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A re-evaluation of evidence attributing the difference in cleavage rates of restriction endonuclease at different sites in the substrate to differences in K_m values

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The only result supporting the hypothesis that the differences in restriction endonuclease cleavage rates at various target sites are caused by differences in K_m values was reported by Forsblom, Rigler, Ehrenberg, Petterson & Philipson [(1976) Nucleic Acids Res. 3, 3255–3269]. The present work shows that the kinetic analysis in that paper is based on incorrect derivation and in fact provides no support for the hypothesis mentioned.

INTRODUCTION

Ever since the first observation that the rates of DNA cleavage by restriction endonuclease at various target sites were different, as reported by Thomas & Davis [1], the question has existed concerning the cause of these differences. On the level of macroscopic kinetic constants, they may be caused (1) by K_m, (2) by $V_{max.}$ or (3) by both. The result of Forsblom *et al.* [2] obtained with adenovirus type 6 DNA is the only result cited for the support of the first possibility mentioned [3, 4, 5]. The aim of the present report is to show that this result is based on unjustifiable application of some mathematical procedures.

THEORY

The authors of [2] start from an obvious two-step scheme:

$$E+S \underset{k_d}{\overset{k_r}{\rightleftharpoons}} ES \xrightarrow{k_c} P+E \qquad (I)$$

where E is free endonuclease, S its target site, ES enzyme–substrate complex, and P products. [A free enzyme molecule on the right side of (I) is omitted from the original paper.]

They assume [E] remaining constant during the reaction and give (without deriving it) the determinant equation:

$$\lambda^2+(k_r\cdot[E]+k_d+k_c)\cdot\lambda+k_r\cdot k_c\cdot[E]=0 \qquad (1)$$

Then they assume a rapid equilibrium of the first step in (I) and identify one of the eigenvalues of (1) with the experimentally determined cleavage rate constant:

$$k=-\lambda_1=\frac{k_r\cdot k_c\cdot[E]}{k_r\cdot[E]+k_d+k_c} \qquad (2)$$

After a rearrangement of this relationship to:

$$k=\frac{[E]\cdot k_c}{[E]+K_m} \qquad (3)$$

where $K_m=(k_d+k_c)/k_r$, they draw a plot of $1/k$ against $1/[E]$ (with the relative input concentration of endonuclease *Eco*RI substituted for [E]) and interpret it by the means outlined below.

This calculation shows the following mistakes. The authors assume the concentration of free endonuclease [E] as remaining constant and a rapid equilibrium of the first step in (I). Under these conditions, the derivatives $d[E]/dt$ and $d[ES]/dt$ are zero. The determinant equation (1) derived from the respective differential equation has therefore no physical sense. Under the assumption given, the system (I) can be solved without the construction of differential equations:

$$v=k_c\cdot[ES] \qquad (4)$$

$$E_0=[E]+[ES] \qquad (5)$$

$$K\cdot[ES]=[E]\cdot[S] \qquad (6)$$

where v is the reaction rate, E_0 is the input enzyme concentration, and $K=k_d/k_r$ is the dissociation constant of the enzyme–substrate complex. Combining (4), (5) and (6) we obtain:

$$v=\frac{k_c\cdot[E]\cdot[S]}{K+[S]} \qquad (7)$$

which is a classical Michaelis–Menten equation. The plot $1/v$ against $1/E_0$ must give a straight line with the intercept being zero, not $1/k_c$ as believed by the authors of [2]. This was really the case (see Fig. 4 in [2]). The authors interpret the result incorrectly as k_c values being equal for all target sites.

If the assumption about [E] being constant is not taken into consideration, the time course of [ES] is given by the equation:

$$\frac{d[ES]}{dt}=k_r\cdot[E]\cdot[S]-(k_d+k_c)\cdot[ES] \qquad (8)$$

The reaction rate and its time derivative are:

$$\frac{d[P]}{dt}=k_c\cdot[ES] \qquad (9)$$

$$\frac{d^2[P]}{dt}=k_c\cdot\frac{d[ES]}{dt} \qquad (10)$$

Combining (8), (9) and (10) together with the conservation equation for DNA:

$$S_0=[S]+[ES]+[P] \qquad (11)$$

we obtain a second-order differential equation:

$$\frac{d^2[P]}{dt^2}+\frac{d[P]}{dt}\cdot(k_r\cdot[E]+k_d+k_c)+[P]\cdot k_r\cdot k_c\cdot[E]=k_r\cdot k_c[E]\cdot S_0 \quad (12)$$

This is solvable only for [E] being constant, which we had to exclude for constructing (8), or $[E] \gg S_0$ (free enzyme concentration can be identified with its total concentration). This was not the case in the experiments reported. Nevertheless, let us continue as if the condition had been fulfilled. The determinant equation (1) can be written and solved. The solutions give the rate constants [not the cleavage rate, as claimed (probably as a result of a misprint) on p. 3263 of the work cited] in the solution of (12):

$$[P]=S_0\cdot\left(1+\frac{\lambda_2}{\lambda_1-\lambda_2}\cdot e^{\lambda_1\cdot t}+\frac{\lambda_1}{\lambda_2-\lambda_1}\cdot e^{\lambda_2\cdot t}\right) \quad (13)$$

The exact solution of (1) is:

$$\lambda_{1,2}=\frac{-k_r\cdot[E]-k_d-k_c\pm\sqrt{\{(k_r\cdot[E]+k_d+k_c)^2-4k_r\cdot k_c\cdot[E]\}}}{2} \quad (14)$$

By what means was this solution simplified into the form (2)? According to the authors of [2], it was done "by assuming a rapid equilibrium of the first step in (I)". But from $k_r \gg k_c$ follow:

$$\lambda_1=0 \quad (15)$$

$$\lambda_2=-k_r\cdot[E]-k_d \quad (16)$$

Generally, the solutions of (1) must fulfil the conditions:

$$\lambda_1+\lambda_2=-k_r\cdot[E]-k_d-k_c \quad (17)$$

$$\lambda_1\cdot\lambda_2=k_r\cdot k_c\cdot[E] \quad (18)$$

Assuming:

$$|\lambda_2| \gg |\lambda_1| \quad (19)$$

we obtain (2) for λ_1 and:

$$\lambda_2=-k_r\cdot[E]-k_d-k_c \quad (20)$$

DISCUSSION

Thus I eventually obtained the expression (2) given by the authors of [2], but in the course of deriving it I had to use an inappropriate assumption, $E_t \gg S_0$. Under this condition, the result has a plausible interpretation, but the condition is not met in [2]. The expression (2) can be derived much more conveniently by a steady-state approach, identifying E_t with [E] and expressing the free substrate concentration as a total concentration corrected for [ES].

An extraordinary briefness of the derivation was probably the cause of a general acceptance of [2] without a profound examination.

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