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

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Dedicated to Professor Jacob Biegeleisen 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:

\[
\text{EA} + B \xrightarrow{k_2} \text{EAB} \xrightarrow{k_4} \text{EAB}^* \xrightarrow{k_6} \text{EPQ}^* \xrightarrow{k_8} \text{EPQ} \xrightarrow{k_{10}} \text{EQ} \xrightarrow{k_{12}} E, \quad (1)
\]

where the chemical interconversion is between \text{EAB}^* and \text{EPQ}^* so that only \(k_3\) and \(k_5\) are isotope-sensitive \((k_1, k_2, k_4, \text{ and } k_8\) are for conformation changes that convert the open \text{EAB} or \text{EPQ} complexes from which reactants can dissociate to the closed \text{EAB}^* and \text{EPQ}^* forms in which catalysis occurs). In mechanism (1), the isotope effect on \(V/K\) (the apparent first order rate constant at low substrate level) is given by

\[
D_k \frac{v}{k} = \frac{D_k k_3 + c_f + D_k k_6 c_r}{1 + c_r + c_f}, \quad (2)
\]

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

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 \text{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 \(k_5/k_7\) and \(k_6/k_9\), but the initial rate of the burst does not, and thus contains information concerning \(k_3, k_4, k_5\), and \(k_8\) only. It might seem that the isotope effect on the burst rate might be given by

\[
D_k \text{burst} = (D_k k_3 + c_f)/(1 + c_r) \quad (5)
\]

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 \(k_3, k_4, \text{ and } k_5\) in mechanism (1).

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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:

$$\frac{EPQ^*}{E_i} = 1 + A_3 e^{-k_2 t} - A_\beta e^{-k_\beta t},$$

where

$$k_2 = \frac{(p+q)}{2}, \quad k_\beta = \frac{(p-q)}{2},$$

$$p = k_3 + k_4 + k_5, \quad q = \sqrt{p^2 - 4 k_3 k_5},$$

$$A_3 = k_\beta/(k_3 - k_\beta), \quad A_\beta = A_3 + 1.$$
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 $k_x$ and $k_y$ in (6). The numbers in parentheses are the ratios of amplitudes ($A_x/A_y$).

The term in $k_x$ causes a lag or induction period, but this is often not seen because $A_x \ll A_y$. 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 $k_x + k_y$), and the product $k_3 k_5$ (from $k_x k_y$).

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 $k_x$ (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 $k_x/k_5 > 10$, or if $k_3/k_5 > 3$, nearly the full isotope effect is seen on the burst ($c_t < 0.1$; isotope
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 $k_j/k_\beta$ ratio. The number in parentheses (0.1, 0.2, 0.5, 1, 2) is $A_y/A_\beta$, and we assume that when $A_y$ is less than 10% of $A_\beta$ 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 $c_t$ value of 5-8, assuming that the true isotope effect is 6 on the hydride transfer step, or to a $c_t$ 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

$$Dk_\beta = (k_{1H}/k_{1D})(A_y/A_\beta),$$

(7)

where $A$ is the amplitude of the burst, and $k_{1H}$ or $k_{1D}$ is the apparent rate constant from semilog plots of the burst, or from least squares fitting as noted below.

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**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 $k_1/k_2$ in (6) is (A) 1.1, (B) 2, (C) 1.5. The apparent slopes of the semilog plots are (A) 0.9, (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
\[ y = A(1 - e^{-kt}) + C, \] (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
\[ y = A(1 + (b - a) e^{-kt/(1 - b)} - b e^{-kt}) + C, \] (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 uncertain).

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
\[ y = a e^{-kt} + bt + c, \] (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.

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