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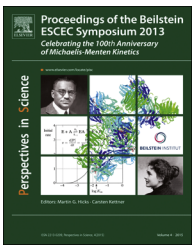

REVIEW

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,

but it was not, as Umbarger's observations (Umbarger (1956), Umbarger and Brown, 1958) were later confirmed by Changeux (1961). In his thesis work, he found that the inhibitory effect of isoleucine was competitive with respect to threonine, even with purified enzyme (Umbarger's experiments were done with crude extracts). This fact led him to postulate that distinct binding groups would exist on the surface of the enzyme, and that it would be possible to desensitise the enzyme, that is, to have a threonine deaminase still active but insensitive to isoleucine. p-Chloromercuri-benzoate proved to be very effective for achieving this (Changeux, 1961). So here are the roots that would lead to the concept of the allosteric site (Monod et al., 1963).

Aspartate transcarbamoylase: inhibition by cytidine derivatives

In parallel to the studies of Umbarger, Yates and Pardee (1956) showed in vitro with crude extracts that cytidine, and especially cytidine-5-phosphate, acted as competitive inhibitors with respect to aspartate for the formation of ureidosuccinic acid (now known as carbamoylaspartate), the first reaction unique to pyrimidine biosynthesis.

This type of inhibition was even more striking than in the previous case, as here a nucleotide end product was able to compete with a structurally very different substrate, an aminoacid. However, as these experiments, like Umbarger's, were done in crude extracts one could argue that the inhibition by cytosine derivatives could be indirect: they could have been transformed in the extract to the real inhibitor. But, as with threonine deaminase, they proved to be real, as some years later, Gerhart and Pardee (1962), using a highly purified enzyme, confirmed that CTP inhibits competitively with respect to aspartate.

Furthermore, CTP appeared to bind to a second site different from the active site, which they called the feedback site, as the enzyme could be desensitised without losing catalytic activity (Gerhart and Pardee, 1962). This led them to postulate that 'the bound end product perhaps inhibits by deforming the enzyme so that the latter has a low affinity for the substrate'.

The term allosteric site, coined in Paris by Monod and colleagues, prevailed over the term feedback site, coined in California by Gerhart and Pardee, probably because of the review of the former group (Monod et al., 1963).

An interesting point is that although the deviation from hyperbolic behaviour of aspartate transcarbamoylase is very obvious to a modern reader, as there is significant and very noticeable co-operativity with respect to aspartate, the authors downplayed this observation: they only make comments such as: 'Despite the complex kinetics…', or 'the unusual sigmoidal dependence of the native enzyme…'. It's a pity that they did not pay more attention to the sigmoidicity because their article also illustrates very clearly the idea that effectors modify the degree of co-operativity with respect to the substrate: inhibitors by increasing it and activators by decreasing it.

The lack of emphasis on the co-operativity may perhaps be because they did not have any explanation for it, whereas they did have a plausible mechanism for explaining the feedback inhibition. The same applies to the studies of Changeux on threonine deaminase.

So, as late as 1962 the observation of sigmoidal dependence was regarded with some suspicion, as it went against the ideas established in the article of Michaelis and Menten, and this despite the fact that the co-operativity of oxygen binding to haemoglobin had been known since 1910 (Hill, 1910). This attitude changed in the middle of the 1960s, when models for explaining enzyme co-operativity appeared.

Something to bear in mind, to fully appreciate the value of all these discoveries, is that the concept of allosteric site or feedback site was in conflict with the conceptual view of that time of enzyme action. The "lock-and-key" image introduced by Fischer (1894) predominated and implied a certain rigidity of enzyme structure; with this image, it was very difficult to conceive of an allosteric site. So, it is not surprising that in the 1950s researchers such as Umbarger (1956) and Yates and Pardee (1956), who tried to understand how the final product of a pathway could inhibit the pathway, were completely astonished to find that the inhibitor acted as a competitive inhibitor of the first enzyme of the pathway, in spite of the big structural difference between substrate and inhibitor. In this respect Koshland's ideas of induced fit, in 1958 (Koshland, 1958) played an important role, because with the introduction of the concept of flexibility in enzyme action, he paved the way to the allosteric concept.

Models to explain co-operativity and allostery

Two principal models appeared in the mid-1960s, which attach a functional importance to multiple conformations (with different kinetic parameters), an idea that originated with Koshland's induced fit hypothesis (Koshland, 1958). These models postulate that the observed kinetic co-operativity is a consequence of a co-operative binding of the substrate to an enzyme with several active sites, i.e., an enzyme with several subunits. They differ in relation to the conformational transition (Figure 1).

(i) The *allosteric model*, also called the symmetry model or the concerted model, proposed by Monod et al. (1965). Here, all the subunits in each tetramer have the same conformation; the conformational transition is concerted and the symmetry is preserved. In this model the ligand (substrate, inhibitor or activator) does not induce a conformational change, but selects a certain conformation displacing the equilibrium. As with haemoglobin the binding of ligand is at equilibrium.

(ii) The *sequential model* proposed by Koshland et al. (1966) a year later. Here, in each tetramer the subunits can be in different conformations, because the conformational transition is not concerted; there is no symmetry. The ligand induces a conformational change, which may or may not change the conformation of the neighbouring subunit, and the conformational equilibrium is disturbed.

Both models considered that the following apply:

i. the conformational transition is fast in relation to the catalytic reaction,

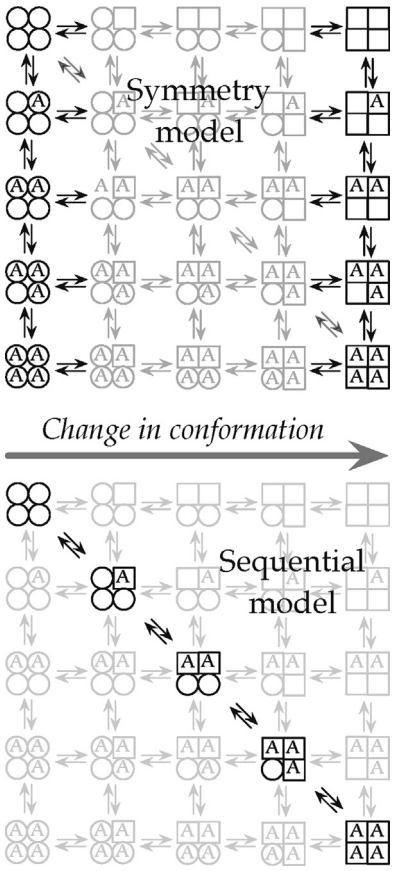

Figure 1 Equilibrium binding models to explain co-operativity and allostery. In the symmetry model proposed by Monod et al. (1965) all the subunits in each tetramer have the same conformation (the transition is concerted). The ligands bind with different affinity to the different conformations and displace the equilibrium; they do not induce a conformational change. In the sequential model proposed by Koshland et al. (1966) the ligand induces a conformational change, disturbing the equilibrium and the transition is not concerted; there is no symmetry. In both models the kinetic co-operativity is a consequence of a co-operative binding of the substrate to the polymeric enzyme.

ii. the binding of ligand is at equilibrium, and
iii. the enzymes that present co-operativity are polymeric, i.e., have multiple subunits.

For further comparison between the two models see a recent review (Cornish-Bowden, 2013).

What about monomeric enzymes? According to these models a monomeric enzyme with just one active site cannot have co-operativity with respect to the substrate. Only if there is a second active site in the same subunit (or a second site able to bind the substrate) could such an enzyme show co-operativity with respect to the substrate.

However, there are monomeric enzymes with co-operative kinetics with its normal substrate and hexokinase D ("glucokinase") is a good example (Cárdenas, 1995; Niemeyer et al., 1975).

"Glucokinase" a monomeric enzyme with kinetic co-operativity

In rat liver there are four isoenzymes able to phosphorylate glucose (González et al., 1964): hexokinases A, B, C and D also called I, II, III and IV, respectively. Hexokinase D or IV is also called "glucokinase" although its specificity is similar to the other three (Cárdenas, 1995; Cárdenas et al., 1984a). However, I shall use here the name "glucokinase", because is almost universal in the literature, even though it gives a misleading impression of the specificity (Cárdenas, 1995; Cárdenas et al., 1984a).

Glucokinase is by far the predominant hexokinase isoenzyme in hepatocytes (Reyes and Cárdenas, 1984) and has attracted attention since its discovery because of the effects of diet and hormones on its level of activity, which are due to changes in the amount of enzyme, i.e., in gene expression; it is unique with respect to its molecular mass, tissue distribution, structure, kinetic properties and function (Cárdenas, 1995). The glucose uptake by glucokinase in hepatocytes and pancreatic islets is an essential physiological process, crucial for glucose homoeostasis. Glucose-stimulated insulin release is tightly regulated by islet glucokinase, which acts as a molecular sensor to couple glucose metabolism to insulin release (Matschinsky, 1990). So it is not surprising that glucokinase has acquired progressively increased interest and importance with the rise in recent decades of diabetes type 2 as a major problem of human health (Iynedjian, 2009; Matschinsky, 2009).

When this enzyme was first characterised the kinetic co-operativity with glucose was not reported, but the two groups that were working on this enzyme detected some departure from the hyperbolic behaviour (González et al., 1967; Parry and Walker, 1967). This could have been expected, as to detect co-operativity it is necessary to do experiments over a large range of substrate concentrations, which requires analytical methods sensitive enough to measure the product at low concentrations, and even more important, to be able to do the experiments in conditions in which the enzyme is stable. In the 1960s obtaining pure enzymes and even partially purified ones was still difficult, and progress required knowledge of how to stabilise enzymes, and the development of purification techniques and of such appropriate materials as ion-exchange resins and filtration gels. Affinity chromatography, which contributed greatly to the field, only developed in the 1970s. For further discussion about this point see Cárdenas (2013). In general, intracellular enzymes tend to be unstable, and it is the case of glucokinase as it is very sensitive to oxidation; so it was crucial to learn how to stabilise it. All this explains why the kinetic co-operativity of glucokinase with respect to glucose was only reported by Niemeyer et al. (1975), a decade after its first characterisation; this result was confirmed a year later with pure enzyme and with the evidence that glucokinase was monomeric (Storer and Cornish-Bowden, 1976; Holroyde et al., 1976). Afterwards, we proved that it persists as a monomer in the assay conditions (Cárdenas et al., 1978), a crucial fact from the point of view of a plausible co-operative mechanism. The form of enzyme with a higher molecular mass mentioned in the paper of 1975 may have been a complex of glucokinase with the regulatory protein, discovered several

years afterwards (Van Schaftingen, 1989). A similar degree of co-operativity is obtained with mannose as substrate (Cárdenas et al., 1984b). In contrast, with 2-deoxyglucose the kinetic behaviour is hyperbolic (Monasterio and Cárdenas, 2003).

As the degree of co-operativity was small (Hill coefficient of 1.5–1.6) there was initially the worry that it could be artefactual, as glucose could be acting as a stabilising factor. So we did several experiments to make sure that it was real. In addition, in contrast to what was discussed in the previous section, no feedback inhibitor had been described for this enzyme. So, this appeared to be a very special case: a monomeric enzyme with co-operativity with respect to its substrate, and in addition not allosteric: no feedback site or a second site for binding glucose. All my efforts to desensitise the enzyme were in vain. Contrary to the cases of threonine deaminase (Changeux, 1961) and aspartate transcarbamoylase (Gerhart and Pardee, 1962), as long as glucokinase retained activity it also retained the co-operativity (Cárdenas, 1995). There were, however, several facts that supported the real existence of co-operativity, with a possible physiological meaning, and in addition contributed to give hints to postulate a possible mechanism (Cárdenas, 1995, and references therein):

(i) The co-operativity is a very well preserved feature through evolution. All vertebrate glucokinases that have been described show co-operativity with glucose and mannose, with Hill coefficient values between 1.4 and 1.7. This is reviewed in Cárdenas (2004).
(ii) The degree of co-operativity depends on the concentration and identity of the nucleotide substrate. Thus, if the concentration of MgATP is low enough the co-operativity can be abolished. There is no co-operativity if the nucleotide substrate is MgITP.
(iii) It can be eliminated by the presence of competitive inhibitors with respect to glucose (mannose, fructose, 2-deoxyglucose, N-acetylglucosamine).

Kinetic models to explain co-operativity in monomeric enzymes

The limitation imposed by the models based on co-operative binding mentioned above, encouraged some people in the 1960s, e. g., Rabin (1967), to seek models that could explain kinetic co-operativity without needing co-operative binding: this could be based, for example, on enzyme isomerisation during the course of the reaction (Rabin, 1967). This was initially a sort of intellectual challenge as no monomeric enzymes showing deviations from hyperbolic behaviour had been described experimentally, at least, none with natural substrates. This view changed in 1975 with the discovery of the kinetic behaviour of glucokinase (Niemeyer et al., 1975), but it was not easy to arrive to a model, and I considered more than one possibility (Cárdenas et al., 1979, 1984b).

Basically, the existence of kinetic co-operativity implies the existence of at least two different pathways able to accomplish the reaction and release the products, and with different kinetic parameters, so the point is how these different pathways could be generated. They could derive from the existence of at least two enzyme conformations, E and E′ with different activities, whose relative proportion depends on substrate concentration, and which are not in equilibrium. Two main models were postulated to explain the kinetic co-operativity of glucokinase: the mnemonical model (Ricard et al., 1974; Storer and Cornish-Bowden, 1977) and the slow-transition model (Cárdenas et al., 1984b; Ainslie et al., 1972), as we have reviewed (Cornish-Bowden and Cárdenas, 1987, 2004). In both models the enzyme exists in two distinct forms, E and E′ that are interconverted relatively slowly, with the more stable form E′ predominating in the absence of glucose. In the mnemonical model the less stable form E is the one that is released at the end of the catalytic cycle. The slow-transition model is somewhat more complicated, as both conformations can accomplish a catalytic cycle, but with different kinetic parameters (Figure 2). In both models, as glucose binds in two different steps, the full rate equation contains terms in the squared concentration of glucose, thereby allowing deviations from Michaelis–Menten kinetics.

Before structural information was available, there existed several different types of information to support the existence of conformational changes of glucokinase induced by glucose (Cárdenas, 1995 and references therein):

(i) *Spectroscopic*. Glucose binding enhances intrinsic fluorescence of glucokinase. This fluorescence enhancement is slow and the half-time of the transition depends on the glucose concentration. This is consistent with a slow isomerisation between two forms of glucokinase.
(ii) *Kinetics*. Transient states can be detected in the assay in the presence of glycerol. A burst if the enzyme has been preincubated with high glucose concentration or a lag if it has been preincubated without glucose.

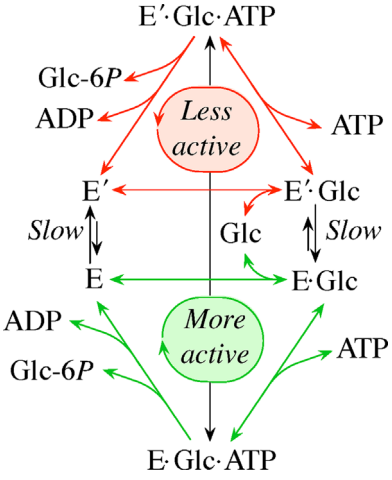

Figure 2 Slow-transition model as applied to glucokinase, a monomeric enzyme. In the absence of the substrate, glucose, the enzyme exists in a stable conformation E′. The presence of glucose induces a conformational change to a more active form E. This transition is slow in relation to catalysis and the proportion of E increases with the glucose concentration.

Figure 3 Scheme showing the conformational change of glucokinase induced by the binding of glucose. The open form corresponds to conformation E′ in Figure 2 and the closed form to conformation E, the more active form. Adapted from Kamata et al. (2004).

When the glucokinase structure was finally available it revealed that the free enzyme has a more open conformation than that of free hexokinase A (brain hexokinase) or yeast hexokinase; glucose binding induces a big conformational change (Kamata et al., 2004) (Figure 3).

Although until today no natural allosteric effector (inhibitor or activator) has been described, the enzyme has a site where an activator could bind. Thus, Grippo and colleagues (Grimsby et al., 2003) searched for small molecules that could increase the activity of glucokinase by screening a library of 120,000 structurally diverse synthetic compounds. One compound increased the enzymatic activity of glucokinase, and chemical optimisation of this initial molecule led to the synthesis of glucokinase activators (Grimsby et al., 2003) that bind to a site 20 Å remote from the active-site, at the interface between the large and small domains, on the back of the structure with respect of the location of the glucose binding site (Dunten et al., 2004). Activators act as staplers (Kamata et al., 2004) and close the conformation, increasing the affinity of glucokinase for glucose (Grimsby et al., 2003; Brocklehurst et al., 2004). However, no natural activator has been described and probably evolution has not selected one as the substrate, glucose, acts as an activator. In addition, with the type of diet available in the wild, probably there was no necessity for developing an activator, or a feedback inhibitor, and now it is too late. The lack of feedback inhibition appears to have at present dramatic consequences for human health, because activators—which were seen as potential medicines for diabetes as they are effective for decreasing glycaemia, at least temporarily—produce undesirable effects in the long term (Matschinsky, 2013). This is probably because there is no feedback that controls the system.

Concluding remarks

Today it has been well established that glucokinase can exist in more than one conformation and that glucose induces a conformational transition, that is slow in relation to the catalytic reaction, and consequently, the different conformations are not in equilibrium. This departure from equilibrium allows the existence of co-operativity in monomeric enzymes with only one active site. It has been a long road where development of technology has played a crucial role giving rightness to the opinion of Carl Woese that "without an adequate technological advance the pathway of progress is blocked, and without an adequate guiding vision there is no pathway, there is no way ahead" (Woese, 2004).

Conflict of interest

The author declares that there is no conflict of interest.

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