Specificity of Non-Michaelis−Menten Enzymes: Necessary Information for Analyzing Metabolic Pathways†

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The specificity of an enzyme obeying the Michaelis−Menten equation is normally measured by comparing the k_{cat}/K_m for different substrates, but this is inappropriate for enzymes with a Hill coefficient h different from 1. The obvious alternative of generalizing K_m in the expression as $K_{0.5}$, the substrate concentration for half-saturation, is better, but it is not entirely satisfactory either, and here we show that $k_{cat}/K_{0.5}^h$ gives satisfactory results for analyzing the kinetic behavior of metabolic pathways. The importance of using $k_{cat}/K_{0.5}^h$ increases with the value of h, but even when h is small, it makes an appreciable difference, as illustrated for the mammalian hexokinases. Reinterpretation of data for the specificity of these enzymes in terms of the proposed definition indicates that hexokinase D, often believed highly specific for glucose, and accordingly called "glucokinase", actually has the lowest preference for glucose over fructose of the four isoenzymes found in mammals.

Introduction

Enzyme specificity is fundamental in physiology, as metabolism would be impossible if enzymes could not distinguish between structurally similar substrates, and all reactions proceeded unchecked toward thermodynamic equilibrium. Fischer[1] noted the great selectivity of enzyme-catalyzed reactions long ago, and his lock-and-key model was influential for the development of understanding of enzyme catalysis. However, it focused on substrate binding, suggesting that high specificity implies a small Michaelis constant, K_m, whereas the reality is less simple.

The rate equations for enzyme-catalyzed reactions have been available for many years, at least since 1913 for one-substrate reactions,[2] later extended to the reversible case,[3] but at the beginning of the 1950s this work had barely advanced beyond the state in which Michaelis and his collaborators had left it more than thirty years earlier. During and after the 1950s, however, understanding of more complex reactions, both reversible and irreversible,[4−7] advanced greatly, thanks in particular to the work of Alberty,[5,6] which touched almost every aspect of enzyme kinetics and mechanism, including pH[8] and temperature dependence,[9] progress curve analysis[10] and rapid-reaction kinetics.[11] The question of how best to define enzyme specificity was not considered seriously until much later, however, and different authors disagreed as to whether the catalytic constant k_{cat}, the Michaelis constant K_m, or the ratio k_{cat}/K_m gave the best measure of specificity. For example, Gutfreund[12] seems to have regarded specificity as synonymous with binding; Brot and Bender[13] called K_m the "relative binding specificity" and k_{cat} the "relative catalytic specificity" but concluded that both of these were inferior to k_{cat}/K_m as measures of specificity. They reached the correct conclusion, building on earlier work[14] and introduced the term "specificity constant",[13] but they were primarily concerned with the problems caused by nonproductive binding and did not consider how an enzyme can discriminate between substrates that are present simultaneously.

As long as a reaction is considered with just one substrate at a time, the definition of specificity is little more than a matter of opinion, but the essential physiological question is the capacity of an enzyme to distinguish between two or more competing substrates when they are mixed together.[15] We shall consider only effects of competing substrates on the rates of individual enzyme-catalyzed reactions, but much more complicated questions also arise in physiology, such as the effects of numerous sugars and other molecules on the synthesis of glycogen in hepatocytes.[16] Although there is some compartmentation of metabolites in cells, this is not sufficient to prevent mixing of competing substrates: for example, all of the common amino acids have equal access to aminoacyl-RNA synthetases, so each of these enzymes needs to be able to select the one substrate that is appropriate for it in a mixture where all 20 are available. When specificity is defined in terms of competition between substrates it follows unambiguously that the ratio k_{cat}/K_m defines this capacity, and Fersht[15] proposed that specificity should be defined in terms of k_{cat}/K_m. The name *specificity constant*[13] was later adopted by the International Union of Biochemistry and Molecular Biology,[17] albeit for a different reason from the original one.[13]

Koshland (whose theory of induced fit[18] had earlier provided the first major advance on the lock-and-key theory of specificity[1]) argued that a specificity constant ought to "provide a means of contrasting the specificities of different enzymes towards their substrates",[19] to compare, for example, the specificity of a kinase for different carbohydrate substrates with that of a proteinase for different peptide substrates. This is an interesting point, but it does not address the question of what happens in vivo with mixtures of substrates. In any case, Koshland's appears to be a minority view, and Fersht's interpretation has become widely accepted.[20] There is, however, a regrettable tendency to use k_{cat}/K_m for comparing different enzymes as catalysts, such as mutant forms of an enzyme obtained by genetic manipulation, and not just for comparing different substrates for the same enzyme.

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This can sometimes lead to incorrect conclusions,[21] because enzymes with the same value of k_{cat}/K_m and acting on the same substrate may have different rate ratios at different substrate concentrations: the enzyme with the larger k_{cat}/K_m will always give the higher rate at low concentrations, but the enzyme with the larger k_{cat} will always give the higher rate at high concentrations. This is a separate question from discrimination between substrates that are simultaneously available. It follows, therefore, that use of k_{cat}/K_m as a measure of specificity should not be generalized beyond the original idea of comparing the kinetics of different substrates for the same enzyme.

Fersht's definition of specificity can be generalized to measure the specificity for different competing substrates in reactions that also require additional cosubstrates and may include inhibitors or activators: in these cases the appropriate measure is the *apparent* values of k_{cat}/K_m for the competing substrates at the physiological concentrations of cosubstrates and effectors.[22] This is not only intuitively satisfying but also experimentally convenient, as it means that extrapolation to physiologically unrealistic concentrations is not only unnecessary but also incorrect. A recent application[23] has shown that zinc is the most specific of several metal substrates of protoporphyrin IX ferrochelatase. Adequate assessment of specificity has acquired a new importance with the increasing emphasis on studies of enzymes as components of metabolic pathways rather than of enzymes in isolation.

Many enzymes found at metabolic branch points are cooperative with respect to their substrates, and this cooperativity is typically induced and increased by allosteric inhibitors, a classic prediction of the symmetry model of Monod, Wyman, and Changeux.[20,24] Nearly all discussion of specificity, however, has referred to enzymes that obey Michaelis−Menten kinetics, i.e., noncooperative enzymes, despite its importance for cooperative enzymes. For example, the specificity of mammalian hexokinases is frequently discussed in relation to their capacity to distinguish between glucose and fructose, and the term *phosphorylation coefficient*[25] was widely used in the hexokinase field for the ratio of k_{cat}/K_m values for fructose and glucose. However, it was not initially realized that hexokinase D (also called hexokinase IV), the isoenzyme characteristic of mammalian liver, is cooperative with respect to glucose, and thus has no K_m for glucose, though it follows Michaelis−Menten kinetics with respect to fructose.[27] This complication, together with its high half-saturation concentrations for both substrates, led to much confusion about its specificity, a confusion that persists in the misleading name *glucokinase* that remains common for this isoenzyme. The lack of an established measure of specificity for a cooperative enzyme led one of us[28] to compare substrates in terms of $k_{cat}/K_{0.5}$. However, this is not satisfactory either, and in this paper we consider how specificity should be defined for enzymes that display cooperativity with respect to one or more of their competing substrates.

Theory

If a reaction catalyzed by an enzyme at total concentration e_0 obeys Michaelis−Menten kinetics with respect to two competing substrates, A at concentration a and B at concentration b, the rates v_A and v_B in the presence of both substrates may be written as follows:

$$v_A = \frac{\left(\frac{k_{cat,A}}{K_{m,A}}\right)e_0 a}{1 + \frac{a}{K_{m,A}} + \frac{b}{K_{m,B}}} \qquad v_B = \frac{\left(\frac{k_{cat,B}}{K_{m,B}}\right)e_0 b}{1 + \frac{a}{K_{m,A}} + \frac{b}{K_{m,B}}} \tag{1}$$

Both expressions have the same denominator, which cancels when one is divided by the other, and the reason for regarding k_{cat}/K_m as the most appropriate measure of specificity then follows naturally:

$$\frac{v_A}{v_B} = \frac{k_{cat,A}}{K_{m,A}} \cdot \frac{K_{m,B}}{k_{cat,B}} \cdot \frac{a}{b} \tag{2}$$

It might seem that this equation could be derived more simply and directly just by considering the kinetics at low substrate concentrations, because in the limit the rate of a reaction obeying Michaelis−Menten kinetics is strictly proportional to k_{cat}/K_m. This would give the same result, but generality would be lost as it would incorporate a major unnecessary assumption, that the equation only described behavior at low concentrations. However, eqs 1 apply at all substrate concentrations, including all concentrations of mixed substrates, and eq 2 also applies, therefore, at all concentrations. This was an essential part of Fersht's original argument,[15] and of the one we develop here.

It is also important to emphasize that to be physiologically meaningful, the parameters that appear in these equations must be the apparent values in physiological conditions,[22] not the theoretical values obtained by extrapolation to saturating concentrations of cosubstrates or activators, or zero concentrations of inhibitors. The ratio of rates is then simply the ratio of substrate concentrations multiplied by the ratio of apparent specificity constants. Assessment of discrimination between substrates is thus far easier than it would be if extrapolated kinetic parameters needed to be used. This relationship applies at all concentrations of substrates: although the specificity constant treated as a second-order rate constant defines the rate only at very low substrate concentrations, as a measure of specificity it applies at any concentrations. Likewise, the use of k_{cat}/K_m to eliminate problems due to nonproductive binding[13] is valuable mainly when nonproductive binding is a major concern, as in studies of proteolytic enzymes with small unnatural substrates, but as a measure of specificity it applies to all enzymes.

How is this analysis changed if the reaction is cooperative with respect to a or b? There was intense interest 40 years ago in quaternary interactions[24,29] as the basis for most cases of kinetic cooperativity: although a few known monomeric cooperative enzymes clearly require purely kinetic explanations of their cooperative behavior,[30] these have always been regarded as exceptional. In practice, therefore, most cases of kinetic cooperativity have been analyzed in terms of equations that are little more than equations for equilibrium binding with slight modifications to take account of the chemical reaction, and even in this form they are too complicated to allow a simple analysis of specificity. However, the modern interpretation[15] of specificity is as a physiological rather than a mechanistic concept,[13] and it is well-known that the Hill equation[31] provides an accurate description of the kinetic behavior in the physiological ranges of substrate concentrations of most cooperative enzymes:

$$v = \frac{k_{cat} e_0 a^h}{K_{0.5}^h + a^h} \quad (3)$$

Here h, the Hill coefficient, is typically a nonintegral value in the range 1−4, and $K_{0.5}$, a generalization of K_m in the Michaelis−Menten equation, is the value of a at which $v = 0.5k_{cat}e_0$. Equation 3 is written here as an irreversible equation, in common with the usual practice, but it can be generalized to reversible cases if required.[32,33] If eqs 1 are modified to take account of eq 3, they become as follows:

$$v_A = \frac{k_{cat,A} e_0 \left(\frac{a}{K_{0.5,A}}\right)^{h_A}}{1 + \left(\frac{a}{K_{0.5,A}}\right)^{h_A} + \left(\frac{b}{K_{0.5,B}}\right)^{h_B}}$$

$$v_B = \frac{k_{cat,B} e_0 \left(\frac{b}{K_{0.5,B}}\right)^{h_B}}{1 + \left(\frac{a}{K_{0.5,A}}\right)^{h_A} + \left(\frac{b}{K_{0.5,B}}\right)^{h_B}} \quad (4)$$

As before, the two expressions have the same denominator, which vanishes when the rates are compared:

$$\frac{v_A}{v_B} = \frac{k_{cat,A}}{k_{cat,B}} \cdot \frac{K_{0.5}^{h_B}}{K_{0.5}^{h_A}} \cdot \frac{a^{h_A}}{b^{h_B}} \quad (5)$$

It follows that $k_{cat}/K_{0.5}^h$ is the appropriate quantity to be regarded as the measure of specificity when the Hill equation is obeyed. It is not ideal, because its units include nonintegral powers of $K_{0.5}$ and vary with the strength of the cooperativity. For example, if $h = 2.3$ then $k_{cat}/K_{0.5}^h$ has units of $s^{-1} \cdot mM^{-2.3}$. However, this will create no problems in practice as long as $K_{0.5}$ is measured in the same units as the substrate concentrations, as normally it will be.

Application to Mammalian Hexokinases

As mentioned already, hexokinase D from mammalian liver is an example of an enzyme that is cooperative with respect to glucose, and noncooperative with respect to fructose,[27] though some authors[34] have reported cooperativity. The specificity of this isoenzyme has been a point of contention since it was discovered,[28,35] and the suggestion of the name "glucokinase"[36] was intended to be provisional until "further characterization of the enzyme could make more appropriate some other name". Subsequently, however, it has continued to be called glucokinase by most researchers long after detailed measurements of its specificity became available,[27] for which some relevant values are listed in Table 1. In the original study specificity was provisionally defined as $k_{cat}/K_{0.5}$ in the absence of a more rigorous definition. Comparing the two right-hand columns of the table shows that although the use of $k_{cat}/K_{0.5}^h$ leads to a somewhat lower preference for glucose over fructose than originally suggested, the change is not very great and the rank order of the four substrates considered is unchanged. Notice also that the difference in specificity between mannose and 2-deoxyglucose is small, and possibly within experimental error, whereas $k_{cat}/K_{0.5}$ suggested that the enzyme had a clearly higher preference for mannose.

Although hexokinase D is certainly more specific for glucose than for fructose, this is no less true of the other mammalian hexokinases, *all* of which are more specific for glucose than for fructose:[27] the name "glucokinase" remains misleading, and there is no basis for singling out just one of the four isoenzymes as a glucokinase. To illustrate this point, Table 2 shows specificity data for the four isoenzymes[27] recalculated with $k_{cat}/K_{0.5}^h$ rather than $k_{cat}/K_{0.5}$ as the measure of specificity. When an appropriate measure of specificity is used, it is seen that far from being the most specific for glucose of the four isoenzymes, hexokinase D is in reality the *least* specific, by a large factor.

In this analysis we have made the simplifying assumption that although different substrates for the same enzyme may have different degrees of cooperativity these are unaffected by the presence of a competing substrate. For example, in Table 1 we have assumed that the value of $h = 1.5$ for glucose would be unchanged in the presence of fructose, and the $h = 1.0$ for fructose would be unchanged in the presence of glucose. The reality is more complicated, however, and in fact, competing substrates can suppress the cooperativity of hexokinase D with respect to glucose, albeit at very high and unphysiological concentrations: 250 mM fructose, 25 mM mannose, or 200 mM 2-deoxyglucose.[28,37]

Discussion

It has long been known[15] that the appropriate measure for the substrate specificity of a noncooperative enzyme is k_{cat}/K_m,

TABLE 1: Specificity of Hexokinase D at pH 8.0[a]

substrate	$K_{0.5}$ (mM)	h	$K_{0.5,hex}/K_{0.5,Glc}$	$k_{cat,hex}/k_{cat,Glc}$	$(k_{cat,Glc}/K_{0.5,Glc})/(k_{cat,hex}/K_{0.5,hex})$	$(k_{cat,Glc}/K_{0.5,Glc}^{h_{Glc}})/(k_{cat,hex}/K_{0.5,hex}^{h_{hex}})$
glucose	5	1.5	1.0	1.0	1.0	1.0
mannose	8	1.5	1.6	0.9	1.8	2.3
2-deoxyglucose	22	1.0	4.4	0.7	6.3	2.8
fructose	325	1.0	65	2.6	25.0	11.2

[a] The subscripts Glc and Fru are standard symbols recommended by the IUPAC-IUBMB Joint Commission on Biochemical Nomenclature[26] for glucose and fructose, respectively; hex is a generic symbol that refers to the hexose that is shown in the first column of the table.

TABLE 2: Preference of Mammalian Hexokinases for Glucose over Fructose at pH 7.5[a]

isoenzyme	$K_{0.5,Glc}$ (mM)	$K_{0.5,Fru}$ (mM)	$K_{0.5,Fru}^{h_{Fru}}/K_{0.5,Glc}^{h_{Glc}}$	$k_{cat,Glc}/k_{cat,Fru}$	$(k_{cat,Glc}/K_{0.5,Glc}^{h_{Glc}})/(k_{cat,Fru}/K_{0.5,Fru}^{h_{Fru}})$
hexokinase A	0.044	3.1	70.5	0.91	64.1
hexokinase B	0.130	3.0	23.1	0.83	19.3
hexokinase C	0.020	1.2	60.0	0.77	46.2
hexokinase D	7.5	420	20.5	0.42	8.5

[a] The subscripts Glc and Fru refer to glucose and fructose respectively, as in Table 1.

TABLE 3: Variation of Specificity with Degree of Cooperativity

	$(k_{cat,1}/K_{0.5,1}^{h_1})/(k_{cat,i}/K_{0.5,i}^{h_i})$ at $h_3 = h_4 = 1$ and $h_1 = h_2$ as shown							
substrate[a]	$h_1 = 0.5$	$h_1 = 1.0$	$h_1 = 1.5$	$h_1 = 2.0$	$h_1 = 2.5$	$h_1 = 3.0$	$h_1 = 3.5$	$h_1 = 4.0$
A_1/A_2	1.40	1.8	2.3	2.84	3.6	4.6	5.8	7.4
A_1/A_3	14.1	6.3	2.8	1.3	0.6	0.3	0.1	0.05
A_1/A_4	55.6	25.0	11.2	5.0	2.2	1.0	0.5	0.2

[a] In each line the parameter for substrate A_2, A_3, or A_4 is compared with that for a reference substrate A_1. These are given anonymous symbols, because the table refers to hypothetical enzymes in which the Hill coefficients have different values from those that apply to the hexokinase isoenzymes in Table 1. However, they can be thought of as glucose, mannose, 2-deoxyglucose, and fructose, respectively, without doing great violence to reality.

or its apparent value if other cosubstrates or effectors are present,[22] but it has not been clear how this ought to be generalized for considering the specificity of cooperative enzymes, and the lack of an adequate measure has helped to perpetuate confusion over the specificity of mammalian hexokinases, three of which (hexokinases A, B, and C) are noncooperative with (as far as is known) all substrates, apart from the complication that hexokinase C is inhibited by excess glucose.[38] The fourth isoenzyme, hexokinase D, is weakly cooperative with respect to some substrates and noncooperative with respect to others. We have shown that as long as the Hill equation provides an adequate representation of the kinetic behavior (which will usually be true for physiological but not for mechanistic studies) the appropriate measure is $k_{cat}/K_{0.5}^h$ and not, as might have been guessed, $k_{cat}/K_{0.5}$. For the mammalian hexokinases this changes the previous interpretation of their specificity[27] rather little, but that just reflects the rather low level of cooperativity. With more highly cooperative enzymes, with Hill coefficients in the range 3−4, the effect is much greater, as seen in Table 3, in which the four substrates are not named, to emphasize that although the column for $h_1 = 1.5$ corresponds to the right-hand column of Table 1, Table 3 refers to hypothetical enzymes. When both substrates have the same degree of cooperativity, as supposed for A_1 and A_2, the effect is not very great and does not alter the rank order of the substrates. However, when they have different degrees of cooperativity, as seen in Table 3 for comparison of A_1 with A_3 and A_4, the effect is very large and needs to be taken into account in assessing the discrimination between competing substrates in physiological conditions. Although we are mainly concerned with positive cooperativity, the column for $h = 0.5$ in Table 3 also illustrates that specificity may be substantially perturbed by negative cooperativity for one or more of the substrates compared. For example, *N*-acetylglucosamine kinase from rat intestinal mucosa shows a pronounced negative cooperativity with respect to glucose that long caused it to be confused with hexokinase D (glucokinase),[39] and the effects of this on discrimination between glucose and *N*-acetylglucosamine at high concentrations of glucose (such as occur in diabetes) ought to be investigated.

These considerations are assuming a new importance with the rapid growth of systems biology, and especially in efforts to understand the kinetic behavior of complete systems of enzymes, such as reconstituted metabolic pathways. For example, aspartate metabolism in plants[40] involves numerous examples of cooperativity and isoenzymes, and without an adequate definition of specificity, it will hardly be possible to ask, let alone answer, questions of how satisfactory discrimination between substrates and, especially, effectors is achieved in such a system. Although, for example, aspartokinase II shows negligible cooperativity with respect to its substrate aspartate in the absence of allosteric inhibitors, threonine at high concentrations can induce cooperativity (G. Curien, personal communication), and this is a property that ought to be taken into account in future simulations.

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