Deuterium Isotope Effects on the Presteady State Burst of an Enzymatic Reaction

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Dedicated to Professor Jacob Bigeleisen on the occasion of his 70th birthday

When a presteady state burst is seen in an enzymatic reaction where a change in absorbance accompanies the isotope-sensitive step, there will be an isotope effect on the initial rate of the burst. The apparent commitment which reduces the size of the intrinsic isotope effect will not be solely the partition ratio of the intermediate undergoing the isotope-sensitive step (as in steady state kinetics), but will also be a function of the ratio of the forward rate constants for the isotope-sensitive step and the one preceding it. A contour map of these relationships is presented.

Introduction

Enzymatic reactions are usually studied under steady state conditions where the enzyme level is much lower than that of the substrate, or than the Michaelis constant, which is the measure of apparent substrate affinity in the steady state. The theory of isotope effects on steady state enzymatic reactions is well worked out [1] and may be illustrated by the mechanism:

$$\mathrm{EA}+\mathrm{B} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \mathrm{EAB} \underset{k_4}{\overset{k_3}{\rightleftharpoons}} \mathrm{EAB}^* \underset{k_6}{\overset{k_5}{\rightleftharpoons}} \mathrm{EPQ}^* \underset{k_8}{\overset{k_7}{\rightleftharpoons}} \mathrm{EPQ} \xrightarrow{k_9} \mathrm{EQ} \xrightarrow{k_{11}} \mathrm{E}\,, \tag{1}$$

where the chemical interconversion is between EAB* and EPQ* so that only k_5 and k_6 are isotope-sensitive (k_3, k_4, k_7, and k_8 are for conformation changes that convert the open EAB or EPQ complexes from which reactants can dissociate to the closed EAB* and EPQ* forms in which catalysis occurs). In mechanism (1), the isotope effect on V/K (the apparent first order rate constant at low subtrate level) is given by

$${}^{\mathrm{D}}(V/K)=\frac{{}^{\mathrm{D}}k_5+c_\mathrm{f}+{}^{\mathrm{D}}K_\mathrm{eq}\,c_\mathrm{r}}{1+c_\mathrm{f}+c_\mathrm{r}}\,, \tag{2}$$

where the leading superscript D indicates a deuterium isotope effect. ${}^{\mathrm{D}}k_5$ is the intrinsic isotope effect on k_5 (that is, k_5 for unlabeled substrate divided by k_5 for deuterated substrate), and ${}^{\mathrm{D}}K_\mathrm{eq}$ is the isotope effect on the equilibrium constant (that is ${}^{\mathrm{D}}k_5/{}^{\mathrm{D}}k_6$). The isotope effect on the maximum velocity is given by a similar equation where c_f is replaced by new constant, c_Vf, which consists of the sum of the ratios of $k_3\,k_5/(k_3+k_4)$ and k_3, $k_7\,k_9/(k_8+k_9)$, k_9, and k_{11}.

In (2), c_f and c_r are called forward and reverse commitments, and consist of partition ratios for intermediates:

$$c_\mathrm{f}=(k_5/k_4)\,[1+k_3/k_2]\,, \tag{3}$$

$$c_\mathrm{r}=(k_6/k_7)\,[1+k_8/k_9]\,. \tag{4}$$

In reactions such as those of pyridine nucleotide-linked dehydrogenases the chemical step is accompanied by a change in absorbance (at 340 nm in this case), so that the formation of EPQ* and subsequent complexes is readily measured in a rapid reaction apparatus. It often happens, when EA and a saturating level of B are used in such an apparatus, that a burst of absorbance is seen before the steady state rate is achieved. The amplitude of the burst depends on partition ratios such as k_6/k_7 and k_8/k_9, but the initial rate of the burst does not, and thus contains information concerning k_3, k_4, and k_5 only. It might seem that the isotope effect on the burst rate might be given by

$${}^{\mathrm{D}}k_\mathrm{burst}=({}^{\mathrm{D}}k_5+c_\mathrm{f})/(1+c_\mathrm{f}) \tag{5}$$

but c_f in this equation will not be given by (3), and it is the purpose of the present paper to show how the apparent value of c_f in (5) varies with the values of k_3, k_4, and k_5 in mechanism (1).

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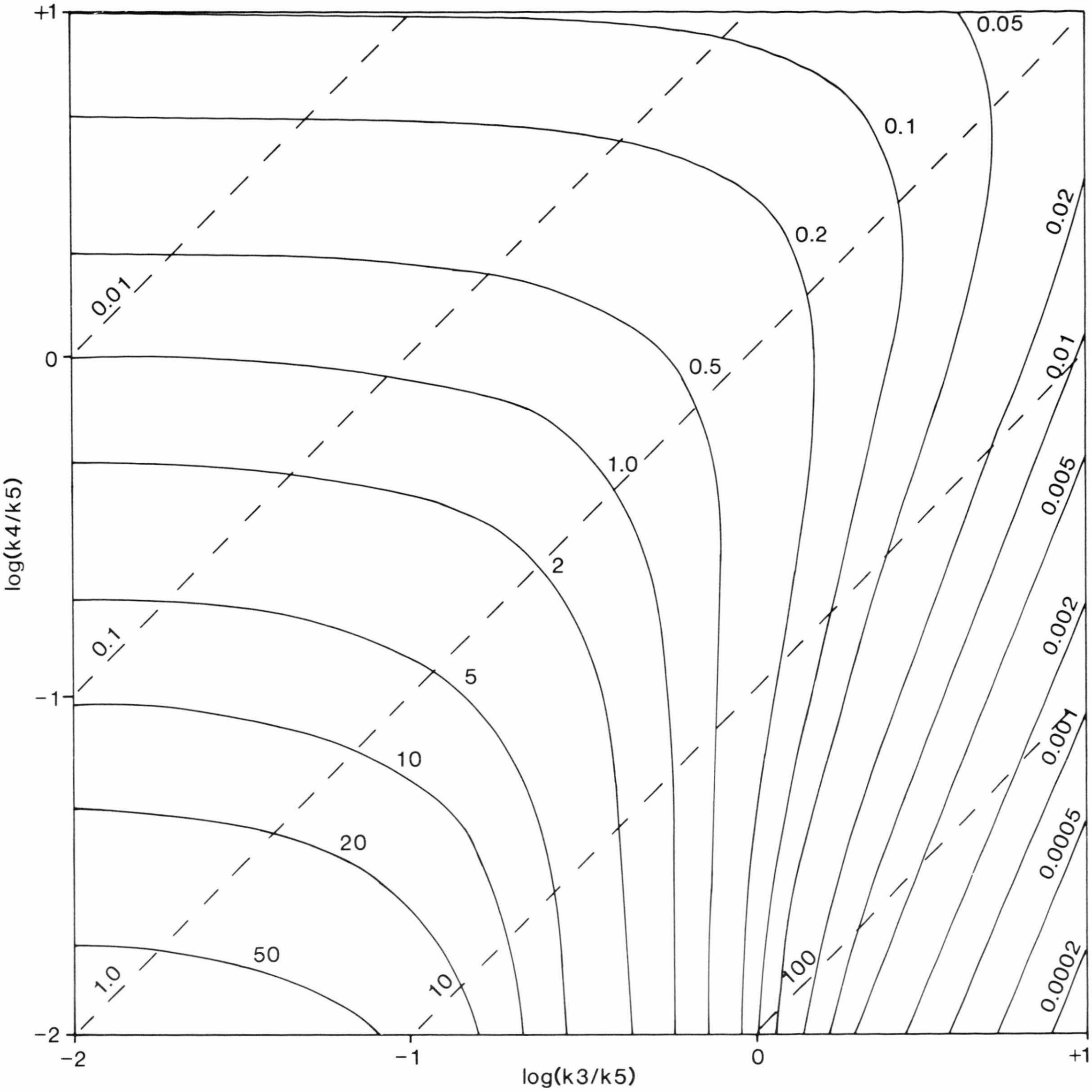

Fig. 1. Apparent commitments for an isotope effect on the initial velocity of the presteady state burst of an enzme-catalyzed reaction when saturating substrates and enzyme are rapidly mixed. The intrinsic isotope effect is assumed to be 6. The rate constants k_3 and k_4 are for forward and reverse directions of the step prior to the isotope-sensitive one.

Results and Discussion

We will assume that deuterated and undeuterated substrates are used separately and the apparent initial rates of the bursts are compared, discounting any lag. Actual data handling will be discussed below.

The general solution for observation of a burst in mechanism (1), starting with EAB, is an equation with two exponential terms:

$$\mathrm{EPQ}^*/E_t = 1 + A_\alpha\, e^{-k_\alpha t} - A_\beta\, e^{-k_\beta t},$$

where

$$k_\alpha = (p+q)/2, \qquad k_\beta = (p-q)/2,$$
$$p = k_3 + k_4 + k_5, \qquad q = \sqrt{p^2 - 4 k_3 k_5},$$
$$A_\alpha = k_\beta/(k_\alpha - k_\beta), \qquad A_\beta = A_\alpha + 1.$$

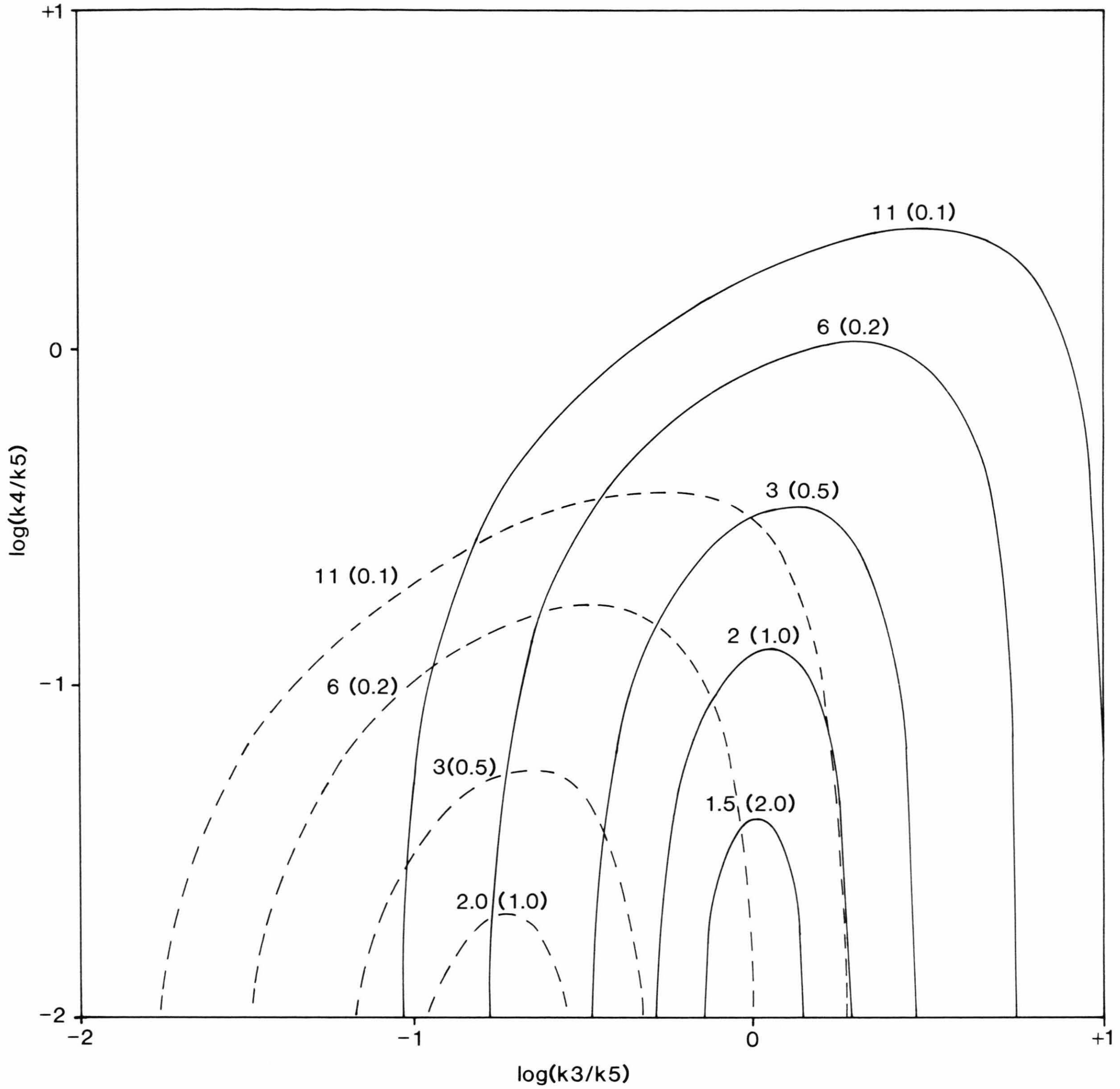

Fig. 2. Contours showing where lag will be observed in the presteady state burst of an enzyme-catalyzed reaction. The solid lines are for unlabeled substrate, and the dashed ones for deuterated substrate. Other details as in Figure 1. The first numbers on each contour are the ratios of k_α and k_β in (6). The numbers in parentheses are the ratios of amplitudes (A_α/A_β).

The term in k_α causes a lag or induction period, but this is often not seen because $A_\alpha \ll A_\beta$. Also the initial portion of the burst is the hardest to observe experimentally, and the lag is often buried in the mixing time of the instrument. Even when the lag is seen, the time course does not have enough information in it to determine unique values of k_3, k_4 and k_5. One can calculate only the sum of the three constants (from $k_\alpha + k_\beta$), and the product $k_3 k_5$ (from $k_\alpha k_\beta$).

Figure 1 has $\log(k_4/k_5)$ on the vertical axis, and $\log(k_3/k_5)$ on the horizontal axis. These ratios are for k_{5H}. The contours running from 50 to 0.0005 are apparent commitments that will be observed in (5) for an isotope effect of 6.0 on k_5 (the contours vary only slightly with the size of the isotope effect, and thus this graph may be used for any primary deuterium isotope effect). Thus if $k_4/k_5 > 10$, or if $k_3/k_5 > 3$, nearly the full isotope effect is seen on the burst ($c_f < 0.1$; isotope effect >5.5). Shore's data for liver alcohol dehydrogenase fit this pattern [2].

The diagonal straight lines in Fig. 1 represent k_3/k_4 values, or the equilibrium constant for the first step.

The contours in Fig. 2 define the regions where a lag will be seen before the burst. The first numbers on the contours (11, 6, 3, 2, 1.5) represent the k_α/k_β ratio. The number in parentheses (0.1, 0.2, 0.5, 1, 2) is A_α/A_β, and we assume that when A_α is less than 10% of A_β you cannot see the lag. Thus two clear exponentials are seen only in the region defined by the 0.1 contour. The contours for the deuterated substrate are plotted diagonally to the lower left of the ones for the unlabeled substrate (displaced by log 6 both vertically and horizontally in the present case; for an isotope effect different from 6, these contours will be displaced a different amount), since k_{5D} is 6 times less than k_{5H}. Note that it is possible to see a lag for unlabeled substrate and not for the labeled one, and vice versa. Fisher's data for glutamate dehydrogenase showing an isotope effect of 1.5–1.8 on the burst [3] correspond to a c_f value of 5–8, assuming that the true isotope effect is 6 on the hydride transfer step, or to a c_f value of 4–7 if the isotope effect is 5. No lag seems visible for the unlabeled substrate, but a small one may be present for the deuterated molecule. The data suggest that k_3 and k_4 are about equal, and both about 0.1 of k_5.

By contrast, the steady state isotope effects on V/K for glutamate are ~1.2, showing that there are reverse commitments in the system as well as forward ones [4].

Experimental Methods

In practice one should make semilog plots of the burst, and determine the amplitude of the burst and the apparent rate constant (see Fig. 3 for what these should look like). The initial velocity is then the product of the amplitude and the apparent rate constant. While to determine absolute values of the initial rates requires knowing the extinction coefficient of EPQ*, one need not know this for determining isotope effects, since the value will cancel out unless deuterium substitution changes the extinction coefficient. Thus the isotope effect on the initial velocity of the burst is given by

$$^{D}k_\beta = (k_H/k_D)(A_H/A_D), \tag{7}$$

where A is the amplitude of the burst, and k_H or k_D is the apparent rate constant from semilog plots of the burst, or from least squares fitting as noted below.

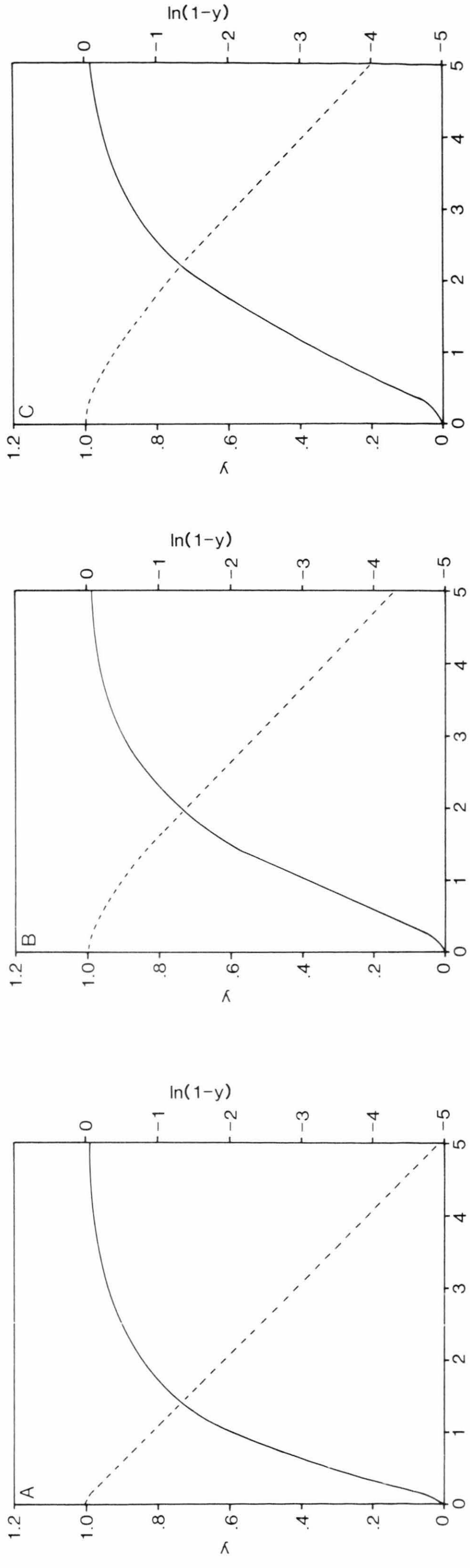

Fig. 3. Time courses (solid lines, left axis) and semilog plots (dashed lines, right axis) of the presteady state bursts of an enzymatic reaction (the steady state rate has been subtracted). The ratio of k_α/k_β in (6) is A) 11, B) 2, C) 1.5. The apparent slopes of the semilog plots are A) −1.0, B) −0.98, C) −0.92 (the slope of the asymptote in each case is −1.0).

When only one exponential term is seen (no lag), one can use a semilog plot, or better, fit the data to

$$y = A(1 - e^{-kt}) + C, \tag{8}$$

where y is the absorbance, A the amplitude of burst, k the apparent rate constant and C the absorbance at $t = 0$. Where a lag is observed, one makes a semilog plot of: ln $[(A - y + C)/A]$ vs. time, and extracts preliminary estimates of k from the slope and b from the vertical intercept, which is ln b. The data are then fitted to the equation

$$y = A(1 + (b-1)\,e^{-kt/(1-1/b)} - b\,e^{-kt}) + C, \tag{9}$$

which will supply good estimates for A, k, and b, even for values of b as large as 3 (with test data, good values of A and k are obtained even when b is large and somewhat undertain).

The above analysis assumes that the burst is so much faster than the subsequent steady state rate that the latter can be ignored, or that a single turnover experiment is being observed. In the more usual case, a steady state rate is observed after the burst. This can be handled by extrapolating the steady state rate to $t = 0$, and subtracting from each point the absorbance developed by the steady state rate alone. The remaining absorbance can then be analyzed as a single burst as noted above, and the initial velocity from this analysis added to the steady state rate to give the true initial velocity. A better procedure is to fit the data to

$$y = a\,e^{-kt} + bt + c, \tag{10}$$

where $(b - ak)$ is the initial velocity and $(a + c)$ the initial absorbance at $t = 0$. For a burst, a is negative, and b and c positive. If an initial lag is clearly present, $d\,e^{-(k_2)t}$ (where $k_2 > k$) can be added to this equation (d will be positive); $(b - ak)$ is still the desired initial velocity. Computer programs to make the fits mentioned in this article have been used in this lab.

Acknowledgement

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