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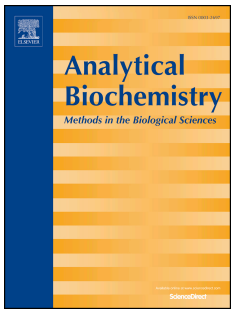

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PII: S0003-2697(19)30779-1

DOI: https://doi.org/10.1016/j.ab.2020.113595

Reference: YABIO 113595

To appear in: *Analytical Biochemistry*

Received Date: 13 August 2019

Revised Date: 10 January 2020

Accepted Date: 20 January 2020

Please cite this article as: E. Zangelmi, L. Ronda, C. Castagna, B. Campanini, M. Veiga da Cunha, E. Van Schaftingen, A. Peracchi, Off to a slow start: Analyzing lag phases and accelerating rates in steady-state enzyme kinetics, *Analytical Biochemistry* (2020), doi: https://doi.org/10.1016/j.ab.2020.113595.

Zangelmi et al. YABIO_2019_625

Author Contribution Statement

Erika Zangelmi: Methodology, Investigation, Writing - Reviewing and Editing, Visualization

Luca Ronda: Resources, Investigation, Writing - Reviewing and Editing.

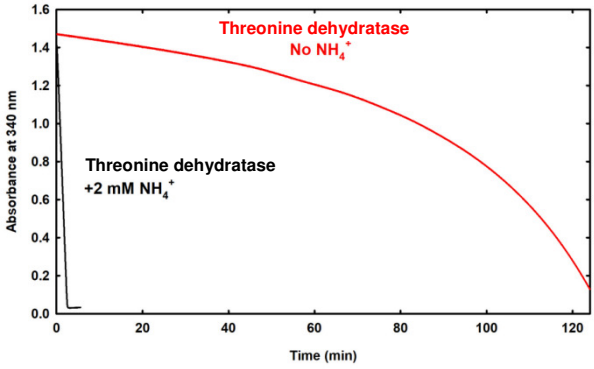
Camilla Castagna: Investigation, Writing - Reviewing and Editing.

Barbara Campanini: Resources, Writing - Reviewing and Editing.

Maria Veiga da Cunha: Resources, Writing - Reviewing and Editing.

Emile Van Schaftingen: Resources, Writing - Reviewing and Editing.

Alessio Peracchi: Conceptualization, Methodology, Formal Analysis, Writing – Original Draft



Off to a slow start: analyzing lag phases and accelerating rates in steady-state enzyme kinetics

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Short title: Interpretation of lag phases and kinetic delays in enzyme assays

Keywords: enzyme kinetics; lag phase; substrate inhibition; product activation; coupled assay; ornithine aminotransferase; phosphoserine aminotransferase; threonine ammonia-lyase

Abbreviations:

mmOAT, mouse ornithine δ-aminotransferase; PYCR, pyrroline 5-carboxylate reductase; hsPSAT, human phosphoserine aminotransferase; ecTD, *Escherichia coli* threonine dehydratase; RidA, 2-iminopropanoate deaminase; LDH, lactate dehydrogenase; MDH, malate dehydrogenase; GDH, glutamate dehydrogenase; BSA, bovine serum albumin; PLP, pyridoxal 5'-phosphate; α-KG, α-ketoglutarate; GSA, glutamate 5-semialdehyde; Glx, glyoxylate; GSH, glutathione; dGSH, deaminated glutathione; TEA, triethanolamine; MES, 2-(N-morpholino)ethanesulfonic acid.

SUMMARY

Steady-state enzyme kinetics typically relies on the measurement of 'initial rates', obtained when the substrate is not significantly consumed and the amount of product formed is negligible. Although initial rates are usually faster than those measured later in the reaction time-course, sometimes the speed of the reaction appears instead to increase with time, reaching a steady level only after an initial delay or 'lag phase'. This behavior needs to be interpreted by the experimentalists. To assist interpretation, this article analyzes the many reasons why, during an enzyme assay, the observed rate can be slow in the beginning and then progressively accelerate. The possible causes range from trivial artifacts to instances in which deeper mechanistic or biophysical factors are at play. We provide practical examples for most of these causes, based firstly on experiments conducted with ornithine δ-aminotransferase and with other pyridoxal-phosphate dependent enzymes that have been studied in our laboratory. On the side to this survey, we provide evidence that the product of the ornithine δ-aminotransferase reaction, glutamate 5-semialdehyde, cyclizes spontaneously to pyrroline 5-carboxylate with a rate constant greater than 3 s^{-1}.

INTRODUCTION

As enzymes play key roles in virtually any biological process, their kinetic characterization is often required during the study of biological systems. Nearly always, such a characterization entails the execution of steady state assays, in either a low- or high-throughput format, and the analysis of the ensuing results [1,2]

A cornerstone of steady-state enzyme kinetics is the concept of 'initial rates', whose importance was first recognized by Michaelis and Menten in the derivation of their famous equation [3]. Initial rates are, in the lab practice, rates measured at the very beginning of the reaction time-course, when the substrate is not consumed to a significant extent and the amount of product formed is negligible. Since the progress of the reaction may bring about inhibition by accumulated products, substantial decrease of the substrate concentration or spontaneous inactivation of the enzyme, it is usually implicitly assumed that such initial rates are faster than the rates attainable at longer times. On occasion, however, the opposite phenomenon is observed; that is, for some enzymes and under some conditions the reaction rate undergoes an apparent acceleration with time, often resulting in time courses characterized by an obvious, initial 'lag phase'.

Lag phases may be relatively short (sometimes just a few seconds) so they are most easily appreciated in continuous (e.g., spectrophotometric or spectrofluorometric) assays. When lag phases or slow-starting kinetics are observed, this behavior may hint to some interesting properties of the enzyme or of the reaction under examination; however, a correct interpretation of the datum entails a thorough analysis to rule out a number of possible artifacts. Artifacts and kinetic complexities may occur in all types of assays, but they are particularly facile in coupled assays, which are very common in enzymology and analytical biochemistry [4].

Herein, we describe and analyze the variety of reasons that may lead to the observation of progressively accelerating enzyme kinetics – reasons that range from trivial artifacts to complex mechanistic phenomena. An extensive list of such reasons, organized in categories, is given in Table 1, which also represents a roadmap for the content of the whole paper. In fact, in the Results and Discussion section, the entries in Table 1 will be presented in more detail and illustrated whenever possible by practical examples, based on kinetic experiments performed in our lab. These experiments involve enzymes dependent on pyridoxal 5'-phosphate (PLP) and chiefly recombinant mouse ornithine δ-aminotransferase (mmOAT), which we have characterized recently. As a matter of fact,

our interest in kinetic delays was initially aroused by a paper published in Analytical Biochemistry and reporting the systematic occurrence of lag phases in the kinetics of ornithine δ-aminotransferase monitored through a coupled assay [5].

Ultimately, the present paper aims at providing a guide and reference for researchers who observe kinetic delays and lag phases in their enzymatic assays and who may be seeking to sort out the significance of such an observation.

Table 1 – Possible reasons for the observation of accelerating enzyme kinetics
The present list assumes that the enzyme under examination is free of contaminating enzymes/activities. Furthermore, it considers only reactions occurring in solution, rather than at interfaces (on membranes, micelles etc).

Cause	Explanation	Diagnosis/ Troubleshooting
Instrumental/setup artifacts		
1 - Temperature change over time.	Starting the reaction before the assay mixture has reached the desired temperature, can yield an inconstant (usually increasing) reaction rate	Allow enough time to reach thermal equilibration of the reaction mixture.
2 - Non-linear or slow response of the detecting system	Under certain conditions, the detecting system (e.g., a spectrophotometer) may not respond linearly to the change in concentration of the product.	Test beforehand to be working under conditions that ensure a linear and rapid instrumental response. In some instruments the slow response may reflect a slow initial mixing (e.g., [6])
3 - Incompletely mixed or turbid reaction solution (particularly in spectrophotometric assays)	Incompletely mixed solutions can diffuse and particles in suspension can form or settle as the reaction proceeds, leading to erratic changes of the apparent reaction rate.	Kinetic traces are generally irreproducible. If turbidity is the culprit, it may be visible to the naked eye [1].
Artifacts due to contaminants		
4 - Contamination of the enzyme stock with a bound inhibitor that dissociates slowly.	High-affinity inhibitors may be released slowly from the enzyme upon dilution into the assay mixture. This will give rise to a lag phase, lasting until the inhibitor dissociation reaches its equilibrium.	The lag phase may disappear when the enzyme is pre-incubated in the reaction mixture and reaction is started by adding the substrate last, or when the enzyme is extensively dialyzed prior to reaction. Occasionally however the inhibitors remain tightly bound in the absence of substrate and can be released only after drastic treatments (e.g., precipitation of the enzyme [7]), at which point they can be detected and quantitated.
5 - Contamination by a preferred substrate that is either slow-reacting or whose	The reaction mixture may contain (as an impurity) an alternative, better-binding substrate that either reacts slowly or whose reaction is not	If the alternative substrate contaminates the stock of the intended substrate, different batches may yield different lags. If it contaminates the buffer, changing

reaction is not detected by the assay	detected by the assay. The alternative substrate effectively acts as a competitive inhibitor and is removed over time by the reaction itself.	buffer system will solve the issue. In certain redox reactions, dissolved oxygen may act as an alternative electron acceptor [8].
6 - Contamination by substances that interfere with the detection of the reaction product.	The reaction mixture may contain some substance that reacts rapidly with the product of an enzymatic reaction, competing with its detection until the contaminant is consumed.	May be suspected when the reaction product to be measured is particularly reactive or unstable (e.g., a radical; [22]).

Mechanistic reasons

7 - Diminishing substrate inhibition	If the initial substrate concentration in the assay mixture is appreciably inhibitory, the reaction rate will tend to increase with time as substrate is consumed.	Substrate inhibition is identifiable by a biphasic dependence of initial rate vs. substrate concentration [9].
8 - Activation by product	The product of a reaction may activate the enzyme, e.g., by binding to an allosteric site. Product buildup requires some time, hence the lag phase.	Adding some product in the reaction mixture will yield an increased initial rate and possibly a disappearance of the lag phase (e.g., [10,11]).
9 - Slow activation by the substrate	A reaction time-course that accelerates over time may be observed if one of the substrates of the reaction is a (slow-binding) activator.	The phenomenon can be suspected for allosteric enzymes (e.g., [12,13]). The lag may disappear if the enzyme is preincubated with the substrate and reaction is started by addition of a co-substrate (when possible) [14].

Structural/biophysical reasons

10 - Hysteresis (slow adaptation of the enzyme structure or conformation to new reaction conditions)	The enzyme, when added to the assay mixture, may slowly adopt a more active form, due to the different pH, ionic conditions, dilution, presence of reducing agents [15], etc.	The lag or delay of the kinetics should disappear upon pre-incubation of the enzyme in the reaction mixture (minus the substrate). Evidence for a conformational change in the reaction mixture may be sought using spectroscopic techniques (e.g. [16]).
11 - Slow covalent self-modification by the enzyme	Self-processing of the enzyme (e.g., by autoproteolysis, self-phosphorylation…) may yield a more active catalyst.	The phenomenon can be suspected in particular for enzymes that act on protein substrates [17,18]. Evidence for the self-modification can be provided by analytical methods (e.g., mass spectrometry).

Artifacts or kinetic complexities specific of coupled assays

12 - Suboptimal amount of indicator enzyme.	Inadequate amounts of indicator (coupled) enzyme may delay the attainment of an effective steady-state	The initial lag phase disappears upon increasing the amount of the indicator enzyme(s) present.
13 - Interference between the reactions of the assayed and	One product of the coupling enzyme is a substrate or an activator of the enzyme to be studied. One substrate of the coupling enzyme is an inhibitor	When using a different (e.g., non-coupled) assay, the lag is not observed.

indicator enzymes	or a slowly-binding activator of the enzyme under examination.	
14 - The assayed and coupled reactions are separated by a spontaneous (uncatalyzed) step	If the product must undergo a spontaneous chemical transformation (e.g., cyclization, hydrolysis) to yield a signal, an irreducible lag phase may ensue in the kinetic traces.	Lags should be eliminated only in the presence of an enzyme that catalyzes the intermediate step (e.g., [19]).

MATERIALS AND METHODS

Materials

The cloning of mmOAT (lacking the first 32 amino acids, corresponding to the signal peptide sequence), and its expression in *E. coli* has been described previously [20]. Recombinant mmOAT carried an N-terminal hexahistidine tag and was purified by affinity chromatography on a His-Select® cobalt affinity resin (Sigma-Aldrich) following the manufacturer's instructions. Fractions with purity higher than 90% (as judged by SDS-PAGE) were pooled, dialyzed against storage buffer (50 mM potassium phosphate pH 7.5, 100 mM NaCl, 0.5 mM EDTA, 1 mM DTT) plus 5 μM pyridoxal 5'-phosphate (PLP). The purified, dialyzed enzyme was supplemented with 10% glycerol and conserved at -80°C.

The mmOAT in apo form was obtained by reacting the enzyme with phenylhydrazine (80 mM) in 0.5 M potassium phosphate pH 7 for an hour at 25°C in the dark [21], followed by extensive dialysis against storage buffer.

Human phosphoserine aminotransferase (hsPSAT) was produced in recombinant form as described [22]. Cloning and expression of the deaminated glutathione amidase from *E. coli* (product of the ybeM gene) has also been described previously [20]. Other enzymes from *E. coli*, in particular pyrroline 5-carboxylate reductase (PYCR, the product of the proC gene), biosynthetic threonine dehydratase (ecTD, product of the ilvA gene) and 2-iminopropanoate deaminase (product of the ridA gene) were expressed from ASKA clones [23]. All these enzymes were purified by affinity chromatography as above. Glutamate dehydrogenase (GDH, beef liver), L-lactate dehydrogenase (LDH, rabbit muscle) and L-malate dehydrogenase (MDH, pig heart) were from Sigma.

L-ornithine, L-glutamate, L-aspartate, L-serine, glutathione (GSH), α-ketoglutarate (α-KG), glyoxylate (Glx), triethanolamine (TEA), 2-(N-morpholino)ethanesulfonic acid (MES), pyridoxal-5'-phosphate (PLP), bovine serum albumin (BSA) were from Sigma-Aldrich. NADPH was from Calbiochem. L-glutamine and NADH were from Alfa Aesar. Cysteamine hydrochloride was from Fluka.

Spectrophotometric enzyme assays

The mmOAT-catalyzed transamination of ornithine was mostly measured through a coupled assay with pyrroline 5-carboxylate reductase (PYCR) from *E. coli*, using α-ketoglutarate as a co-substrate [5].

Solutions for the coupled assay (1 ml final volume) typically contained 50 mM buffer (TEA-HCl, MES-NaOH or potassium phosphate), 0.5 mg/ml BSA, ~0.25 mM NADPH, in addition to the enzymes and substrates. Unless otherwise indicated, reactions were carried out at 20°C and started by the addition of mmOAT. The reaction was measured by monitoring the disappearance of NADPH at 340 nm on a Cary 400 thermostatted spectrophotometer (Varian).

Alternatively, the transamination between ornithine and α-KG was measured directly by following the decrease in absorbance at 210 nm, as described in the results and in Fig 1. For these assays, a quartz cuvette was used and the reaction mixture contained 50 mM phosphate buffer in addition to mmOAT and substrates.

The slow mmOAT-catalyzed transamination of glutathione, using glyoxylate (Glx) as the amino group donor, was monitored through a coupled assay with deaminated glutathione (dGSH) amidase and GDH, as described previously [20] and summarized in the Results. In the present study, the dGSH amidase used was the product of the ybeM gene from *E. coli*, functional orthologue of the mammalian Nit1 [20].

The hsPSAT-catalyzed transaminations of L-glutamate and L-aspartate, using Glx as the amino group acceptor, were measured by coupled assays with GDH and with MDH, respectively.

The ecTD-catalyzed deamination of L-serine was monitored via a coupled assay with LDH. Unless otherwise specified, the assay mixture also contained 2-iminopropanoate deaminase (RidA), to accelerate the formation of pyruvate from the immediate product of the ecTD reaction, 2-aminoacrylate/2-iminopropanoate, as described in the text.

Kinetic data were analyzed by nonlinear least-squares fitting to the appropriate kinetic equation (e,g., the Michaelis-Menten equation) using Sigma Plot (Systat Software Inc.).

Calculation of 'instantaneous' reaction rates

The instantaneous rate of a reaction at a given point in time, t, is formally defined as the first derivative of the progress curve (product concentration vs. time) in that point. In our coupled assays with NAD(P)H-dependent enzymes, the instantaneous rate was

estimated from the kinetic profile (absorbance at 340 nm vs. time) as follows. First, we determined the absolute change of absorbance over a narrow interval around t (typically 6 s - from t-3 s to t+3 s) and computed the slope of the curve in that interval. Second, we divided such slope by the extinction coefficient of NADH (0.00622 $\mu M^{-1}cm^{-1}$) to obtain a reaction rate expressed in $\mu M\ min^{-1}$.

This operation was repeated all along the kinetic curve, yielding a plot of the rate as a function of time. The mathematical treatment of the data and their graphical representation were performed using Sigma Plot (Systat Software Inc.).

Fluorescence measurements

Emission spectra of NADPH and time-dependent emission kinetics were recorded using a Horiba Fluoromax 3 (Jobin Yvon, Kyoto, Japan) spectrofluorimeter equipped with a circulating water bath. Samples were contained in a thermostatted quartz cuvette (1 cm optical path). To measure the kinetics of mmOAT reaction (in coupled assay with PYCR) the excitation wavelength was set at 340 nm and the emission was collected at 460 nm. The slits width was set to 2 nm and the integration time to 0.3 s to optimize the signal-to-noise ratio.

Stopped-flow measurements

Fast kinetic measurements of the mmOAT reaction were carried out using a SX-18MV stopped flow apparatus (Applied Photophysics) equipped with a 75-watt Xenon lamp and coupled to an MS 125TM 1/8-m spectrograph and Instaspec II photodiode array (Lot-Oriel) for multi-wavelength measurements. The temperature of the loading syringes and of the stopped-flow mixing cell compartment was kept at 20°C with a circulating water bath.

Reactions were carried out in 50 mM potassium phosphate buffer pH 7. mmOAT (10 μM) was mixed with 60 mM L-ornithine and 1 mM α-ketoglutarate. Spectra of the reaction mixture (in the 296-462 nm interval) were collected every 8.4 ms, and kinetics at various single wavelengths were extracted from spectral series. The instrumental dead time was 1.5 ms.

RESULTS AND DISCUSSION

As stated in the introduction, the model enzyme system used here to provide most examples of slow-starting kinetics is ornithine δ-transaminase from *Mus musculus*

(mmOAT). mmOAT is a mitochondrial enzyme that catalyzes the transfer of the δ-amino group of L-ornithine to α-KG, to yield L-glutamate and glutamate 5-semialdehyde (GSA), which subsequently cyclizes, forming pyrroline 5-carboxylate (P5C) (Figure 1A).

The reaction of mmOAT can be monitored spectrophotometrically by following the decrease in absorbance at 210 nm that accompanies conversion of the substrates into products (Figure 1B). However working at such a low wavelength (where sensitivity of most spectrophotometers is low and where many organic compounds show an intrinsic absorbance) imposes the use of quartz cuvettes, limits the kind of buffers that can be used and is not compatible with high concentrations of α-KG (which absorbs intensely at 210 nm; [22,24]).

Accordingly, in this study the assays involving mmOAT were primarily conducted using a coupled reaction with pyrroline 5-carboxylate reductase (PYCR; Figure 1A and 1C). Coupled assays are a commonplace in enzyme studies and, as summarized in Table1, at least some of the causes for (apparently) accelerating kinetics are specific to this kind of assays.

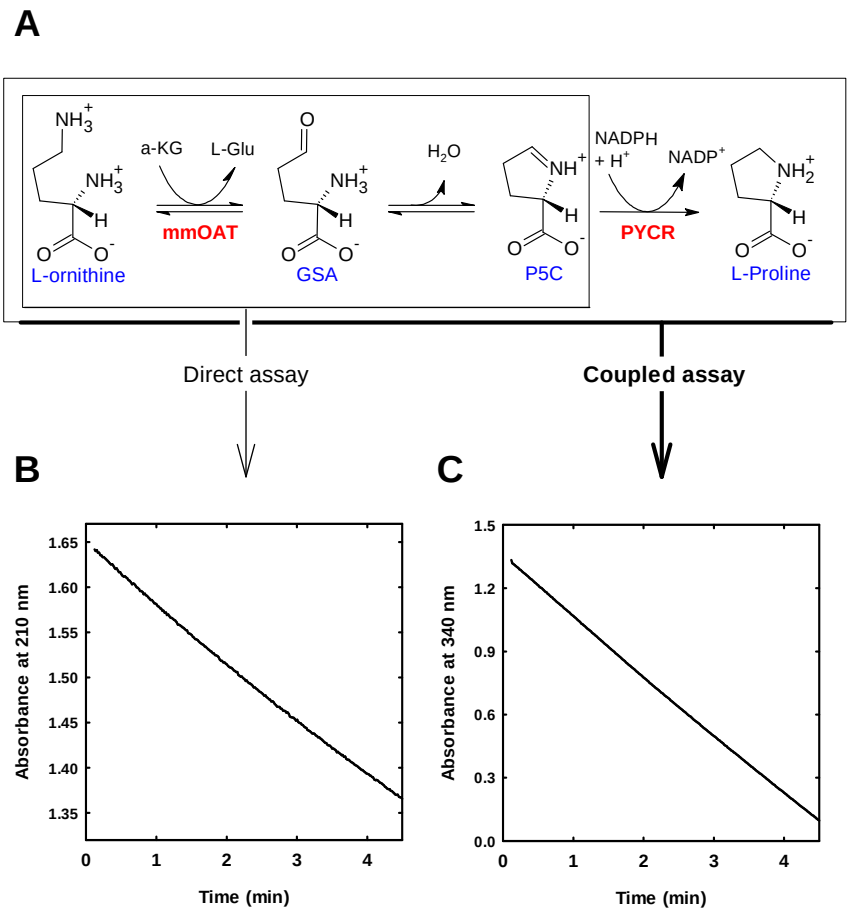

Figure 1 – Kinetic assays for mmOAT. (A) Scheme of the ornithine transaminase reaction and of the coupled reaction with PYCR. (B) The mmOAT reaction monitored spectrophotometrically at 210 nm. Conditions: 8 mM L-ornithine, 0.5 mM α-KG, 0.16 μM mmOAT in 50 mM potassium phosphate buffer pH 7.9, 20°C. (C) The reaction monitored through the coupled assay at 340 nm. Conditions as above, except for the presence of PYCR (0.5 μM), BSA (0.5 mg/ml) and NADPH (~250 μM). By comparing the slope observed in this time course (0.287 OD/min) with that in panel B (0.0674 OD/min) one can estimate a $\Delta\varepsilon$ of 1460 M^{-1} cm^{-1} at 210 nm associated to the conversion of the substrates (L-ornithine and α-KG) to products.

1 - Temperature variation during assay

When conducting enzyme assays, the stocks of reagents and enzymes are often maintained at temperatures colder than the reaction temperature (e.g., on ice), so that the assembled reaction mixture will also be initially colder than desired for the assay. As the activity of most enzymes increases with temperature, starting the reaction before reaching thermal equilibration can result in an artifactual acceleration of the observed reaction rate. An example is given in Figure 2.

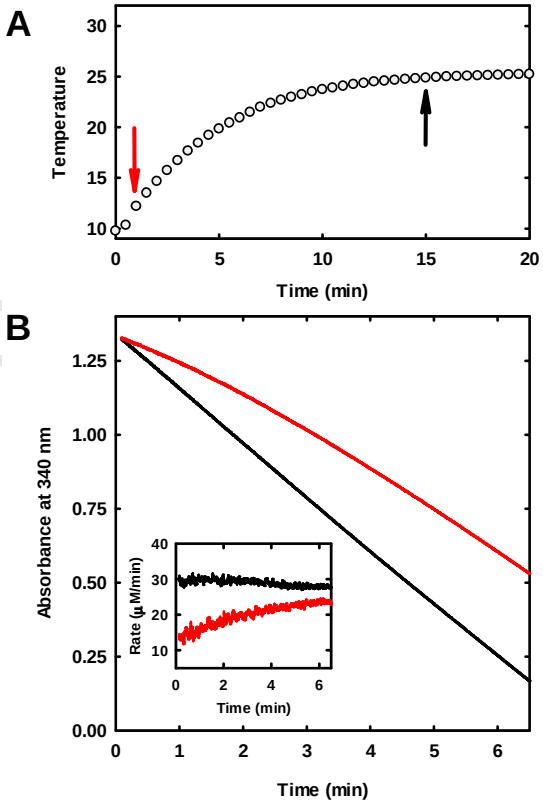

Figure 2 – Kinetics in a solution that either has or has not been thermally equilibrated. (A) Time-dependent change of the temperature inside a spectrophotometer cuvette: The cuvette holder of the spectrophotometer used in this experiment (Cary 400, Varian) was kept at 25°C. One ml of a solution pre-equilibrated at 6°C (30 min in a water bath) was transferred to a disposable plastic cuvette and placed into the spectrophotometer, after which its temperature was monitored for 30 min. (B) Reaction time-course for the transamination reaction (coupled assay) started before (red curve) or after (black curve) attaining thermal equilibration of the reaction mixture. The reaction

mixture (50 mM TEA-HCl pH 8.0, 6 mM L-ornithine, 1 mM α-KG, 0.5 mg/ml BSA, ~250 μM NADPH, 0.5 mM DTT, 0.2 μM PYCR) was incubated at 6°C for 30 min, prior to transferring 1 ml to a plastic cuvette and inserting the sample in the spectrophotometer, thermostatted at 25°C. Black line: the reaction was started by adding mmOAT (60 nM) after allowing the cuvette to equilibrate for 15 min in the spectrophotometer (this corresponds to the point signaled by a black arrow in panel A). Red line: the time allowed for thermal equilibration was only 1 min (this corresponds to the point signaled by a red arrow in panel A). Inset: time dependence of the observed reaction rates.

It should be noted that the experiment in Figure 2B was conducted under conditions purposely devised to magnify the initial lag phase. In a more ordinary setting, the time required for pre-equilibration will depend, among other things, on the volume of the sample, on the material in which it is contained (plastic being much less thermal conductive than glass or quartz, for example) and on the efficiency of the thermostatting system, whose proper functioning should be kept under check. It should be further noted that the pH of buffered solutions (particularly that of amine-based buffers, such as TEA) is substantially temperature-dependent [25], which may exacerbate (or, in other cases, attenuate) the observed variation of reaction rate with time.

2 – Non-linear or delayed instrumental response

The artifactual appearance of a lag in the kinetic profile may also arise in continuous assays when the detecting system does not respond linearly (or responds only belatedly) to the change in concentration of the product. For example, the coupled reaction of mmOAT and PYCR can be monitored not just by following the decrease in absorbance of NADPH at 340 nm, but also the decrease in emission of the same compound at ~460 nm (upon excitation at 340 nm). However, the fluorescence signal is strongly subject to inner filter effects, which in this case mainly depend on absorption of the incident light prior to reaching the point of the cuvette where emission is detected [26]. As a consequence of inner filtering, the measured fluorescence will not depend linearly on the NADPH concentration, especially if such concentration is high (Figure 3A). If not accounted for, inner filter effects are conducive to distorted kinetics [27,28], and in particular may yield reaction profiles that seemingly accelerate over time, as exemplified in figure 3B.

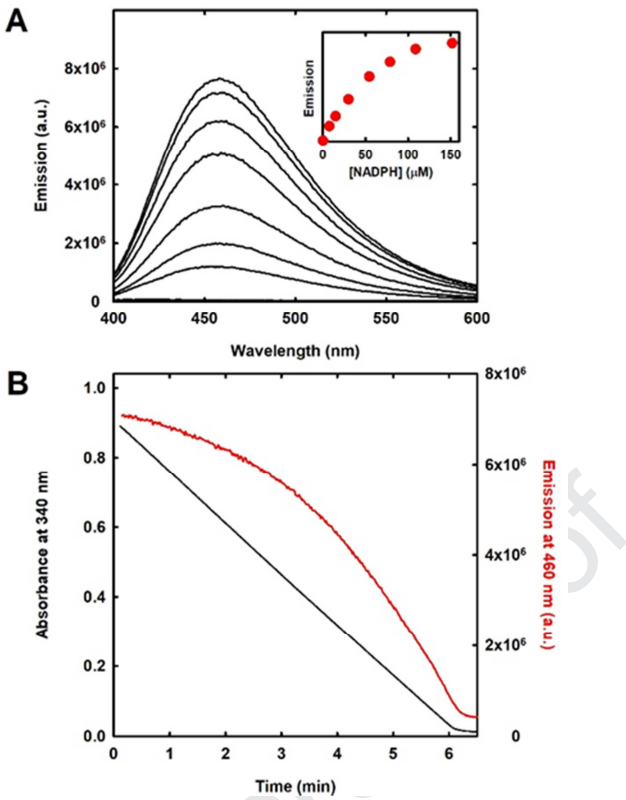

Figure 3 – Inner filter effects and their consequences on the kinetics of the mmOAT reaction measured spectrofluorometrically. (A) Emission spectra of NADPH at increasing concentrations, from 0 to 152 μM (conditions: 50 mM TEA-HCl buffer pH 8.0, 21°C; λ_{ex}=340 nm). The inset shows the strongly non-linear dependence of the observed 460 nm emission as a function of [NADPH] (as a rule of thumb, linearity is lost when the optical density of the solution at the excitation wavelength is higher than 0.1 OD [26]). (B) Kinetics of the coupled mmOAT/PYCR reaction, monitored by following either the NADPH absorbance (black curve) or emission (red curve). Reaction conditions: 6 mM L-ornithine, 1 mM α-KG, 0.5 mg/ml BSA,150 μM NADPH, 0.5 μM PYCR, 60 nM mmOAT in 50 mM TEA-HCl pH 8.0, 21°C.

3 – Artifacts associated to mixing

In enzyme assays, the reaction is usually triggered by mixing together a solution containing the enzyme and one containing the substrate(s). If initial mixing is incomplete (e.g., due to substantial differences in viscosity between the two solutions) a subsequent, slow diffusion of the reagents can give rise to observed rates that change anomalously with time. Similarly, if the assay is based on a spectrophotometric measurement, the presence of turbidity (insoluble particles that are dispersed in the reaction mix and can slowly settle afterwards) can lead to erratic changes in absorption. Turbidity can sometimes be due to aggregation of the enzyme upon adding it to the reaction mixture. All these artifacts may occasionally yield an apparent lag phase in the reaction profile; they are particularly trivial and generally irreproducible and examples are not provided here.

4 - Slow release of an enzyme-bound inhibitor

Inhibitors with high affinity may be present in the enzyme stock (e.g., because they co-purify with the enzyme [7,29–31]) and dissociate only slowly upon dilution of the enzyme into the assay mixture. This will give rise to a lag phase, lasting until the inhibitor dissociation reaches its equilibrium [32,33]. As a proxy for this case, we examine the reaction kinetics of mmOAT aliquots that were preliminarily treated with sub-millimolar concentrations of cysteamine. This small amine is capable of reacting with the PLP cofactor of mmOAT, yielding a thiazolidine adduct [34]. Formation of this adduct is evident from the spectral changes in the absorption of the cofactor (Figure 4A) [35] and impedes reaction with the standard substrates. Formation of the thiazolidine is not irreversible, but reversal of the adduct is slow. An enzyme stock treated with 0.5 mM cysteamine and then diluted into the reaction mixture would slowly release cysteamine and regain activity. Indeed, kinetic traces of cysteamine-treated mmOAT show a reaction rate that becomes substantially faster over time (Figure 4B).

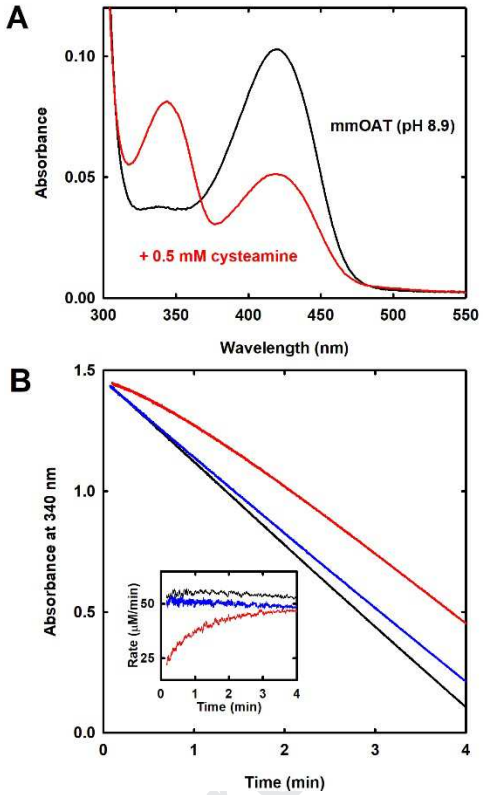

Figure 4 – Kinetics of cysteamine-treated mmOAT (A) Spectra showing that cysteamine (0.5 mM) reacts with the PLP cofactor of the enzyme, leading to the formation of a thiazolidine adduct, absorbing at ~340 nm. (B) Reaction time-courses (measured through the PYCR-coupled assay) for the unmodified mmOAT (160 nM; black line) and the same enzyme preincubated ~10 min with 0.5 mM cysteamine (red line). The blue line shows the reaction rate of the cysteamine-treated mmOAT incubated in reaction buffer for 10 min before starting the reaction by the addition of ornithine. For the pre-treated enzyme, the final concentration of cysteamine (upon dilution of mmOAT in the reaction mixture) was 1 μM. Other conditions: 50 mM TEA-HCl buffer pH 8.0, 6 mM L-ornithine, 1 mM α-KG, 0.5 mg/ml BSA, ~0.25 mM NADPH, 0.5 μM PYCR. Inset: time dependence of the observed reaction rates.

5 - Contamination of the reaction mixture by a tightly-binding alternative substrate

Conceivably, artifacts can arise when the reaction mixture contains traces of a substrate (different from the intended substrate) that binds with higher affinity to the enzyme but either reacts slowly or yields products that are not detected by the assay in use. In these cases, the tightly-binding substrate effectively acts as a competitive inhibitor of the reaction under examination. Reaction with the intended substrate will then be slowed down until the tightly-binding substrate is consumed [36].

An example, purposely devised to illustrate this point, is the reaction of human phosphoserine aminotransferase (hsPSAT) with L-aspartate, in the presence of minor amounts of L-glutamate. Glutamate is a standard substrate of hsPSAT and shows a K_M in the micromolar range. On the other hand hsPSAT, being a rather promiscuous enzyme [22], can transaminate with lower efficiency other amino acids, including L-aspartate. This

latter reaction can be selectively monitored through a coupled assay with malate dehydrogenase (Figure 5A) revealing a K_M for L-aspartate much higher than that for L-glutamate (Figure 5B). When the reaction of hsPSAT with 30 mM aspartate is measured in the presence of 0.3 mM glutamate (simulating a 1% contamination of the aspartate stock), the reaction shows an initial delay which is absent when glutamate is omitted (Figure 5C).

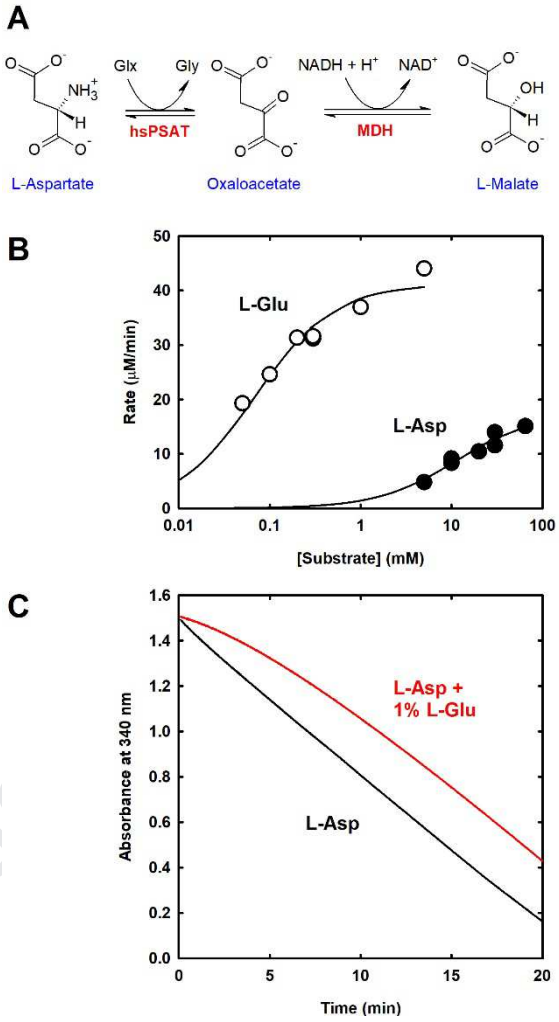

Figure 5 – hsPSAT reaction with L-Asp. (A) Reaction scheme, including the coupled reaction with MDH. (B) hsPSAT activity as a function of the concentration of L-aspartate (black circles, activity assayed through the coupled reaction with MDH) compared with the activity in the presence of L-glutamate (white circles, activity assayed through a coupled assay with GDH). Other reaction conditions: 0.64 μM hsPSAT, 5 mM Glx, 0.5 mg/ml BSA, ~0.25 mM NADH in 50 mM TEA-HCl buffer pH 8.0, 20°C. The reaction mixture for the glutamate reaction also contained 20 mM ammonium chloride. (C) Kinetics collected using 30 mM L-aspartate, in the presence (red) or in the absence (black line) of 0.3 mM L-glutamate.

The kinetic theory related to reactions with alternative, 'silent' substrates has been elaborated by Case and coworkers and can be found in ref. [36]. In the practice of enzyme assays, 'alternative' substrates may come from different sources. E.g., oxygen may act as

an alternative electron acceptor in certain redox reactions, hence when these reactions are not conducted under anaerobic conditions, formation of the expected reduced product may be slowed down until the dissolved O_2 is consumed [8].

6 – Presence of contaminants that prevent detection of the product

If the reaction mixture contains substances that react rapidly with the product of an enzymatic reaction, competing with its detection, a lag phase will be observed, lasting until the contaminant is consumed.

A practical example could perhaps refer to oxidases and hydroxylases that generate H_2O_2, which in turn can be detected by a coupled reaction with peroxidase. In these instances the presence of trace thiols (e.g., DTT) in the reaction medium would react with hydrogen peroxide, competing with its detection [37]. Another example refers to spectrophotometric assays for measuring the activity of peroxidase itself in cellular extracts, where the presence of reducing compounds in the extract may counteract the formation of the expected chromophoric products, resulting in apparent lags [38].

7 – Time-dependent decrease in substrate inhibition

Substrate inhibition, albeit often regarded as a sort of kinetic oddity, is far from rare in biochemistry and it may have important biological implications [9]. For enzymes showing substrate inhibition, the activity rises as substrate concentration increases, up to a maximum value attained at some 'optimal' substrate concentration, after which activity begins to diminish.

This behavior is common with aminotransferases; for example, the dependence of mmOAT activity on α-KG shows clear signs of substrate inhibition, which is particularly striking at pH<7. At pH 6.5, in the presence of 5 mM L-ornithine, the enzyme is already inhibited by concentrations of α-KG in the hundreds of micromolar range, while the 'optimal' α-KG concentration is estimated at around 20 μM (Figure 6A). Under these conditions, when mmOAT activity is tested in the presence of 200 μM α-KG (initial concentration) a progressive acceleration is observed, with the reaction rate reaching a maximum when the concentration of α-KG drops to approximately 20 μM (Figure 6B).

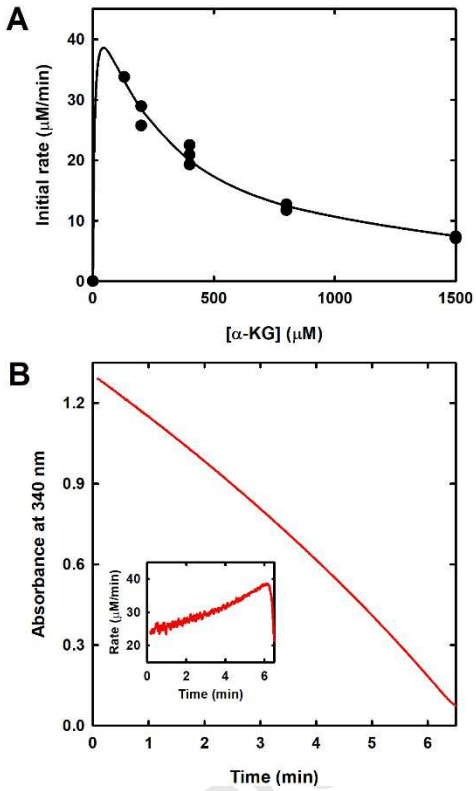

Figure 6 – Relief of substrate inhibition by α-KG during mmOAT kinetics. (A) Dependence of the initial rate of ornithine transamination by mmOAT (0.64 μM) as a function of α-KG. The reaction rate was measured through the coupled assay in 50 mM MES-NaOH pH 6.5, 20°C. Other conditions: 5 mM L-Ornithine, 0.5 mg/ml BSA, ~0.25 mM NADPH, 0.7 μM PYCR. (B) The time-course of the reaction, measured in the presence of 200 μM α-KG, shows a progressive increase of rate (as reported in the inset).

8 – Activation by the reaction product

Product activation is a phenomenon whereby the product of an enzyme reaction stimulates the activity of the enzyme itself. As product buildup requires some time, this results in a more or less pronounced lag phase in the reaction kinetics.

The first mention of product activation in Pubmed refers to a PLP-dependent enzyme, threonine dehydratase (TD; threonine ammonia-lyase) from yeast. This enzyme, which catalyzes the conversion of L-threonine and L-serine to the corresponding ketoacids and ammonium (Fig. 7A) is in turn allosterically stimulated by the latter product [39]. Such a behavior is shared by other TD enzymes. A striking example is TD from *Escherichia coli*, whose reaction with L-serine in the absence of ammonium (or other small monovalent cations) is characterized by an extended lag phase, which disappears when the reaction is carried out in the presence of ammonium chloride (Figure 7B).

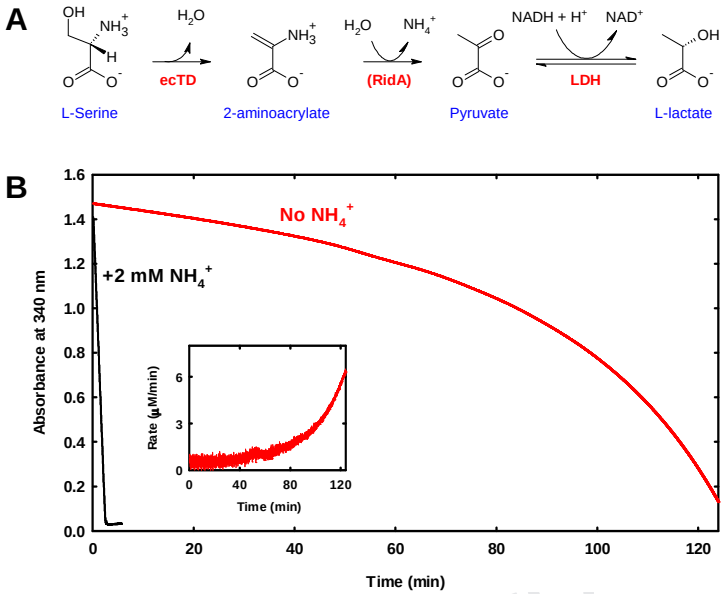

Figure 7 – Product activation in the reaction of *E. coli* threonine dehydratase (ecTD) with L-serine. A) Reaction scheme, including the coupled reaction with lactate dehydrogenase (LDH). The ecTD immediate product, 2-aminoacrylate, spontaneously reacts with water to yield ammonia and pyruvate (this hydrolysis can be accelerated by 2-iminopropanoate deaminase, RidA). The production of pyruvate can be easily monitored using LDH, whereas ammonium is a known activator of ecTD. (B) Kinetics of the reaction of ecTD (870 nM) with 10 mM L-serine, collected in a buffer devoid of small monovalent cations (red) or in the presence of 2 mM ammonium chloride (black). Reaction conditions: 50 mM TEA-HCl pH 8.0, 21°C,1 mM DTT, ~0.25 mM NADH, 0.4 μM LDH and 0,76 μM RidA. LDH and RidA, as well as ecTD, were dialyzed extensively against TEA-HCl before use, to remove traces of monovalent cations such as Na^+ or K^+. A qualitatively similar, but less spectacular lag phase was observed when L-threonine was used as a substrate in the absence of NH_4^+. Inset: the 'instantaneous' velocity increases near-exponentially with time, as expected for a self-promoting reaction.

Product activation, despite being relatively rare, has been described in other well-known enzymes. For instance rat liver glutaminase, not unlike ecTD, is activated by ammonia [10,40] whereas mammalian phosphofructokinases are activated by the product fructose-1,6-bisphosphate, presumably by binding to the same site where the allosteric activator fructose 2,6-bisphosphate binds [41]. Other examples of product-activated enzymes are maize ADP-glucose pyrophosphorylase [42] and the human phosphatidylinositol-(3,4,5)-trisphosphate phosphatase PTEN [43]. Activation is often allosteric but can also rely on indirect mechanisms. In the much-studied case of tyrosinase, the immediate product of the reaction (e.g., dihydroxyphenylalanine) spontaneously forms a quinone which reacts rapidly with the copper center of the enzyme, reactivating it [11].

9 – Slow activation induced by the substrate

Accelerating kinetics may be observed if one of the substrates of the reaction is a (slow-binding) activator of the enzyme. Such a phenomenon may be suspected for enzymes that show positive cooperativity or allosteric regulation, and may be particularly difficult to pin down and analyze.

Slow substrate activation has much in common with the 'hysteretic' phenomena described below (Reason 10), but in this case the lag phase cannot be eliminated by preincubating the enzyme in the reaction medium (minus the substrate). Furthermore, while the duration of the lag phase should in general decrease when the substrate concentration increases (because the second-order binding would be faster), this prediction is often not met because substrate binding to the activating site is preceded or followed by a rate-limiting conformational or structural transition [44].

One classic example of slow binding of the substrate to an activating (allosteric) site is pyruvate decarboxylase (e.g., see [12]). Glucokinase (a monomeric but cooperative enzyme, for which at least two conformations have been described [45]) also shows a lag phase due to a slow conformational change associated to glucose binding; this lag phase virtually disappears when the enzyme is pre-incubated in glucose and the reaction is started by adding the co-substrate ATP [14].

10 – Slow activation of the enzyme upon transfer to the reaction medium (Hysteresis)

Hysteresis is an umbrella term, including many different phenomena, which however have in common the occurrence of slow structural transitions or conformational changes that affect the overall reaction rate [46]. In practice the enzyme, when added to the assay mixture, may slowly convert into a more active form, due to the different pH [47], ionic conditions, dilution, reducing potential [15] etc. Accordingly many different subtypes of 'hysteresis' could be described. Since however the enzymes which show a true hysteretic behavior are often also allosteric and structurally dynamic, several types of hysteretic phenomena may be occurring at the same time and they may be very difficult to untangle.

Liver phosphofructokinase is a pertinent example. When the enzyme is assayed under near physiological conditions (where its allosteric properties are most apparent), the shape of the progress curves depends on the order of substrate addition [48]. When the enzyme is preincubated with ATP-Mg (a substrate, but also an allosteric inhibitor) and the

reaction is initiated by the addition of fructose-6-phosphate, the initial rate is slow and it accelerates progressively in the following minutes. The opposite behavior is observed when the order of substrate addition is inverted, i.e. a high initial rate is followed by a progressive deceleration. These time-dependent changes have been ascribed to changes in subunit association: active liver phosphofructokinase is a tetramer, which loses activity upon dissociation to dimers and monomers, but can gain activity when it forms high molecular weight aggregates [49]. The association to high molecular weight aggregates is favored by fructose-6-phosphate and fructose-2,6-bisphosphate [50], while their dissociation is promoted by ATP-Mg.

One more straightforward but still relevant subcase may occur when the enzyme is activated upon binding metal ions or organic cofactors which are present in the assay mixture, either as contaminants (e.g., [51]) or as supplements to maximize activity. As a proxy for this situation, we report the slow reactivation of apo-mmOAT by the cofactor PLP, added in different amounts to the reaction mixture (Figure 8). As it can be seen, activity accelerates sharply after a lag phase whose duration decreases when the concentration of exogenous PLP increases. Less pronounced, but still appreciable lags can be expected when, initially, only part of the enzyme is in apo form.

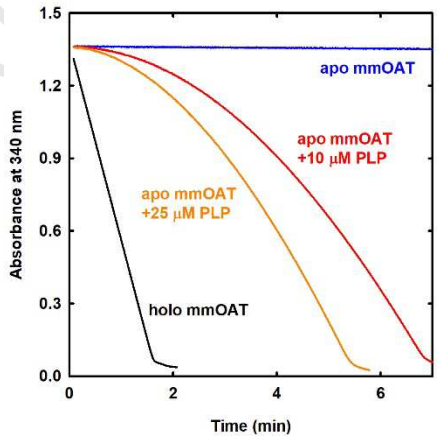

Figure 8 – Slow reactivation of apo mmOAT in the presence of exogenous PLP. Kinetics were measured (through the PYCR coupled assay) using 0.4 μM mmOAT in 50 mM TEA-HCl buffer pH 8.0, 20°C. The concentrations of α-KG and L-ornithine were 1 mM and 6 mM, respectively. The concentration of PLP in the assay mixture was 0 μM (blue line), 10 μM (red line) or 25 μM (orange line) – in these cases the reaction was initiated adding the apo mmOAT last. For comparison, the black line identifies an experiment in which the apoenzyme was incubated for ten minutes in the reaction mixture (containing 25 μM PLP and lacking ornithine) before starting the reaction by adding ornithine. Other conditions: 0.5 mg/ml BSA, ~0.25 mM NADPH, 0.6 μM PYCR.

11 – Slow covalent self-modification of the enzyme

Some enzymes are capable of self-modification reactions, due to which they become more catalytically active. This autoactivation process is typical of enzymes (in particular proteases [52,53] and protein kinases [54,55]) that use other proteins as canonical substrates, but exceptions exist [56,57]. In cases when this mechanism of activation is suspected, analytical techniques such as mass spectrometry may be needed to confirm the occurrence of self-modification. Furthermore, it may be important to distinguish between intermolecular and intramolecular activation mechanisms; to this end, mathematical models and simulations of activity curves have been devised, particularly in the case of kinases [17].

12 – Suboptimal amount of indicator enzyme

Coupled assays, due to a setup that is inherently complex, are particularly prone to artifacts [4]. If we consider the simplest case, in which the reaction of interest is monitored in the presence of a single coupling enzyme (also termed ‘secondary’ or ‘auxiliary’ or ‘indicator’ enzyme in the literature), it is essential that the activity of such coupling enzyme never becomes rate-limiting, to ensure that the measured rate represents in fact the rate of the enzyme under study [58]. This can be checked by confirming that the measured activity is not increased by increasing the amount of the coupling enzymes present.

Furthermore, coupled assays are generally *expected* to show a lag phase, which is required to allow an intermediate species (e.g., oxaloacetate in the coupled assay of Figure 5A) to build up to a steady-state level, where the rate for the intermediate’s formation matches the rate for its conversion to the final product [59]. A quantitative analysis of lags in coupled assays can be found in Cleland [60] and Garcia-Camona and coworkers [61]. By using high amounts of the coupling enzyme, the duration of the lag phase can be reduced so that it is completed during the mixing time. On the other hand, inadequate amounts of the coupling enzyme (as well as of its coenzymes and cosubstrates, since their concentrations will affect the efficiency of the coupled reaction) may delay achievement of the steady-state phase, as detailed among others by Tipton [1].

Lag phases associated to an inefficient coupling reaction are frequently reported in the literature (e.g. [62,63]). We illustrate this case in Figure 9, which shows how the observed rate of the coupled mmOAT-PYCR reaction varies depending on the concentrations of the reductase.

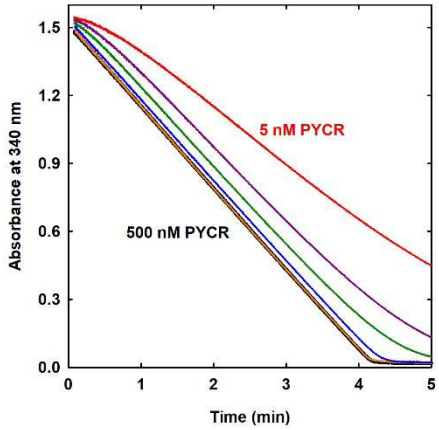

Figure 9 – Effect of variable concentration of PYCR on the coupled assay of mmOAT activity. Kinetics were conducted using 0.16 μM mmOAT in 50 mM TEA-HCl buffer pH 8.0, 20°C containing 6 mM L-ornithine,1 mM α-KG, 0.5 mg/ml BSA and ~0.25 mM NADPH. The concentration of PYCR in the assay mixture was 5 nM (red line), 10 nM (purple), 20 nM (green), 70 nM (blue), 200 nM (orange) or 500 nM (black). The last two curves are almost perfectly superimposed, confirming that under these conditions the PYCR reaction is not rate-limiting.

13 – Interference or crosstalk between the reactions of the coupling and assayed enzymes

Other artifacts, sometimes difficult to predict, may be encountered specifically in coupled assays and depend on the effects that substrates or product of the coupling enzyme(s) can have on the assayed enzyme. For example, the product of the coupling enzyme may be an activator of the enzyme under examination; also, the coupling enzyme may eliminate a compound that is inhibitory for the enzyme of interest. In both cases, the observed kinetics are going to accelerate over time.

One practical example refers to a coupled assay in which the ADP produced by phosphofructokinase is detected via the coupling activities of pyruvate kinase and LDH. The assay requires the presence of phosphoenolpyruvate (the co-substrate of pyruvate kinase) which however is an allosteric inhibitor of most phosphofructokinases [4]. For these enzymes, a lag in the progress curve is observed [1,64].

One even more complex example can be described in connection to a side reaction carried out by mmOAT, namely the transamination of glutathione (GSH) to yield deaminated glutathione (dGSH) [20]. mmOAT catalyzes this reaction much less efficiently than the transamination of substrates such as L-ornithine or L-glutamate. The mmOAT-catalyzed GSH transamination (when using an amino group acceptor different from α-KG – for example glyoxylate) can be conveniently detected by coupling it to two subsequent reactions. First, the reaction of dGSH amidase (YbeM), which cleaves dGSH into α-KG

and cysteinylglycine; and second, the reaction of GDH, which converts α-KG to glutamate while oxidizing NADH (Figure 10A). In such a coupled assay, however, the glutamate ultimately generated by GDH, being a good mmOAT substrate, will be preferentially transaminated and reconverted to α-KG (Figure 10A, dashed arrow), which in turn will be reduced again by GDH. The result of this kinetic short circuit is an artifactual, progressive acceleration of the observed rate (Figure 10B), which becomes higher than the actual rate of GSH transamination.

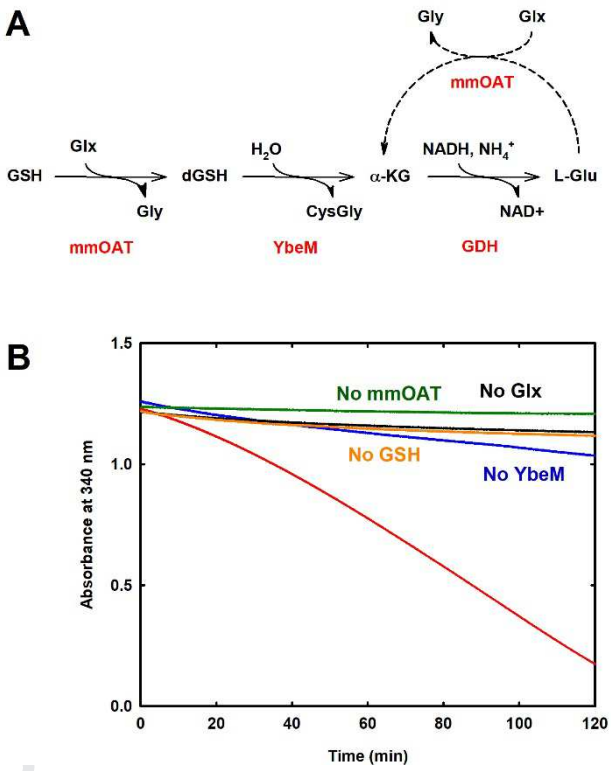

Figure 10 - Assay of glutathione transaminase activity by mmOAT, through a coupled assay with deaminated glutathione amidase (YbeM) and GDH. (A) Reaction scheme. (B) GDH activity (reflecting the release of α-KG in the reaction mixture) was detected upon addition of mmOAT, and only if the mixture contained GSH, glyoxylate and mmOAT. Omission of any of these reagents essentially prevented the decrease in absorbance at 340 nm. In the presence of the complete reaction mixture (red curve; the mixture contained 15 mM GSH, 5 mM glyoxylate and 2 μM mmOAT) the observed kinetics accelerated progressively with time due to the fact that the glutamate generated by GDH was being reconverted by mmOAT to α-KG, which in turn could serve again as a GDH substrate, causing an artifactual amplification of the signal. Other reaction conditions (red curve): 50 mM TEA-HCl buffer pH 8.0, 22°C, 0.5 mg/ml BSA, ~0.25 mM NADH, 2 mM DTT, 5 mM ammonium chloride, 0.48 μM YbeM and 3 units/ml GDH.

14 – Slow, uncatalyzed intermediate step in a coupled assay

If the product of the reaction under examination must spontaneously convert into something different to yield a signal, an irreducible lag phase may ensue. This problem may be encountered for example in assays based on certain synthetic substrates, whose

reaction products must undergo a cyclization/elimination reaction to yield chromophoric/ fluorescent species [65] but it is also common in coupled enzyme reactions [60]. An example may be that of methylmalonyl-CoA decarboxylase assayed through a coupled assay with phosphoenolpyruvate carboxylase and MDH [19]. Methylmalonyl-CoA decarboxylase produces CO_2 but the coupling enzyme phosphoenolpyruvate carboxylase requires bicarbonate as the substrate. This results in an extended lag phase in the observed kinetics, due to the slow spontaneous hydration of CO_2. Such a lag phase can be eliminated by adding a sufficient amount of carbonic anhydrase to the assay mixture [19].

In the reaction of ecTD with L-serine (see Fig. 7A), something similar occurs. The immediate product of the enzyme is 2-aminoacrylate, which must react with water to yield the final products, ammonia and pyruvate (which in turn is a LDH substrate). The spontaneous hydrolysis of 2-aminoacrylate may take seconds, resulting in a small but appreciable lag in the kinetics of the coupled assay (Figure 11). Such a lag, however, can be eliminated by adding to the reaction mixture an enzyme (the product of the *E. coli* gene ridA) that accelerates the hydrolysis step (Figure 11) [66].

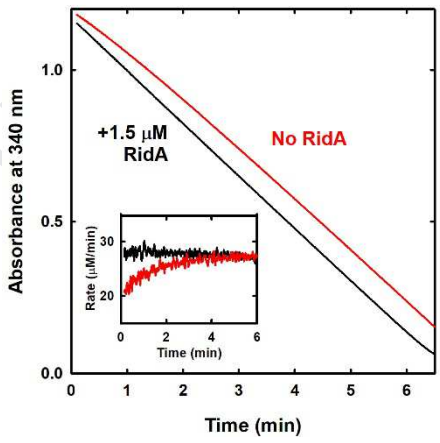

Figure 11 – Reaction of ecTD with L-serine: effect of the intermediate hydrolysis step on the kinetics measured through the LDH-coupled assay. The assay is illustrated in Figure 7A. Activity was measured in the presence of the activator ammonium; to limit any possible hysteretic behavior, the enzyme was preincubated 15 min in the reaction mixture (minus the substrate) before starting the reaction by adding L-serine. The red line represents a reaction carried out in the absence of the 2-aminoacrylate-hydrolyzing enzyme RidA, while the black line refers to a reaction otherwise identical, but carried out in the presence of abundant RidA (1.4 μM). Other conditions: 50 mM TEA-HCl pH 8.0, 21°C, L-serine 10 mM, 2 mM ammonium chloride, 1 mM DTT, ~0.25 mM NADH, ecTD 0.87 μM, 1 μM LDH. Inset: time dependence of the 'instantaneous' reaction rates.

Could something similar occur in the mmOAT reaction, monitored by the PYCR-coupled assay (Fig 1A)? In such an assay cyclization of glutamate semialdehyde (GSA) could conceivably be slow enough, that it might (under some conditions) produce a lag phase, irrespective of the concentration of PYCR. Clearly, the kinetics of mmOAT measured through a standard spectrophotometer do not show a delay (see figures 1C, 2B, 3B, 9). But since manual mixing requires several seconds, one may not exclude that a lag phase could take place in the first (unmonitored) five or ten seconds of the reaction.

In contrast to the hypothesis above, the kinetics of mmOAT coupled with PYCR, measured with a stopped-flow, do not show any lag phase. Subsequent to the first ~300 ms, which is the approximate time required by mmOAT to complete it first turnover under the conditions of the assay, the data appear completely linear (Figure 12). This allows us to estimate that the time required to complete the cyclization of GSA (to form pyrroline 5-carboxylate) is less than 0.3 seconds.

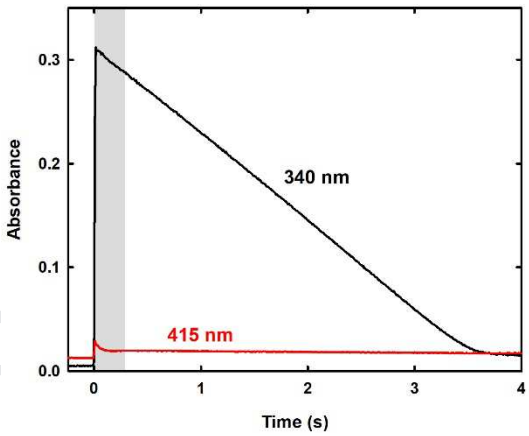

Figure 12 – Stopped flow reaction showing no lag phase in a mmOAT coupled assay. The conditions of the assay (phosphate buffer pH 7, 60 mM L-ornithine, 1 mM α-KG) were chosen to maximize the rate of the mmOAT reaction while possibly slowing the spontaneous cyclization of GSA, mmOAT's immediate product (cyclization to P5C requires a deprotonated form of the α-amino group. Execution of the experiment at pH 6.5 was unfeasible, due to the tendency of mmOAT to precipitate). Under the conditions of the assay the turnover rate of mmOAT is about 3.3 s^{-1}, that is, the first turnover of the enzyme is expected to occur in 0.3 s (corresponding to the shaded area of the graph). If cyclization of GSA to P5C occurred at a slower rate (e.g., 0.5 s^{-1}) an irreducible lag phase of about 2 s should be observed, irrespective of the concentration of PYCR. As no lag was observed, this suggests that cyclization occurs spontaneously at a rate substantially greater than 3.3 s^{-1}. The red line refers to changes in absorbance at 415 nm, where the PLP cofactor of mmOAT absorbs appreciably.

CONCLUSIONS

Lag phases and slow-starting kinetics may be observed in enzyme assays due to several different reasons. Herein at least ten (mechanistically distinct) cases of this behavior have been illustrated by examining the kinetics of just three enzymes – mmOAT, hsPSAT and ecTD. This suggests that the observation of the phenomenon is very common, more so than usually thought. Indeed, while kinetic delays are frequently described in the scientific literature, very often the authors choose to ignore the initial lag phases (particularly when they are short, not very pronounced and/or difficult to explain), focusing instead on the following, linear part of the kinetic profile. This implicitly assumes that the observed lag phases are just artifacts, or relegates them to undefined and uninteresting 'hysteretic' behaviors. This attitude may be reasonable in many instances; however, it misses two points. First, the rates measured after the lag cannot be considered actual initial rates. Second, by ignoring the phenomenon some interesting properties of the enzyme may be left uncovered.

To assist the researchers in the analysis of lag phases, we have summarized in Table 1 the possible basis for (apparently) accelerating enzyme kinetics and provided some initial criteria to discriminate between different causes. As a rule of thumb, identifying conditions under which delays are no longer observed (e.g., by changing the reaction conditions, changing the way in which the reaction is started, or using a different assay) can be of great help in identifying the possible undelying mechanisms. Note that the list in Table 1 is extensive but arguably not exhaustive: for example, it does not consider enzymes involved in interfacial catalysis (e.g., phospholipases), where observation of lag phases is quite the rule [67]. Furthermore, the classification adopted in Table 1 must not be taken too rigidly: sometimes different types of phenomena may concur to produce a kinetic delay (e.g., see [47]) whereas in other cases the complex mechanisms behind an observed lag phase may defy a univocal categorization (e.g., [68]).

Several possible causes of slow-starting kinetics had already been treated in previous publications [1,4], but this is the first study in which they have been systematically analyzed, offering practical examples for most of them. Practical examples are useful to the experimentalist, in particular because they provide an immediate sense of how different causes can bring about different types of lag phases and kinetic delays. This can be appreciated for example by comparing the consequences of relieving substrate inhibition (Figure 6) to those of releasing an enzyme-bound inhibitor (Figure 4).

As mentioned in the introduction, one initial stimulus for conducting this study was a paper reporting the systematic occurrence of lag phases in the kinetics of ornithine δ-

aminotransferase monitored through the coupled assay with PYCR [5]. Although we could not replicate exactly the original experiments (due to the different enzymes used here), we have shown, by performing activity assays, how many different artifacts or mechanisms can give rise to lag phases and delays in mmOAT kinetcs. We further have ruled out that the observed lag could be due to a slow cyclization of GSA, which instead appears to occur faster than the turnover rate of mmOAT itself. This confirms that this intramolecular reaction is inherently very fast and presumably does not require to be catalyzed *in vivo* [69]. Reaching an estimate of the lower limit for cyclization based on kinetic experiments (Figure 12) suggests that sometimes it is interesting not only interpreting lag phases when they are observed, but also explaining their absence when they could reasonably be expected.

ACKNOWLEDGEMENTS

This work has benefited from the framework of the COMP-HUB Initiative, funded by the 'Departments of Excellence' program of the Italian Ministry for Education, University and Research (MIUR, 2018-2022).

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Highlights

- Slow-starting kinetics are observed frequently in enzyme assays.
- We survey the reasons why the rate of an enzyme reaction may accelerate with time.
- The possible causes of delays and lag phases range from trivial to profound.
- We provide first-hand examples for most of these causes.
- We offer practical suggestions to discriminate between different possible causes.