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REVIEW

Understanding mechanisms of enzyme co-operativity: The importance of not being at equilibrium ☆

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Abstract
The discovery at the end of the 1950s and the beginning of the 1960s that there were enzymes like threonine deaminase and aspartate transcarbamoylase that failed to follow the expected hyperbolic behaviour predicted by the Michaelis–Menten equation, raised several questions and induced the development of mechanisms to explain this peculiar behaviour. At that time it was already known that the binding of oxygen to haemoglobin did not follow a hyperbolic curve, but a sigmoidal one, and it was thought that a similar situation probably existed for enzymes with sigmoidal kinetics. In other words, the observed kinetic behaviour was a consequence of co-operativity in the substrate binding. Two main models were postulated: those of Monod, Wyman and Changeux in 1965 and of Koshland, Némethy and Filmer in 1966. Both consider that the different conformations are in equilibrium and that there is a rapid equilibrium in the binding, which implies that co-operativity could only exist if there is more than one substrate binding site per enzyme molecule, that is, if the enzyme is an oligomer. What about monomeric enzymes, could they show kinetic co-operativity? Yes, but only through mechanisms that imply the existence of enzyme conformations that are not in equilibrium, and have different kinetic parameters. There are, in fact, very few examples of monomeric enzymes showing kinetic co-operativity with a natural substrate. The case of "glucokinase" (hexokinase D or hexokinase IV), a monomeric enzyme with co-operativity with respect to glucose, will be discussed.

Contents

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Introduction

The year 2013 was important for enzymologists for two reasons: on the one hand we celebrated the first centenary of the equation of Michaelis and Menten (1913), a cornerstone in the development of enzymology, and on the other hand the 50th anniversary of the concept of allostery (Monod et al., 1963), which illuminates the field of metabolic regulation.

Michaelis and Menten, like Henri (1903) before, regarded the formation of the enzyme–substrate complex as a process at equilibrium, i.e., the formation of this complex and its dissociation were considered to be much faster than the formation and release of the product. Some years later, Briggs and Haldane (1925) introduced the steady-state hypothesis, which led to a similar equation changing only the significance of the Michaelis constant. In the equilibrium hypothesis K_m can be considered as a dissociation constant (k_{-1}/k_1) which is not the case in the steady-state hypothesis, as another rate constant, k_2, needs to be included ($(k_{-1}+k_2)/k_1$).

Both equations predict the same sort of kinetic behaviour. If the experiments are well done, according to the protocol of Michaelis and Menten the relationship between substrate concentration and velocity is represented by a hyperbola passing through the origin. This type of plot was mentioned by Victor Henri in his thesis, but it was not illustrated. Michaelis and Menten, however, didn't use this plot but a semilogarithmic plot (velocity against log[S]). This plot is very useful to compare mutants or isoenzymes, such as hexokinase isoenzymes, which differ greatly in substrate affinity (Cárdenas, 1995), but it is not often used nowadays. The establishment of a correct experimental protocol was crucial because it meant that any deviation from hyperbolic behaviour was either an artefactual error or needed another explanation. The linear transformations of the Michaelis-Menten equation (Woolf plots) (Woolf, 1932) allowed the possibility of recognizing deviations and the discovery of enzyme co-operativity.

Feedback inhibition and co-operativity: two sides of the same coin

Although the Woolf plots (Eadie–Hofstee plot, Hanes plot and Lineweaver–Burk plot) introduced at the beginning of the 1930s (Woolf, 1932) facilitated the task of detecting deviations from hyperbolic behaviour, more than twenty years passed before any deviation was reported. There are many reasons for this long gap, as previously discussed (Cárdenas, 2013). Probably the main reason was the type of enzymes that were being studied at the beginning of the 20th century: extracellular enzymes that are not subject to feedback control. As feedback inhibition and co-operativity are in fact two sides of the same coin (Cárdenas, 2013), this restricts the possibilities of observing real deviations. Thus, it is not by chance that deviations from Michaelian behaviour were only detected when people started to try to understand feedback control and to study intracellular enzymes.

In the 1950s there were indications that feedback control could exist in living organisms: for example, in *Escherichia coli* the presence of isoleucine in the culture medium prevented threonine from being metabolised to isoleucine (Abelson, 1954). Among the first enzyme reactions known not to follow the classical hyperbolic behaviour were threonine deaminase (Umbarger, 1956) and aspartate transcarbamoylase (Gerhart and Pardee, 1962)); these enzymes also showed feedback inhibition. The deviations from hyperbolic behaviour were observed while studying feedback inhibition and were received with surprise and worry, as it was not easy to show that they were not artefacts. Umbarger (1956), studying threonine deamination, referred to 'peculiar kinetic behaviour' because when the double-reciprocal plot of Lineweaver and Burk was employed, it was necessary to square the substrate concentration; the inhibition by isoleucine appeared not to be hyperbolic either. This led Umbarger to say that: "This property of the data would be expected if the enzyme combined with two molecules of substrate or inhibitor. Further experiments are in progress in an effort to decide whether this peculiar kinetic behaviour is apparent or real."

In other words co-operativity and feedback inhibition were discovered at the same time and both phenomena required an explanation. This article of Umbarger, of one single page, constituted a real revolution in enzyme kinetics, and opened the field of regulation by feedback inhibition through allosteric regulation, although this term was not yet coined. It reported the following main kinetic characteristics of an enzyme subject to feedback inhibition:

(i) isoleucine prevents utilisation of threonine by *E. coli*, due to inhibition by isoleucine of the deamination of threonine, the first step in its utilisation.
(ii) in spite of the structural differences between threonine and isoleucine, isoleucine behaves as a competitive inhibitor with respect to threonine.
(iii) the kinetic behaviour of threonine deaminase with respect to its substrate is not hyperbolic.
(iv) the inhibition by isoleucine is not hyperbolic either.

As these studies were done in crude extracts, this peculiar kinetic behaviour could have been an artefact,