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Product inhibition in mechanisms in which the free enzyme isomerizes

In any enzyme mechanism, the form of free enzyme left after the last product has been released may be different from the one to which the first substrate binds, so that the catalytic cycle is completed by an enzyme isomerization to regenerate the original form. Efforts to detect such isomerization met with little success, however, until the introduction of the tracer perturbation technique by Britton (1966, 1973), which was used by him and his associates to study a number of mutases (Britton and Clarke, 1968, 1972; Britton et al., 1971, 1972); more recently it has been applied to proline racemase (Fisher et al., 1986), triose phosphate isomerase (Raines and Knowles, 1987), and fumarase (Rebholz and Northrop, 1993). Britton and associates refer to the technique as 'induced transport', but the term 'tracer perturbation' proposed by Albery and Knowles (1987) gives a clearer indication of what it involves, and will be used here.

In principle, free-enzyme isomerization can also be detected by product inhibition, because it generates a term in *ap* in the denominator of the rate equation, where *a* and *p* are the concentrations of first substrate and last product respectively. It is obvious that if the isomerization is very fast this term will be negligible, as has long been realized (Cleland, 1963; Ray and Roscelli, 1964; Taraszka and Alberty, 1964). Britton (1973) drew attention to the less obvious point that it also becomes negligible when the isomerization is slow, so that product inhibition cannot provide a reliable indication of whether a kinetically significant isomerization occurs.

Rebholz and Northrop (1993) have recently contested this analysis, claiming that 'Britton (1973) was frustrated by the lack of a definite relationship between his kinetic constants and the relative rates of isomerizations', and explaining this hypothetical frustration in terms of two supposed errors in his analysis. In a note added in proof, they add that 'Britton's intuition that inhibition will appear competitive in the presence of slow isomerization appears to be correct', but they do not explain why they ascribe this conclusion to Britton's intuition rather than to his algebraic analysis, nor why they thought it useful to devote several pages to a laboured account of their efforts to arrive at an opposite (and wrong) conclusion. Here I show that the claims of Rebholz and Northrop (1993) to have detected errors in Britton's analysis are unfounded.

The three-step model used by Britton (1973) to analyse product inhibition in a mechanism with isomerization of the free enzyme is shown at the left-hand side of Scheme 1. One of the criticisms by Rebholz and Northrop (1993) was that this three-step model fails to cover the range of possible behaviour for this kind of mechanism, because of the absence of an explicit chemical step converting the enzyme–substrate complex into the enzyme–product complex. The corresponding four-step model, with this step included, is therefore shown at the right-hand side of Scheme 1. The two models are laid out, and their rate constants numbered, in such a way as to facilitate comparison between them; specifically, the steps in the three-step model are numbered 1, 3 and 4, and there is no step 2. In all algebraic expressions, primed rate constants refer to the three-step version and unprimed rate constants to the four-step version.

The rate equation, which can be obtained by standard methods such as that of King and Altman (1956), has exactly the same form for both versions of the mechanism, and is as follows:

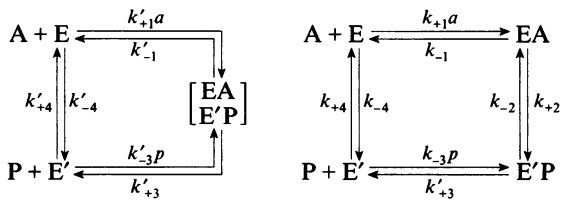
$$v = \frac{\dfrac{V_A a}{K_{mA}} - \dfrac{V_P p}{K_{mP}}}{1 + \dfrac{a}{K_{mA}} + \dfrac{p}{K_{mP}} + \dfrac{ap}{K_{AP}}} \qquad (1)$$

The parameters consist of two limiting rates, V_A and V_P, two Michaelis constants, K_{mA} and K_{mP}, and a constant K_{AP} that determines whether there is any significant uncompetitive component in the product inhibition; their definitions are different for the two versions of the mechanism and are listed and compared in Table 1. The numerical values given in Table 1 refer to the discussion of Table 2 below.

The controversy concerns the circumstances in which the term in *ap* becomes negligible. Britton (1973) considered a symmetrical set of rate constants in which $k'_{+1} = k'_{-3}$, $k'_{+4} = k'_{-4}$ and $k'_{+3} = k'_{-1}$, and studied the effect on K_{AP} of the ratio k'_{+4}/k'_{-1}, i.e. the rapidity of the free-enzyme isomerization compared with the rate constants for substrate and product release. He found that, when this ratio was varied in such a way that the Michaelis constants K_{mA} and K_{mP} remained fixed, the value of K_{AP} showed a curved dependence on this ratio, with a minimum at $k'_{+4}/k'_{-1} = 1$, approaching infinity not only when k'_{+4}/k'_{-1} became very large (as was expected from previous work), but also when it became very small (as was not).

According to Rebholz and Northrop (1993), this analysis contains two errors: first, they believe that K_{AP} is strictly proportional to a ratio *m*, defined as $m = k'_{+4}/k'_{-1}$; second, they consider that the treatment was seriously incomplete in the sense that if the chemical step had been included, as in the right-hand side of Scheme 1, a wider range of behaviour would have been possible. Both of these points are false.

In their efforts to reconstruct Fig. 1 of Britton (1973), Rebholz and Northrop (1993) overlooked the fact that Britton had held K_{mA} and K_{mP} constant while he varied *m*: to do this it is not



Scheme 1

Table 1 Definitions of kinetic constants in terms of rate constants

The Table shows the definitions of the parameters of eqn. (1) in terms of both the three-step and four-step model for a reaction with isomerization of free enzyme (left-hand side and right-hand side of Scheme 1 respectively). The numerical values refer to the calculations discussed in relation to Table 2.

Parameter	Three-step model	Four-step model	Example
V_A	$\frac{k'_{+3}k'_{+4}e_0}{k'_{+3}+k'_{+4}}$	$\frac{k_{+2}k_{+3}k_{+4}e_0}{k_{-2}k_{+4}+k_{+2}k_{+3}+k_{+2}k_{+4}+k_{+3}k_{+4}}$	8.954×10^{-2}
V_P	$\frac{k'_{-1}k'_{-4}e_0}{k'_{-1}+k'_{-4}}$	$\frac{k_{-1}k_{-2}k_{-4}e_0}{k_{-1}k_{-2}+k_{-2}k_{-4}+k_{-1}k_{-4}+k_{+2}k_{-4}}$	8.625×10^{-4}
K_{mA}	$\frac{(k'_{-1}+k'_{+3})(k'_{-4}+k'_{+4})}{k'_{+1}(k'_{+3}+k'_{+4})}$	$\frac{(k_{-1}k_{-2}+k_{-1}k_{+3}+k_{+2}k_{+3})(k_{-4}+k_{+4})}{k_{+1}(k_{-2}k_{+4}+k_{+2}k_{+3}+k_{+2}k_{+4}+k_{+3}k_{+4})}$	6.811
K_{mP}	$\frac{(k'_{-1}+k'_{+3})(k'_{-4}+k'_{+4})}{k'_{-3}(k'_{-1}+k'_{-4})}$	$\frac{(k_{-1}k_{-2}+k_{-1}k_{+3}+k_{+2}k_{+3})(k_{-4}+k_{+4})}{(k_{-1}k_{-2}+k_{-2}k_{-4}+k_{-1}k_{-4}+k_{+2}k_{-4})k_{-3}}$	9.587
K_{AP}	$\frac{(k'_{-1}+k'_{+3})(k'_{-4}+k'_{+4})}{k'_{+1}k'_{-3}}$	$\frac{(k_{-1}k_{-2}+k_{-1}k_{+3}+k_{+2}k_{+3})(k_{-4}+k_{+4})}{k_{+1}(k_{-2}k_{+2})k_{-3}}$	94.05

Table 2 Correspondence between the three- and four-step models

The Table shows the relationships between the rate constants of the four-step model and those of the three-step model. The numerical values shown in the right-hand column were obtained by assigning the following arbitrary values to the rate constants of the four-step model: $k_{-1} = 6.3 \times 10^{-3}$, $k_{+1} = 2.7$, $k_{-2} = 4.7 \times 10^{-2}$, $k_{+2} = 2.9 \times 10^{-1}$, $k_{-3} = 3.8$, $k_{+3} = 42$, $k_{-4} = 26$, $k_{+4} = 1.3 \times 10^{-1}$.

Three-step model	Four-step model	Example
k'_{-1}	$\frac{k_{-1}k_{-2}}{k_{-1}+k_{-2}+k_{+2}}$	8.625×10^{-4}
k'_{+1}	$\frac{k_{+1}(k_{-2}+k_{+2})}{k_{-1}+k_{-2}+k_{+2}}$	2.650
k'_{-3}	$\frac{(k_{-2}+k_{+2})k_{-3}}{k_{-2}+k_{+2}+k_{+3}}$	3.025×10^{-2}
k'_{+3}	$\frac{k_{+2}k_{+3}}{k_{-2}+k_{+2}+k_{+3}}$	0.2877
k'_{-4}	k_{-4}	26.0
k'_{+4}	k_{+4}	0.13

sufficient just to vary k'_{+4} and k'_{-4}, as Rebholz and Northrop (1993) did in drawing their Figure 2; nor is it sufficient to vary k'_{+3} and k'_{-1}, as they did in drawing their Figure 3. When the constraint is taken into account it is easy to show, by combining the expressions for K_{mA} and K_{AP} given for the three-step model in Table 1, that $K_{AP} = K^2_{mA}(1+m)^2/4m$. Thus if K_{mA} is held constant, K_{AP} behaves exactly as found by Britton (1973), with a minimum at $m = 1$, i.e. $k'_{+4} = k'_{-1}$.

To dispose of the second criticism, that the three-step scheme does not allow for the full range of behaviour possible when the chemical step is explicitly included, it is sufficient to note that the two schemes are completely interchangeable so far as the steady-state rate equation is concerned. The substitutions necessary for converting one into the other are listed in Table 2. It is evident that any set whatsoever of positive values for the rate constants of the four-step scheme will generate an equivalent set of positive values for the rate constants of the three-step scheme. Thus no steady-state rate behaviour is possible when the chemical step is explicitly written that is not possible when it is subsumed in the central complex. Although there is a danger of making algebraic errors when one tries to verify the correctness of these relationships, it is easy to check that both schemes generate identical kinetic parameters when one assigns arbitrary numerical values to the rate constants. An example is given in the caption to Table 2, with the values of the rate constants of the three-step scheme shown in the right-hand column. Whichever set of definitions in Table 1 one uses, the resulting numerical values for the kinetic parameters are the same, namely those shown in the right-hand column of Table 1.

It follows, therefore, that the four-step scheme is just a more explicit way of writing the three-step scheme, and that making the chemical step explicit adds nothing to the range of possible steady-state behaviour. Of course, if measurements are made in the transient state, or if kinetic isotope effects perturb the rate constants of the chemical step without influencing the binding steps, the models may become different, but neither of these considerations affects whether the term in *ap* can be detected by product inhibition.

According to Albery and Knowles (1987), 'no kinetic investigation of an enzyme is complete until experiments have been conducted to determine whether the rate-limiting steps in the saturated region are concerned with substrate handling or with free enzyme interconversion'. Such experiments are rarely done, however, possibly because product inhibition has rarely provided any evidence for kinetically significant enzyme isomerization, thereby suggesting that it is not an important aspect of enzyme catalysis, in contrast with the view of Albery and Knowles (1987).

The analysis of Britton (1973), however, explains why product inhibition can fail to detect enzyme isomerization even if it is rate-limiting, and makes it clear that it does not provide an adequate basis for deciding whether there is a compulsory isomerization in the mechanism. The only currently available method that does this unambiguously is the tracer-perturbation technique developed by him.

General acceptance of the erroneous analysis by Rebholz and Northrop (1993) would detract from the study of enzyme mechanisms by encouraging the mistaken belief that product inhibition can always detect kinetically significant isomerizations.

This, in turn, would discourage the use of tracer perturbation and foster the belief that enzyme isomerization is not important. It should be realized, therefore, that the criticisms of Britton (1973) by Rebholz and Northrop (1993) are false and misleading.

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Albery, W. J. and Knowles, J. R. (1987) J. Theor. Biol. **124**, 137–171
Britton, H. G. (1966) Arch. Biochem. Biophys. **117**, 167–183
Britton, H. G. (1973) Biochem. J. **133**, 255–261
Britton, H. G. and Clarke, J. B. (1968) Biochem. J. **110**, 161–183
Britton, H. G. and Clarke, J. B. (1972) Biochem. J. **130**, 397–410
Britton, H. G., Carreras, J. and Grisolia, S. (1971) Biochemistry **10**, 4522–4533
Britton, H. G., Carreras, J. and Grisolia, S. (1972) Biochemistry **11**, 3008–3014
Cleland, W. W. (1963) Biochim. Biophys. Acta **67**, 104–137
Fisher, L. M., Albery, W. J. and Knowles, J. R. (1986) Biochemistry **25**, 2538–2542
King, E. L. and Altman, C. (1956) J. Phys. Chem. **60**, 1375–1378
Raines, R. T. and Knowles, J. R. (1987) Biochemistry **26**, 7014–7020
Ray, W. J., Jr. and Roscelli, G. A. (1964) J. Biol. Chem. **239**, 3935–3941
Rebholz, K. L. and Northrop, D. B. (1993) Biochem. J. **296**, 355–360
Taraszka, M. and Alberty, R. A. (1964) J. Phys. Chem. **68**, 3368–3373

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Problems in defining limits: how slow is very slow?

I regret the error in our paper (Rebholz and Northrop, 1993) and apologize to Britton (1973) for misreading his paper. As pointed out by Cornish-Bowden (1994), I overlooked the constraint of holding K_S and K_P constant. Britton's (1973) Fig. 1 contains a plot of K_{SP} versus m, with a Figure legend containing '$K_S = K_P = 1$ mM' and '$k_{+3} = k_{-3} = mk_{-1} = mk_{+2}$.' The former is the only indication of the constraint, and the latter led me to believe that the plot was an expression of K_{SP} as a function of only these rate constants. Britton makes several statements about K_{SP} approaching infinity, accompanied by discussions which include only these rate constants. I could not verify the assertion that K_{SP} approached infinity when $m \rightarrow 0$, which then became the subject of our paper. That assertion is rendered conditionally correct, however, if K_S and K_P are held constant, which requires allowing other rate constants to vary as well. Also, part of the discussion of K_{SP} approaching infinity appears before Fig. 1, and it seems rather irregular to impose a constraint retroactively. Nevertheless, if all references to the magnitude of K_{SP} are understood to mean the ratio K_{SP}/K_SK_P, then I would agree with Britton's (1973) assertion. As we stated in the 'Note added in proof' to our paper, the magnitude of this ratio (which is equivalent to K_{iip}/K_p in the nomenclature of Cleland, 1963) determines whether non-competitive product inhibition will be detected, not K_{SP} alone. We belatedly found that the ratio approaches infinity as $m \rightarrow 0$ and realized that Britton (1973) was therefore correct in stating that product inhibition will appear competitive in the presence of a very slow isomerization, but we continued to disagree with his assertion that K_{SP} also approached infinity. Thanks to Cornish-Bowden (1994), we now understand.

I still contend that Britton's (1973) paper overemphasizes the failure of product inhibition to detect very slow isomerizations, because the emphasis suggests that product inhibition in general is a poor method for identifying iso-mechanisms. I do not believe this is true, despite being mistaken for the reason why. Four enzymes out of seven examined have given positive results with this method, and three of these iso-mechanisms have been confirmed by other methods. The only enzyme known to have a slow isomerization is carbonic anhydrase, yet Steiner et al. (1976) observed non-competitive product inhibition and reported kinetic constants of $K_S = 8$ mM, $K_P = 60$ mM and $K_{SP} = 2 \times 10^{-3}$ M^2 for CO_2 hydration catalysed by human carbonic anhydrase C. The K_{SP} term for this enzyme has hardly begun to approach infinity, despite the fact that the isomerization has been shown to be rate-limiting in a variety of kinetic studies (Silverman and Lindskog, 1988). Apparently Britton's (1973) assertion is realized only in a theoretical limit that is not reached experimentally with any known enzyme.

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Britton, H. G. (1973) Biochem. J. **133**, 255–261
Cleland, W. W. (1963) Biochim. Biophys. Acta **67**, 104–137
Cornish-Bowden, A. (1994) Biochem. J. **301**, 621–623
Rebholz, K. L. and Northrop, D. B. (1993) Biochem. J. **296**, 355–360
Silverman, D. N. and Lindskog, S. (1988) Acc. Chem. Res. **21**, 30–36
Steiner, H., Jonsson, B. H. and Lindskog, S. (1976) FEBS Lett. **62**, 16–20

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