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A kinetic method for the determination of the multiple forms of microsomal cytochrome P-450

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The identification and characterization of the multiple forms of cytochrome P-450 associated with liver microsomes are currently being extensively studied [1–8]. Such studies may lead to the categorization of the various enzymes involved into activity profiles which in turn would provide information as to their relative roles as detoxifying catalysts or as catalysts which generate toxic electrophilic intermediates.

The study of the multiple forms of cytochrome P-450 has been approached by two main lines of investigation. The first and most direct has been biochemical investigations which have focused on the physical isolation, purification and characterization of both normal and inducible forms [9–13]. The second method has approached the problem indirectly by measuring changes produced in the enzymatic profiles of the system by some perturbation, typically the use of inducing agents such as phenobarbital (PB) and 3-methylcholanthrene (3-MC) and inhibitors such as carbon monoxide or SKF-525A [14–20]. In interpreting such changes in terms of multiplicity, most investigators have operated under the same set of assumptions, but to our knowledge neither the assumptions nor the rules for their applications have been explicitly stated.

The purpose of this communication is to state the assumptions initially for a system involving a single substrate, a single enzymatic site and multiple products. Given these assumptions, a systematic framework for the interpretation of changes in microsomal enzymatic profiles of such a system brought about by various perturbations to the system will be proposed. In developing the framework, we assume that product formation is irreversible and that the steady state kinetics of Briggs and Haldane [21] are applicable.

For a single substrate, single enzymatic site, multiple product system, the following two cases are possible.

Case 1. The substrate combines with an enzyme to form a single enzyme–substrate complex which dissociates to multiple products, that is

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E\cdot S \begin{cases} \xrightarrow{k_2} P_1 + E \\ \xrightarrow{k_3} P_2 + E \\ \xrightarrow{k_4} P_3 + E \end{cases}$$

Deriving the expression for $E\cdot S$ by the method of King and Altman [22] as described by Segel [23] and rearranging to the form of the Michaelis–Menten equation yields

$$E\cdot S = \frac{(E_0)(S)}{\dfrac{k_{-1} + k_2 + k_3 + k_4 + S}{k_1}}$$

Therefore, a single K_m

$$K_m = \frac{k_{-1} + k_2 + k_3 + k_4}{k_1}$$

characterizes the entire system and is independent of either enzyme or substrate concentration. Since the individual velocities for each of the products are $dP_1/dt = k_2(E\cdot S)$, $dP_2/dt = k_3(E\cdot S)$ and $dP_3/dt = k_4(E\cdot S)$, the ratio of any two will be constant and independent of either the concentration of E or S.

Case 2. The substrate combines with an enzyme to form energetically distinct $E\cdot S$ complexes each of which dissociates to a different product, that is

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E\cdot S \xrightarrow{k_4} P_1$$

$$\underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E\cdot S' \xrightarrow{k_5} P_2$$

$$\underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} E\cdot S'' \xrightarrow{k_6} P_3$$

Once again, deriving the expressions for $E\cdot S$, $E\cdot S'$ and $E\cdot S''$ by the method of King and Altman [22] and rearranging in the form of the Michaelis–Menten equation yield three equations:

$$E\cdot S = \frac{\dfrac{k_1(k_{-2} + k_5)(k_{-3} + k_6)[E_0][S]}{R}}{K_m + S}$$

$$E\cdot S' = \frac{\dfrac{k_2(k_{-1} + k_4)(k_{-3} + k_6)[E_0][S]}{R}}{K_m + S}$$

$$E\cdot S'' = \frac{\dfrac{k_3(k_{-1} + k_4)(k_{-2} + k_5)[E_0][S]}{R}}{K_m + S}$$

Where

$$R = k_1(k_{-2} + k_5)(k_{-3} + k_6) + k_2(k_{-1} + k_4)(k_{-3} + k_6) + k_3(k_{-1} + k_4)(k_{-2} + k_5)$$

and

$$K_m = \frac{(k_{-1} + k_4)(k_{-2} + k_5)(k_{-3} + k_6)}{R}$$

The K_m again characterizes the entire system. Since the velocities for product formation are $dP_1/dt = k_4(E \cdot S)$, $dP_2/dt = k_5(E \cdot S')$ and $dP_3/dt = k_6(E \cdot S'')$ and since the ratio of

$$(dP_1/dt)/(dP_2/dt) = \frac{k_4k_1(k_{-2} + k_5}{k_5k_2(k_{-1} + k_4)},$$

the ratio of any two products is a constant which is independent of either enzyme or substrate concentration. It should be noted that in deriving the rate laws for Case 1 and Case 2 the only assumptions that were made were that steady state kinetics were applicable and that product formation was irreversible. It was not necessary to assume that product formation is rate limiting.

Consider a microsomal preparation which acts on a single substrate to yield multiple products. Information regarding the enzymatic multiplicity of the preparation can now be gained by perturbing the system. Fundamentally two types of perturbations are possible: (1) those that affect enzyme or substrate concentration without altering the active site or individual rate constants, and (2) those that can alter the active site or individual rate constants.

Type-1 perturbers include such factors as inhibitors, inducers and substrate concentration studies. For such perturbations any statistically valid difference in the measured K_m or any change in product ratio for either Case 1 or Case 2 systems indicates the existence of at least two independent enzymatic sites. Conversely lack of changes in K_m or product ratios are not conclusive evidence for the presence of a single enzymatic site but become increasingly convincing as the number of perturbations studied is increased.

Type-2 perturbers include such factors as pH (by affecting ionizable groups at the active site), ionic strength (change solvation of the active site), temperature (shift the steady state constants) and allosteric interactions (by inducing conformational changes at the active site). For such perturbations, all combinations of changes in K_m and product ratios are possible, that is, three results are possible. (1) K_m and product ratios do not change. This result indicates that the perturbation did not affect either the rate constants or the active site. (2) The K_m changes but product ratios do not (e.g. a temperature effect) or product ratios change and K_m does not (e.g. an allosteric interaction). These results indicate a single enzyme but are not conclusive (to have the situation where two independent enzymes gave exactly the same product ratios but had different K_m values or the converse would be highly fortuitous). (3) Both K_m and product ratios change. This result yields no information regarding multiplicity.

Based on the above analysis and assuming the model is applicable, it would appear that information regarding the multiple forms of microsomal cytochrome P-450 can most readily be obtained by perturbing the system by factors which only affect enzyme or substrate concentrations. The application of this technique and its extension to multiple substrates will be discussed in a future publication.

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