BIOCHEMICAL JOURNAL LETTERS

Specificity constants for two-substrate reactions

Engel [1] has proposed a "true combined specificity constant" for two-substrate reactions. Unfortunately, his proposal is only applicable to reactions which proceed through a ternary complex. There is no equivalent constant for reactions which proceed through a substituted enzyme (ping–pong) mechanism [2,3 (page 112)]. Although specificity constants are often said to measure catalytic efficiency and to have fundamental significance, these statements are only true in the context of measurements *in vitro* when initial velocities are measured with enzyme concentrations much less than those of the substrate and much less than the K_m, and when product is absent, conditions not always found *in vivo* ([4] and references therein). Because of the arbitrary aspect of this constant, a consistent definition is desirable.

The definition of specificity constant given by the IUB Nomenclature Committee [5] for a single-substrate reaction is the apparent second-order rate constant for a reaction at very low substrate concentrations, and thus defined it is applicable whatever kinetics the enzyme displays with higher substrate concentrations. For Michaelis–Menten kinetics, this second-order rate constant is identical with the ratio $k_{cat.}/K_m$. Cornish-Bowden [3 (page 84)] has pointed out that for a pair of alternative substrates A and B present together (and Michaelis–Menten kinetics) the ratio of velocities is given by:

$$\frac{v_A}{v_B} = \frac{V^A K_m^B [A]}{V^B K_m^A [B]} \quad (1)$$

where V and K are the parameters obtained for each substrate separately at the same enzyme concentration, so that for *any* equal substrate concentrations the ratio of velocities is the ratio of specificity constants.

For two-substrate reactions, when a second substrate B is competing with A in its reaction with C, equations for initial velocities are readily derived using steady-state assumptions (except for the random order/ternary complex mechanism which requires equilibrium assumptions to force a Michaelis–Menten outcome). The equations are a little more complex than those for competing one-substrate reactions, but the ratio of velocities reduces to eqn. (1). Except when C is the second substrate to add in an ordered reaction forming a ternary complex, the result is independent of [C]. In the exceptional case it is true only when C is saturating. (It should be stressed, as Engel has done [1], that K_m^A and K_m^B are K_m values properly determined for saturating C [3 (page 111)], even though they are then applied more generally.) For the Theorell–Chance mechanism [6], eqn. (1) is valid when the competing substrates are the first to add to form an enzyme–substrate complex (at any concentration of C), but when the competing substrates are the second to react the concept of specificity constant has no meaning. In this case the ratio of velocities is simply $k_A[A]/k_B[B]$, where k_A and k_B are second-order rate constants for the reaction of the enzyme–C complex with A and B respectively.

Because Cornish-Bowden's competition concept [3] for

enzymes displaying Michaelis–Menten kinetics is applicable to two-substrate reactions at any equal concentrations of the competing substrates, is applicable to all two-substrate reaction mechanisms, and uses the common operationally defined K_m for a substrate, I suggest that the specificity constant defined by $k_{cat.}/K_m$ be retained for all reactions. For some two-substrate reactions we may have to consider two alternative pairs of substrates: for example, two competing substrates for a dehydrogenase which can react with two competing cofactors (commonly ordered addition/ternary complex mechanisms), or two amino acids competing for transamination with two competing 2-oxoacids (substituted enzyme mechanisms when the transaminase has a tightly bound pyridoxal phosphate cofactor). If A or its alternative B can react with C or its alternative D, the substrates may be considered three at a time. For example, if any equal concentrations of A and B compete in a reaction with C and give velocities v_{AC} and v_{BC} respectively, these will have the ratio $k_{cat.}^{AC} K_m^{BC}/k_{cat.}^{BC} K_m^A$. The superscripts identify the reactions for which $k_{cat.}$ and K_m have to be separately determined, and there will be three other ratios (v_{AD}/v_{BD}, v_{AC}/v_{AD}, and v_{BC}/v_{BD}) each with its appropriate $k_{cat.}$ and K_m values. Specificities will then be apparent from a comparison of the four velocity ratios. (The actual work involved is not greater than that required for Engel's proposed combined constant [1].)

This treatment does not consider relative velocities when all four substrates are present together, but that is not the purpose of specificity constants as used, for example, in studies on the effects of point mutations on catalytic efficiency and substrate specificity. The effects of mutations *in vivo* may require evaluation of rates with all four substrates and products present, but the effects of changes in individual rate constants (to which changes in $k_{cat.}$ and K_m are secondary) have not yet been analysed fully ([7] and references therein).

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Enzyme specificity in reactions of more than one co-substrate

In a recent letter, Engel (1992) discusses the meaning of the specificity constant of an enzyme with two co-substrates. He makes several important points, especially in relation to the 'overall catalytic efficiency' introduced by Feeney et al. (1990)

for expressing their efforts to alter the nucleotide specificity of *Bacillus stearothermophilus* lactate dehydrogenase. Engel (1992) concludes that this quantity 'has no easily identifiable physical significance, and to describe it as a measure of overall catalytic efficiency is misleading'. To this one can only agree, but he takes the discussion further, concluding that the appropriate quantity to call the 'true combined specificity constant' is the limit at infinite dilution of the co-substrate of the apparent specificity constant divided by the co-substrate concentration. This is more arguable, because it takes as fundamental a property of the specificity constant that is not directly related to specificity.

For a one-substrate reaction obeying Michaelis–Menten kinetics the specificity constant k_A of a substrate A is the ratio of its catalytic constant and its Michaelis constant, i.e. $k_A = k_{cat.}/K_m$, and as Engel (1992) points out, it has a clear physical meaning as the constant of proportionality relating rate to substrate and enzyme concentrations at low concentrations. However, it was not this particular physical meaning that led the Nomenclature Committee of the IUB (1983) to recommend the name 'specificity constant' for k_A. Rather it was the fact that the ratio of specificity constants k_{A_1} and k_{A_2} for two competing substrates A_1 and A_2 defines the ratio of the rates v_1 and v_2 when A_1 and A_2 are mixed together, i.e. $v_1/v_2 = (k_{A_1}[A_1])/(k_{A_2}[A_2])$. This relationship applies at any concentrations of A_1 and A_2, not just at low ones (see, e.g., Fersht, 1985). Because the meaning of the specificity constant as the limit of a rate constant is in general much more widely known, it is unfortunately easy to forget that the meaning in relation to specificity is not just a limit and remains valid at high concentrations.

Many authors have seen the specificity constant as having additional meanings; for example as a measure of catalytic efficiency (e.g. Feeney et al., 1990). However, in deciding how to employ the term 'specificity constant' in reactions of more than one co-substrate one should not lose sight of the idea of specificity, i.e. the ability of the enzyme to discriminate between two competing substrates that are mixed together. I have discussed the meaning of specificity in such cases elsewhere (Cornish-Bowden, 1984), and will not repeat the algebra here, but will simply summarize the main conclusions in relation to the general equation for a reaction with two co-substrates, written as by Engel (1992) with the symbolism of Dalziel (1957):

$$\frac{e}{v} = \phi_0 + \frac{\phi_A}{[A]} + \frac{\phi_B}{[B]} + \frac{\phi_{AB}}{[A][B]}$$

where v is the initial rate at a total enzyme concentration e and concentrations [A] and [B] of the substrates. The constants ϕ_0, ϕ_A and ϕ_B are the reciprocals of the catalytic constant and the specificity constants, i.e. $\phi_0 = 1/k_{cat.}$, $\phi_A = 1/k_A$, $\phi_B = 1/k_B$. Engel (1992) calls $1/\phi_{AB}$ the true combined specificity constant.

Analysis of this equation may appear complicated, but its results are sufficiently simple and intuitively reasonable that they may seem obvious. The most important is that the constants that determine how well an enzyme can discriminate between two competing substrates A_1 and A_2 [e.g. NADH and NADPH, as in the example considered by Feeney et al. (1990)] at some concentration of a co-substrate B (e.g. pyruvate), are the apparent specificity constants for A_1 and A_2 considered separately at that same concentration of B, i.e. $k_{A_i}^{app.} = 1/(\phi_{A_i}\phi_{A_iB}/[B])$, where $i = 1$ or 2. It is not only not necessary to extrapolate the concentration of B either to zero ($k_{A_i}^{app.} \to [B]/\phi_{A_iB}$) or to saturation ($k_{A_i}^{app.} \to 1/\phi_{A_i}$); it is also wrong, if the objective is to measure specificity. It follows that $1/\phi_{AB}$ does not have the fundamental property of a specificity constant, because it defines discrimination between

competing substrates only under limiting conditions, not in general.

A second point is that a co-substrate that reacts only in the part of a mechanism that is common to two competing reactions (such as the first substrate in a compulsory-order ternary-complex mechanism) has no effect on the discrimination between two competing substrates, because in this case the ratio of apparent specificity constants is equal to the ratio of limiting specificity constants at all concentrations. (Both individual apparent specificity constants can still vary with the concentration of co-substrate, but they vary in constant ratio.) However, the concentration of a co-substrate that reacts outside the common part of the mechanism (such as the second substrate in the same mechanism) can, and in most cases probably does, affect discrimination between the competing substrates. This immediately suggests, of course, a use for specificity measurements in distinguishing between mechanisms.

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Specificity constants for two-substrate enzymes

In his response [1] to my letter [2] about specificity constants, Cornish-Bowden reminds us of the properties of specificity constants for single substrates, but in doing so misses the original point. The issue arose [3] in the context of protein engineering experiments on lactate dehydrogenase, which catalyses a compulsory-order two-substrate reaction. As conventionally defined, the specificity constant for coenzyme would be $1/\phi_{NAD} = k_1$, i.e. it would equal the 'on' rate constant for binding the leading substrate, NAD^+. This does indeed give a figure for comparison in assessing the selectivity between NAD^+ and an alternative coenzyme, and does so in a way that is independent of coenzyme concentration. The point, however, is that it takes absolutely no account of the second substrate, which is assumed to be saturating. (The converse is not true: in this mechanism the conventional specificity constant for the second substrate to bind *does* bear the imprint of the first substrate.)

Suppose that $NADP^+$, though a poor coenzyme with lactate as substrate, was quite a good coenzyme with 2-hydroxybutyrate as substrate. The conventional specificity constants for the two coenzymes would entirely conceal this important aspect of specificity. Feeney et al. [3] were searching for a way to address this deficiency. If one abandons the focus on specificity for a single substrate and considers instead the *substrate pair*, e.g. NAD^+–lactate, then the constant $1/\phi_{AB}$, the apparent third-order rate constant at low concentrations of both substrates, would appear to have the necessary properties to compare relative catalytic efficiencies. One may argue over what the IUB should call this constant, but it seems a useful addition to the