The question of thermal deactivation of enzymes is therefore of strong interest for food scientists, with the scope of a better description of the time course of the phenomenon and understanding of the relevant mechanisms. A similar concern exists in food technology when an enzyme is used at a certain step of an industrial process, with the need to "kill" the catalyst at a further step. This explains the large number of works devoted to the description of the deactivation of enzymes during the past three decades.

The thermal deactivation of enzymes has been and is still now often described as a "one step - two states" process (Fig. 1, scheme 1) where the native (active) form is transformed in the denaturated (inactive) form by a first order unimolecular irreversible reaction. Kinetically speaking, the decay in enzyme activity, expressed as the ratio of the measured activity (A)t at time t of heat treatment to the initial (control) activity (A)o is described by the simple exponential equation of a first-order process with k as the rate constant:

$$(A)t/(A)o = \exp(-k \cdot t) \quad (1)$$

In some cases, authors consider the possible partial retention of activity by the transformed species. This is often admitted for hemoenzymes as peroxidases [1] but it may also reflect an insufficient duration of the thermal process and a lack of sensitivity in the measure of enzyme activity in relation with the existence of a thermoresistent form of the enzyme.

In many other situations, the simple exponential model

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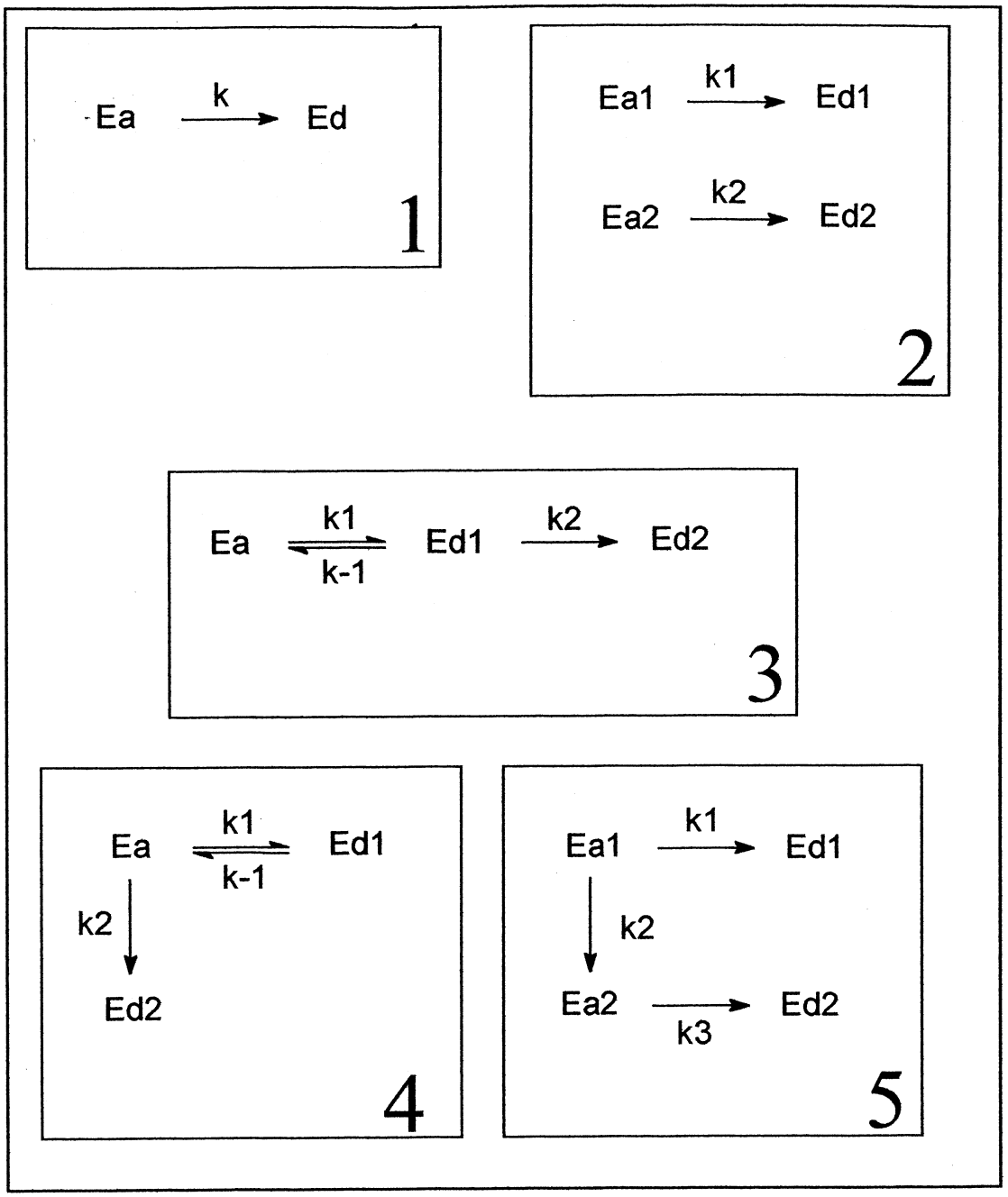

Fig. 1. Some mechanisms of thermal inactivation of enzymes
Scheme 1: simple “one step - two states” reaction
Scheme 2: simplest heterogeneous system, with two enzymatic species (“parallel” deactivation)
Scheme 3: sequence of two consecutive reactions (“series” deactivation)
Scheme 4: competitive (simultaneous) reactions
Scheme 5: mixed competitive-consecutive reactions

cannot fit to the experimental data and the “one step - two states” mechanism must be abandoned. More or less complicated alternative models have been derived and classified. Their kinetic expressions are more complicated than the simple monoexponentiel equation. During near to three decades, detailed integrated rate expressions for various complex mechanisms have been made available in academic textbooks and publications. When individual rate constants are considered, complex expressions are obtained, even if simplifying assumptions are made, such as:

-individual steps in any mechanism are first-order unimolecular reactions

-measured activity is directly proportional to concentrations of active enzyme forms

A short description of the more frequently encountered mechanisms will give the necessary theoretical background for this article:

-"parallel models" (Fig. 1, scheme 2) where the enzyme is in fact a mixture of at least two active forms (isoforms, isoenzymes) with different heat sensitivities (and eventually catalytic properties), each following its own first order, unimolecular evolution, with its proper velocity constant. The scheme can be complicated by retention of partial activity by the final species. The enzymatic parameters kcat (rate constant of the limiting step in the catalytic process) and K*m* (Michaelis constant) for the two active forms may be equal or not, but when the enzyme activity is measured at "saturating substrate concentration," the possible difference in K*m* values for the two active forms is no more to be considered.

The "parallel" label used for such a mechanism [2–3] is ambiguous, since the same word designs for a chemist a mechanism starting from a common precursor, evolving in the same time and the same medium to different directions by different reactions. To unify the terminology between chemical kinetics and enzyme deactivation kinetics, "parallel" could be replaced by "concomitant."

From a kinetics point of view, the mechanism considered here is the simpler among the "complex" ones. Kinetic expression for the enzyme activity decay is well known (see for example [3–5]). For the simple situation with two active isoenzymes, decay in activity is described by:

$$(A)t/(A)o = a \cdot \exp(-k1 \cdot t) + (1 - a) \cdot \exp(-k2 \cdot t) \quad (2)$$

k1 and k2 are inactivation rate constants. If the catalytic properties of the two forms are the same, a is the proportion of the active form Ea1, (1-a) being of course the proportion of Ea2. If they are not, a and (1-a) are no longer the initial proportions of the two active forms but rather the relative activities, standardized with respect to the total activity of the extract.

- "series models" (Fig. 1, scheme 3) where the initial active form produces the ultimate inactive state of evolution through a sequence of first order reactions.

Reversibility of some steps of the sequence can be considered, as well as a retained partial activity by intermediates and/or final state, thus giving "sub-cases" of increasing complexity. A simple series scheme is only with two steps, three species: starting from the initial active form Ea, the form Ed2 is obtained via the intermediate Ed1 and two unimolecular, first-order reactions. The first step can be reversible or not and in this case the second step will be considered as an always irreversible reaction (the true fully reversible denaturation of proteins/enzymes is quite rare and generally observed in a narrow spectrum of physico-chemical conditions). Ed1 and Ed2 may retain or not some activity. The complete kinetic expression for the decay of activity when k-1 = 0 is well known, it can be found for instance in [4], [6–9]. Beyond the apparent complexity, a 4 parameters -biexponential formalism can be recognized (with a constant term to be added if Eb retains some activity):

$$(A)t/(A)o = A \cdot \exp(-\alpha \cdot t) + B \cdot \exp(-\beta \cdot t) \quad (3)$$

In this simplified writing, the four parameters are complex terms related to individual rate constants–but α and β retain the sense of apparent first-order rate constants.

-"competitive" or "simultaneous" models (Fig. 1, scheme 4) where a precursor (active enzyme) is inactivated via two independent reactions. In chemical kinetics, such a scheme is also labeled as "parallel." When one of the competitive reactions is reversible, the system can also be considered as a series model, the relevant exact classification to adopt depends on the position of the precursor (initial reactant = active enzyme) - compare schemes 3 and 4. The kinetic expressions for all species in a mechanism depicted in scheme 4 or more complicated ones are known. An example of kinetic analysis coming from biochemisty can be found in [10]. Admitting that enzyme activities and concentrations of enzyme active species are proportional, the kinetic expression of the decay of enzyme activity can be easily derived from the integrated kinetic expressions, after some transpositions and usual assumptions (for example, a t = 0, no other species are present except the initial reactant = active enzyme). In these conditions, here again, a 4-parameters biexponential equation is obtained, exactly in the same general form as above (equation 3), with the same remarks about the parameters.

-more complex hybrid mechanisms, mixing series and competitive (so-called "parallel") mechanisms. Scheme 5 in Fig. 1 corresponds to what a chemist would label as competitive-parallel or series-parallel hybrid (mixed) mechanism, with the same comment about the sense of the word "parallel," used in enzymology with an other meaning. Several reports of deactivation mechanisms depicted in scheme 5 can be found in the Literature - [11] is a recent example. The kinetic expression of activity decay also reduces (with some simplifying assumptions) to a 4-parameters biexponential equation (equation 3). The four parameters are, as before, complex terms related to individual rate constants, and α and β are apparent first-order rate constants.

We show here that at the price of simple algebraic manipulations the kinetic equation for enzyme deactivation in a diversity of models can be arranged in a single simpler equation with only three parameters, when conditions for using a 4-parameters biexponential relation prevail. Astonishingly, this simplification was not pointed out in the very numerous available descriptions of complex kinetics, in chemistry or biochemistry textbooks and research publications.

The universal character of this 3 parameters - biexponential expression was demonstrated by "back processing" data from a number of publications. Current work in our

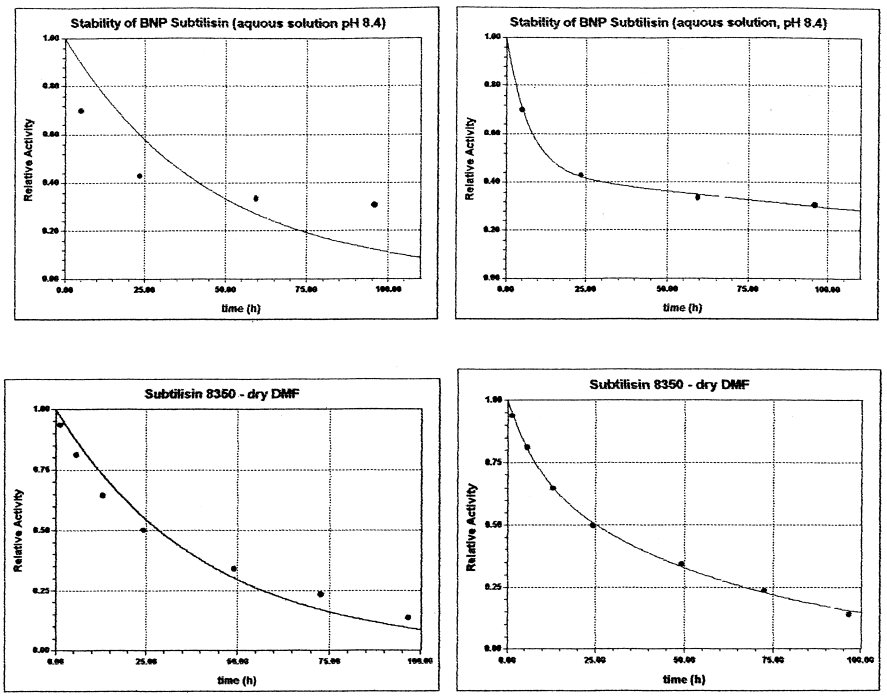

Fig. 2. Graph of the data from Fig. 4 in WONG C.H., (1990), replotted in the same coordinate system. The equation of the fitted curves is the simple exponential equation for a first-order loss of activity (left) or the 3-parameters biexponential equation derived in the text (right). Upper panels are for the wild form of the enzyme, lower panels are for the mutant form of the enzyme

laboratory also gave an opportunity to apply this kinetic equation (results to be published).

This simplification is of interest for people with practical purposes in mind, focusing attention on phenomenological rather than mechanistic description of the kinetics of enzyme deactivation - specially in the context of biotechnology (stabilization of enzymes through molecular modification, immobilization, choice of solvent . . .).

It also gives the occasion to recall that inference of reaction mechanism from kinetic data are always difficult, since a single simple equation can suit to a variety of mechanisms. Insufficient awareness of this kinetic homeomorphism makes questionable the numerous reports in the biochemical literature on the presence of isoenzymes in extracts, stated only from deactivation kinetics.

2. Materials and methods

Data for exemplifying this article came from publications. Our own experimental results will be reported later. As original experimental values were not available, they were "captured" from scanned images of figures in publications, using a simple procedure that we call "pixel conversion," consisting in moving the cursor of the mouse to the points of interest on the scanned image displayed on the screen of a computer for reading the positions, in pixels (this is allowed by most of drawing programs). Conversion of these readings in original X-Yvalues is then a simple matter of calculation (AYMARD and SHIRTS, *Journal of Chemical Education*, in the press) - in most cases, errors in evaluation of original values is less than 3–4%. A full computerized version of this procedure is available ("Clicker" PC program written on the basis of our publication by Pr E.F HEALD, at Thiel College, Greenville, PA 16125 - USA). Not very expensive but very efficient commercial packages must also be mentioned: UNGRAPH from BIOSOFT (USA) and UN-SCAN-IT from SILKSCIENTIFIC are examples. . . .

This approach was used a number of times, with published results - only two examples will be given here: data extracted from Fig. 4 in [12] and data extracted from Fig. 2 in a recent publication ([9]).

As recommended since a long time by several authors [13–16], processing of data were done by direct curvilinear regression, without any transformation. The points of awareness periodically given in the Literature about the bias introduced by logarithmic transformation and linear regression seem not very widely accepted by researchers, who currently plot the data (activity versus time) in a semi-logarithmic graph, a breakdown in the regression line being considered as the diagnostic of a "biphasic" behavior. Complicated graphical procedures ("peeling off" technique for instance) remain in favor to analyze these so-called "biphasic" kinetics.

In our concern, curve fitting by direct least-squares curvilinear regression was performed with a computerized program using the LEVENBURG-MARQUARDT algorithm.

The regressor was the time, the regressand was the relative residual activity, i.e. the ratio of the activity (A)t after a given duration t of heat treatment to the initial activity (A)o. The regression model was the simple three parameters biexponential equation that we will show to be a common form of transformation of more complex kinetic expressions for different deactivation mechanisms.

3. Results

3.1. Derivation of the simple three parameters kinetic model

In spite of their diversity, most of the complex mechanisms can lead to a common 4-parameters biexponential kinetic expression for the activity decay (equation 3), easily recognized behind the complex kinetic equations given in textbooks and research publications.

The parameters of this equation are complex functions of individual rate constants, but α and β have always the dimension of first-order rate constants. The sense of parameters and their expressions as function of individual rate constants differ according to the considered mechanism. The situation with isoenzymes is a very special situation where A and B are generally (but not always) related to the proportions of active species whereas α and β are true rate constants and not apparent constants as in the other cases.

This "convergence" toward a common mathematical formalism (equation 4) has been insufficiently pointed out in textbooks or research publications, where the kinetic equations are given in full details, with individual rate constants. A noticeable exception is a very recent publication [17] where the 4-parameters - biexponential formalism is presented as common for kinetics of the two main simplest situations among complex mechanisms.

We show here that when considering the full expressions (with individual constants) prevailing for different mechanisms, a simple algebraic manipulation leads to a simpler equation, since it is with only three (complex) parameters:

$$(A)t/(A)o = A \cdot \exp(-\alpha \cdot t) + (1 - A) \cdot \exp(-\beta \cdot t) \quad (4)$$

This is the formalism already known for a system with isoenzymes but in the general sense of this expression, constants in the exponential arguments are no longer true rate constants - they are complex expressions of rate constants. To stress out this point, as usually done, we use here Greek symbols rather than the k-notation. Except in the case of a system with isoenzymes, the A-parameter is also a complex function of individual rate constants.

Simplification of equation 3, the common expression for a diversity of mechanisms, into the simpler equation 4 just needs some algebraic manipulations, with a general strategy consisting in forming the sum of parameters A and B. This leads to (A + B) = 1, thus B = (1 - A) and this parameter drops out.

For just one example among the others we made for reaching the conclusion that a diversity of mechanisms have the common kinetic expression of equation 4, consider the mechanism depicted in scheme 3. Assuming the enzyme activity is supported by Ea only (Ed1 and Ed2 are fully inactive forms), and this activity is proportional to the concentration of the active specie, the expression for the residual relative activity is directly derived from the integrated rate equation for Ea versus time, which can be found in any kinetics textbook:

$$(A)t/(A)o = [(p2 - k1)/(p2 - p1)] \cdot \exp(-p1 \cdot t) + [k1 - p1)/(p2 - p1)] \cdot \exp(-p2 \cdot t) \quad (5)$$

p1 and p2 are complex parameters related to individual rate constants k1, k-1 and k2. In relation with our previous notations, p1 = α and p2 = β

The general 4-parameters biexponential equation is recognized. If we call A and B, respectively, the two pre-exponential complex terms, the sum (A + B) is:

$$A + B = [(p2 - k1)/(p2 - p1)] + [(k1 - p1)/(p2 - p1)]$$
$$= [(p2 - k1) + (k1 - p1)]/(p2 - p1)$$
$$= (p2 - p1)/(p2 - p1) = 1$$

Thus B = (1 - A), it can be canceled in equation 3, which becomes the simpler equation 4

3.2. Use of this simplified equation to analyze deactivation kinetics

For illustration of the interest of the 3-parameters biexponential equation, we will first show its application to two sets of data picked-up in the publication [12]. This example was chosen because the author made only a phenomenological description of the loss of stability of two forms (wild and mutant types) of Subtilisin, in aqueous buffered solution or in dry dimethylformamide (DMF), without any assumption on the underlying mechanism or complete analysis of data (the curves in Fig. 4 in this article are drawn as segments between the experimental points and not after curve-fitting).

A first analysis of data coming from Fig. 4 in the WONG's publication was performed using the simple exponential model, because the author reported unstability as half-lifes and a simple first-order model was thus implicitly assumed (for orders other than 1, half-life is not a constant and depends on the initial concentration of the reactant). As seen in Fig. 2 (graphs on the left), there is some lack of fit with this simple model ($r = 0.7415$ for the wild subtilisin in aqueous solution, $r = 0.98$ for the mutant substilisin in dry DMF). When the same two sets of data are analyzed using the 3-parameters biexponential expression as a correlative

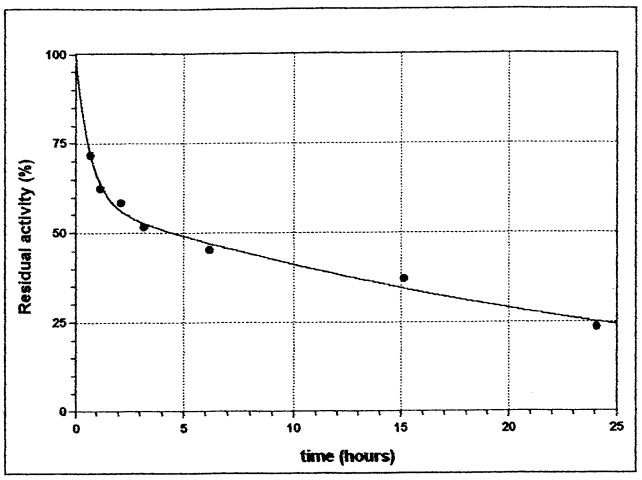

Fig. 3. Graph of the data from Fig. 2 in ARROYO et al.(1999) replotted in the same coordinates. The equation of the fitted curve is the simple three parameters biexponential equation derived in the text.

equation, a perfect fit (graphs on the right) is obtained ($R > 0.9994$ in both cases)

The other examplification of the "fitting power" of the 3-parameters biexponential equation is illustrated in Fig. 3: it shows how well ($r = 0.9973$) this equation fits to data picked-up in the Fig. 2 of [9]. Nonlinear curve fitting leads to $A = 0.4158$ (or 41.58%), and two apparent first-order rate constants: 1.4704 h^{-1} and 0.0352 $h^{-1,}$ respectively - these values compare very well with the values produced by the authors in the Table 2 of their paper.

4. Discussion and conclusion

A large diversity of mechanisms for heat denaturation of enzymes are kinetically described, with simple assumptions (limitation of the number of active forms in the system), by a 4-parameters biexponential equation. The full kinetic expressions with individual rate constants and different senses of parameters (according to the relevant mechanism) may discourage some investigator in attempts to analyze their data in a complete way. For others, the detailed equations are out of scope and interest - for instance in applied enzymology, biotechnology etc . . . when descriptive rather than interpretative studies are performed.

A simplified and "versatile" but still rationale kinetic equation is certainly a need in some fields for certain investigators with practical purposes in mind, focusing on the kinetic aspects and phenomenological description of the thermal deactivation, rather than identifying molecular events and reaction mechanisms, and when access to the individual (true) rate constants is out of scope, as it is often the case for studies in Biotechnology.

We have shown that a simple 3-parameters biexponential equation can describe heat-induced decay in enzyme activity, whatever the mechanism is among the more frequently complex mechanisms involved. It applies to complex mechanisms but also covers the simple "one step - two states" situation: when $A = 0$, we have the exponential decay of enzyme activity, with no partial activity retained by the transformed enzyme, when $\beta = 0$, the transformed form of the enzyme retains some activity (as sometimes assumed for certain enzymes). The proposed expression must be considered as a model-free empiric equation, even if some further analysis of the variation of its parameters with temperature, enzyme concentration etc. may authorize some conclusions on the underlying mechanism.

This simplified form of the general biexponential expression for a variety of complex mechanisms was not introduced before. A recent publication [17] is an illustration: for two complex mechanisms, kinetic equations are detailed with individual rate constants, leading to very long expressions, an elegant graphical method is described to access to the true rate constants, but at no time the simplified form of the general equation, with only 3 parameters, is evoked.

The interest of reducing the number of parameters in a correlative equation is to underline. Beyond the "parcimony principle" (not said in a statistician way, it can be enonced as "do the best with the least") that could by itself justify dropping out a parameter in an equation, the reduction in the number of parameters reduces risks of cross-correlation that may complicate processing of data by nonlinear regression. Cross correlation is the possibility of finding several sets of parameters with equivalent goodness of the fit - at the extreme case (cross-correlation index = 1), the system could remain undetermined. The risk increases with random errors in data and complexity of the correlative equation. In this context of estimation of parameters, using the 4-parameters equation when the 3-parameters equation could apply would lead to values of pre-exponential terms satisfying the $A + B = 1$ relation and correct estimates of rates constants only with error-free data. In real cases, "noise" in data introduces bias in rate constants estimation and may mask the relation between the pre-exponential terms.

Not contradictory with the above plea for the use of the 3-parameters biexponential equation as a first-to-test empiric equation, more in-deep investigations can also be performed. Variation of the parameters when varying experimental conditions (enzyme concentration, temperature . . .) may in some cases allow hypothesis about the molecular events and mechanism of the deactivation. For instance, the pre-exponential (A) parameter not changed at different temperatures is a strong indication in favor to a system with independent different isoenzymes, to be confirmed with further analyses (electrophoresis (zymograms). . . As another example, rate constants in strong deviation to the ARRHENIUS's law when the temperature is changed could be the sign of their complexity (apparent rather than true first-order rate constants).

Introducing this simple equation as a common kinetic expression for a diversity of situations ("kinetic homeomorphism") is also a pertinent recall on the difficulty to make

inference of mechanism from kinetics. Since near to three decades, “biphasic” deactivation kinetics with a 3-parameters biexponential equation fitting to the data are often reported as the proof of the existence of isoenzymes. The need of caution is demonstrated in this paper: the same equation applies for very different deactivation mechanisms and systems with isoenzymes.

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