Enzyme kinetics from a metabolic perspective

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Introduction

The kinetic properties of enzymes have been intensively studied for almost a century, starting soon after Buchner [1,2] demonstrated that alcoholic fermentation was a chemical process, thereby disposing of the spirit of vitalism that had dominated physiological thinking for much of the preceding century [3]. Enzymes have always been studied both out of interest in understanding how they achieve their effectiveness as catalysts and as a way of delineating their role in the regulation of metabolism. However, regardless of whether the primary concern of the experimenter is mechanistic or metabolic, kinetic experiments are nearly always designed as if the primary or only objective were to shed light on mechanisms of action. Everyone knows that most enzymes operate physiologically in complex mixtures with many other enzymes and many metabolites as well as their substrates and products, yet almost the first thing that is done when an enzyme is studied is to purify it, or in other words to remove it from its physiological context. However, desirable though it may be to have some information about the mechanism of action of an enzyme, it is not the only thing one needs to know to understand its physiological role.

It may be objected that this emphasis on mechanism does no great harm: surely, if mechanistically designed experiments lead to a full description of the kinetic behaviour of an enzyme then this full description is perfectly valid for assessing how the enzyme should behave as a component of a system. Up to a point this is true, but only up to a point. Analysing a kinetic mechanism is normally a matter of comparing possibilities and eliminating those that do not satisfy the observations, but in practice different mechanisms lead to very similar predictions that are not easy to distinguish, making it necessary to exaggerate the differences by exploring extreme conditions far removed from those of the cell. For example, the ternary-complex and substituted-enzyme mechanisms for two-substrate reactions predict fairly similar kinetic behaviour at moderate substrate concentrations, but very different behaviour at concentrations high enough for substrate inhibition to be important [4]; from the mechanistic point of view it is therefore useful to compare them at very high substrate concentrations even though these may be far outside the range likely in the cell. Yet it is almost without importance for understanding how an enzyme operates in the cell to know what sort of mechanism it follows or how it behaves at very high concentrations of substrate. On the other hand it may be extremely important to assess the effects of inhibitors that are present in the cell even if they do not appear to shed any particular light on the mechanism of action.

Enzyme kinetics in the cell

To predict the kinetic behaviour of an enzyme in the cell it is not necessary to know how it will respond to concentrations outside the physiological range, and in this sense it is sufficient to study it in the range of concentrations likely to occur in the cell. To this extent it is a far simpler task than the one that faces the student of mech-

anism, but the other side of the coin is that one needs to study the effects of all of the metabolites that have a reasonable likelihood of interacting with it in the cell, and these, of course, need to be selected on the basis of their presence in the living system, not because they are easy to obtain or because they have convenient spectroscopic properties. Moreover, whereas a mechanistic study needs to pay attention to making the measurements with the highest possible accuracy, because otherwise the relatively small differences between mechanisms can become submerged in the experimental error, a metabolic study can be less worried about accuracy. To a first approximation it is fair to assume that properties that are difficult to measure or even to detect are unlikely to have important metabolic consequences.

If I had been writing this article 15 years ago I might have felt that there was little more to be said: studying kinetics for metabolic purposes was easier than for mechanistic purposes and that was that. However, it has become clear that there are important exceptions to the idea that phenomena that are not easily detected in the cuvette are unlikely to be important. These derive in general from the fact that enzymes in the cuvette are usually studied at concentrations controlled by the experimenter, whereas in the cell the metabolic concentrations are just as much properties of the system as are the metabolic fluxes.

Kinetics at constant rate

The usual type of steady-state experiment in the cuvette involves measuring the rate that is produced by setting the concentrations of substrates, products, effectors, etc. at predetermined values. It is crucial to realize that this is not what happens in a living system. Virtually all substrates are products of other enzymes; virtually all products are substrates of other enzymes; their concentrations are all variables that respond to the activities of all the enzymes in this system, not just that of the enzyme towards which the experimenter's attention is directed. As Atkinson [5] pointed out two decades ago, many enzymes operate in a regime far closer to fixed rates than to fixed concentrations, in other words most enzymes are required to turn over their substrates at the rates at which they arrive, and the concentrations of substrates and products have to adjust to whatever values are necessary to sustain these rates.

Here, however, there is an important asymmetry in the kinetic behaviour of an enzyme. This is most easily demonstrated by reference to Michaelis–Menten kinetics, but it is true regardless of the actual kinetic equation, as long as it displays saturation (and as virtually all enzymes do display saturation this is not an important qualification). If the rate equation has Michaelis–Menten form, i.e. the rate v depends on the substrate concentration a according to an equation of the form:

$$v = Va/(K_m + a)$$

then a finite rate is defined by any physically possible substrate concentration: it is impossible to choose a positive value of a such that this equation has no solution for v.

The converse, however, is not true. If we rewrite the same equation to express a in terms of v,

$$a = K_m v/(V - v)$$

then it should be immediately evident not only that there are finite positive values of v that correspond to impossible values of a but also that these are not very different from the rates regarded as 'ordinary'. Many enzymes are believed (albeit often on the basis of little or no evidence) to operate *in vivo* at around half-saturation, i.e. around $a = K_m$ and $v = 0.5V$. If this is true then it is also true that most enzymes work on the edge of a catastrophe: a mere doubling of the rates at which substrates arrive will produce a zero in the denominator of the expression for the substrate concentration, in other words conditions in which no steady state is possible. On this sort of basis Atkinson [5] argued that the need for adequate safety factors would impose major constraints on the possible ways in which metabolic systems might be designed (or might have evolved, for readers who prefer a less teleological phraseology).

The reality is somewhat more complicated than this, first because metabolic fluxes are no more determined externally than metabolic concentrations are; both are properties of the system. Second, the irreversible Michaelis–Menten equation as we have written it ignores effects of products, even though all enzymes are subject to product inhibition at high enough concentrations, and all reactions are ultimately reversible. Thus in reality supplying a substrate at a rate too high for the enzyme that acts on it to sustain will not result in an infinite substrate

concentration but in equilibrium: as the substrate accumulates it will increasingly inhibit the enzyme that produces it. Nonetheless, as a first approximation the constant-flux regime may well provide a more realistic picture than the constant-concentration regime suggested by the usual way steady-state kinetic experiments are designed. Even if the equilibrium concentration of a typical metabolite is not infinite, it may often be several orders of magnitude higher than the concentration the cell can tolerate, and the difference between infinite and enormous may have little practical consequence. A plant that is dying from treatment with Roundup will be little comforted by the thought that the concentration of shikimate in its cells is not infinite but merely a few hundred times higher than normal.

Uncompetitive inhibition

All of this brings us to consideration of uncompetitive inhibition, or more exactly to the uncompetitive component in mixed inhibition. The distinction is important, as pure uncompetitive inhibition is rare enough to be dismissed as having little metabolic importance, whereas mixed inhibition with an uncompetitive component is by no means rare and its metabolic consequences can be equally devastating.

Although competitive inhibition is the only sort of inhibition that is given serious consideration most of the time, it is rarely likely to have significant metabolic consequences. Competition is a symmetric interaction: anything that can compete with a substrate for the active site of an enzyme is something the substrate can compete with. In consequence, almost any effect that is produced by the appearance of a competitive inhibitor in a metabolic system can be annulled by minor adjustments of the concentrations of the metabolites around the inhibited enzyme. To put this quantitatively, the effect of a competitive inhibitor present at a concentration equal to its inhibition constant on an enzyme operating at half-maximal rate can be totally nullified by doubling the substrate concentration, from K_m to $2K_m$.

Uncompetitive inhibition is entirely another matter, because it is potentiated by the substrate. The increase in substrate concentration produced by the inhibition increases the degree of inhibition, and only fairly modest levels of inhibition become irresistible. Again putting this quantitatively, an uncompetitive inhibitor present at a concentration equal to its inhibition concentration will increase the substrate concentration of an enzyme acting at half-maximal rate to infinity. As with Atkinson's view of cellular kinetics this over-simple picture requires a little elaboration to be completely accurate, but it is qualitatively correct [6], especially if we take approaching infinity in practice to mean approaching equilibrium.

It is important to realize that this analysis does not require the existence of pure uncompetitive inhibition, a rare property. The inhibition can be predominantly competitive, but as long as there is a non-trivial uncompetitive component it will have effects. By contrast, the competitive component is largely irrelevant: it can be present or not present and the metabolic consequences will be much the same either way.

Examples of metabolic effects of uncompetitive inhibition

Since attention was first drawn to the potentially catastrophic effects of uncompetitive inhibition *in vivo* [6], several examples have been studied that confirm its potential importance. Although these are apparently quite different from one another, they are all cases where analysis shows that uncompetitive inhibition will produce very large effects in circumstances where the same degree of competitive inhibition would be insignificant.

I have already briefly mentioned the weed-killer Roundup (*N*-phosphonomethylglycine, also known as glyphosate), an uncompetitive inhibitor of 3-phosphoshikimate 3-carboxyvinyltransferase [7], which is believed to owe its effectiveness to the huge increases in metabolite concentrations that are produced in treated plants. An example of clinical importance is the use of Li^+ ions to treat manic depression, the effectiveness of which has been attributed to the uncompetitive inhibition of *myo*-inositol monophosphatase by Li^+ [8]. A recent attempt to apply ideas of this kind to a complicated pathway, glycolysis in *Trypanosoma brucei*, which we modelled with the aid of the program GEPASI [9], led to the conclusion that uncompetitive inhibition of pyruvate export would have a high likelihood of toxic effects, whereas competitive inhibition would not, and that none of the other 19 processes in the model offered a promising target for developing a therapeutic agent [10].

An apparently quite different example is provided by an analysis of the effectiveness of

different kinds of inhibition at generating very large levels of sensitivity in cascades of interconvertible enzymes [11]. Uncompetitive inhibition proved to be an essential component of the conditions necessary for producing enormous sensitivity. More generally, this study indicated that large sensitivity required effects on the limiting rates of the enzymes catalysing the covalent modifications. This aspect of the model led Newsholme and Walsh [12] to point out that it was at variance with what was thought to be known about phosphorylase kinase from rabbit skeletal muscle, prompting them to re-examine this enzyme experimentally, only to discover that the model was correct and the 'facts' were not.

A final example is provided by serine biosynthesis in mammals, which, in contrast to the case in bacteria, is not subject to feedback inhibition. All of the inhibitory effect of serine on its biosynthesis is mediated by ordinary product inhibition back through the (short) pathway [13]. The inhibition by serine of the enzyme that produces it is uncompetitive, and analysis of this regulation showed that this is necessary for effective regulation: competitive inhibition would not work [14].

Conclusions

The essential conclusion to be drawn from these examples is that although it remains true that kinetic properties that are not easily observed *in vitro* are unlikely to have major effects *in vivo*, it is necessary to take account of the fact that the fixed-concentration regime of a typical steady-state experiment is very different from the situation *in vivo*, and that it may mask effects of potentially great importance. In particular, effects on the limiting rates of reactions, such as an uncompetitive component in predominantly competitive inhibition, may pass unnoticed in the cuvette and yet be of profound importance in the cell.

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