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# Use of Isotope-Dilution Phenomenon to Advantage in the Determination of Kinetic Constants $K_m$ and $K_{cat}$ for *Bam*HI Restriction Endonuclease: An Empirical and Iterative Approach

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**An assay using a very small amount of $^{35}$S-labeled deoxyoligonucleotide as a substrate for the determination of $K_m$ and $K_{cat}$ for the restriction enzyme *Bam*HI is described. Two synthetic deoxyoligonucleotides, ATGGCGGATCCGC and ATGGCCGGAGCCGC, containing the cognate and a mismatch *Bam*HI sequence, respectively, were labeled by an end-filling reaction using the Klenow fragment of DNA polymerase and [$^{35}$S]dATP to generate the labeled self-complementary substrates. The dependence of *Bam*HI hydrolysis on substrate concentration was investigated using mixtures of a fixed amount of radiolabeled substrate and varying amounts of cold-labeled substrate over a wide range. The apparent competitive inhibition observed due to the phenomenon of carrier dilution was analytically corrected by an empirical as well as an iterative approach to give $K_m$ values comparable to those reported in the literature. We have found that the values obtained using the empirical formula are very close to the precise values obtained through iteration. Our procedure has used isotopic dilution to advantage to make the assay less expensive and can be applied effectively to any enzyme–substrate reaction in which the substrate and the product have radioactive labels. The method would be especially useful for a rapid analysis and comparison of kinetic constants of various mutant enzymes or substrates.** © 1994 Academic Press, Inc.

Valuable insights into the recognition and catalysis by type II restriction endonucleases have been gained by performing kinetic assays of the nuclease action on synthetic deoxyoligonucleotide substrates having base analogues or mispairs incorporated within the recognition sequence. Usually, the time course of the cleavage of DNA substrates labeled at the 5′ end with $^{32}$P is followed by separation of the cleaved and uncleaved substrates on gel electrophoresis and quantified from the radiolabel. Because this assay is fast, sensitive, and accurate, it has been followed by many workers (1–13). However, the assay requires a considerable quantity of radiolabeled substrate of high specific activity, and the short half-life of $^{32}$P is a disadvantage for the prolonged series of experiments often required. Also, blunt-end kination is not an efficient reaction (14). Ideally, the specific activity should not change during initial velocity measurements at different substrate concentrations. If the radioactive substrate is mixed with cold substrate, then the latter would behave as a competitive inhibitor with the same $K_m$, leading to what is known as the "carrier-dilution effect" and the initial velocity would appear to decrease with the increase in substrate concentration (15). These factors make the assay considerably expensive. In their assay of *Eco*RV, Connolly and co-workers used a mixture of hot- and cold-labeled substrates with a careful manipulation of their ratios and yet had to use a large amount of [$\gamma$-$^{32}$P]dATP for radiolabeling (8). We report here a procedure which avoids these limitations.

During our investigation of *Bam*HI restriction endonuclease, we synthesized deoxyoligonucleotide substrates designed to be labeled by end-filling with the Klenow fragment of DNA polymerase and [$\alpha$-$^{35}$S]-dATP, carried out the assay with a fixed (~10 pmol) amount of radioactive substrate and a varying amount of cold-labeled substrate, and analytically corrected for the carrier-dilution effect. We show here that precise values for $K_m$ and $K_{cat}$ are obtained with our fast and inexpensive procedure, which can be applied generally to any enzyme acting on a radiolabeled substrate.

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## MATERIALS AND METHODS

### *Materials*

Klenow polymerase and *Bam*HI endonuclease were from Boehringer Mannheim; [$\alpha$-$^{35}$S]dATP was from Amersham; and cold dNTPS was from Perkin–Elmer–Cetus. Two deoxyoligonucleotides, 5′-ATGGCGGATCCGC and 5′-ATGGCGGAGCCGC (designated AT and AG oligomers, respectively), were synthesized "trityl on" on an automated DNA synthesizer (Model 391, PCR MATE) using phosphoramidite chemistry (16). The machine and the reagents were from Applied Biosystem (Foster City, CA). After deblocking in $NH_4OH$ for 16 h at 60°C, the oligomers were purified on a Q-Sepharose anion exchanger and desalted on Bio-Gel $P_4$ as described (17). Aliquots of purified oligonucleotides after desalting were lyophilized and stored at −70°C until further use.

### *Labeling Procedure*

Each of the above oligomers can form in solution a duplex structure with three nucleotide overhangs at the 5′ ends, which when end-filled would give complete palindromic sequences as shown below:

5′-ATGGCGGATCCGC _____ 5′-ATGGCGGAT CCGCCAT
CGCCTAGGCGGTA-5′ TACCGCCTAGGCGGTA-5′,

5′-ATGGCGGAGCCGC _____ 5′-ATGGCGGATCCGCCAT
CGCCGAGGCGGTA-5′ TACCGCCTAGGCGGTA-5′.

The end-filling reaction was carried out as follows. Briefly, 0.5 unit of the Klenow fragment of DNA polymerase I was added to a 10-$\mu$l reaction mix containing 100 ng deoxyoligonucleotide (20 pmol 5′ ends) in 10 mM Tris–HCl (pH 8.0), 10 mM $MgCl_2$, 1 mM each of dCTP and dTTP, and 12.5 $\mu$Ci (approx 20 pmol) of [$\alpha$-$^{35}$S]-dATP and incubated at 37°C for 2 h. To generate flush ends, an additional 0.5 unit of Klenow fragment and 1 mM each of the three required dNTPs (cold) were added and incubation was continued for another hour. The extent of incorporation was estimated by spotting 1 $\mu$l of the reaction mixture on DE-81 paper, which was then washed successively once with 0.5 M potassium acetate and twice with TE buffer (10 mM Tris–HCl and 1 mM EDTA, pH 7.5), and the radioactive counts were measured using a beta counter (Beckman Model LS-7000, U.S.A.) before and after the wash. The cold substrates were made similarly using cold dATP.

### *BamHI Endonuclease Assay*

The dependence of *Bam*HI-catalyzed hydrolysis on deoxyoligonucleotide–substrate concentration was investigated to determine the $K_m$ and $K_{cat}$. Assays were performed in 30-$\mu$l volumes of 20 mM Tris–HCl (pH 8.0), 10 mM $MgCl_2$, 7 mM 2-mercaptoethanol, and 100 mM NaCl at 37°C and 50 units of *Bam*HI, 10 pmol of hot oligonucleotide substrates, and varying concentrations of cold substrate. For the native AT sequence 4, 8, 12, 16, and 32 nmol of cold hexadecamer was used and for the AG sequence 0.2, 0.5, 0.75, 2, and 4 nmol of cold hexadecamer was used. The time course varied from 0 to 100 min for the AT and from 0 to 120 min for the AG oligomer (Fig. 2). At appropriate times, 4-$\mu$l aliquots were withdrawn and the reaction was quenched by adding 1 $\mu$l of 0.5 M EDTA solution. The material was evaporated on a vacuum concentrator (Hetovac, Denmark) and 1 $\mu$l of loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol, and 40% glycerol in 6 M guanidine thiocynate) was added. The released product and undigested substrate were separated electrophoretically on urea–PAGE.

### *Gel Electrophoresis*

Polyacrylamide gels were prepared in volumes of 30 ml containing 10× TBE buffer, pH 8.0 (10.8% (w/v) Tris base, 5.5% (w/v) boric acid, 2 mM EDTA), 7 M urea, and 20% polyacrylamide (from deionized stock solution containing 29% acrylamide and 1% bisacrylamide). Polymerization was initiated by addition of 0.2 ml of a freshly prepared 10% (w/v) ammonium persulfate solution and 0.04 ml of *N,N,N,N*-tetramethylethylenediamine. The solution was immediately poured into a Resolvmax water-jacketed gel apparatus (Atto Corp., Japan) with dimensions of 20 cm × 20 cm × 1 mm. Gels were run in 10× TBE buffer. Aliquots of quenched samples of the *Bam*HI endonuclease reaction mixtures were loaded and the gels were run at 50°C and 60 mA constant current until the bromophenol blue dye marker had migrated half the distance of the gel. The use of 6 M guanidine thiocyanate (GSCN) in the loading buffer and running the gel at 50°C were necessary to eliminate anomalous bands of the AG oligomer. The positions of the product and the undigested oligonucleotide were determined by fluorography to enhance the band intensity of weak $\beta$-emitter-like $^{35}$S on the autoradiogram. Fluorography was carried out with the scintillator sodium salicylate as follows (18). After electrophoresis, the gels were soaked in a 1:1 mixture of 10% acetic acid and methanol and 5% glycerol. The gels were washed twice in a large volume of double-distilled water to remove acetic acid and then soaked in 250 ml of 1.0 M sodium salicylate (pH 7.0) for 20 min, dried under vacuum, and placed on Kodak X-OMAT-R film at −70°C for 24 h for autoradiography. The band intensities were measured by densitometry on a Hirshmann–Elsckript 400 (West Germany) apparatus and the percentage of cleavage at different time points was determined as

$$\% \text{ Cleavage} = \frac{\text{Area of the product band}}{\text{Total area of (Product + reactant)}} \times 100.$$

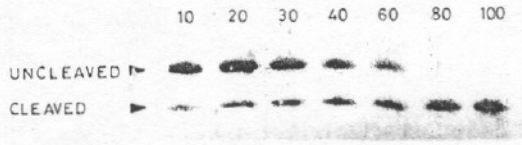


**FIG. 1.** Autoradiogram of the time course of hydrolysis of $^{35}$S-labeled deoxyoligonucleotide substrate 5'-ATGGCGGATCCGCCAT to $^{35}$S-labeled 5'-GATCCGCCAT and cold ATGGCG by restriction endonuclease *Bam*HI.

These values were plotted against corresponding times to evaluate the initial velocities at different initial substrate concentrations. A representative autoradiogram of the time course of hydrolysis of the synthetic DNA substrate AT is shown in Fig. 1, and Fig. 2 shows the variation of initial velocity with substrate concentration for the AT and AG oligomers.

## RESULTS AND DISCUSSION

The efficiency of incorporation of the $^{35}$S label, under the standard conditions of our experiment, was 86% for the AT but only 58% for the AG oligomer. We believe that this marked difference is due to the presence of G·A mispair-induced self-structures (19). When the mismatch AG oligomer was first carefully annealed in the presence of 150 mM NaCl and then end-filled, the incorporation increased to 80%.

It may be noted from Fig. 2 that the velocity curves decrease with increase in initial substrate concentration, although the true velocity, in reality, has increased. This is due to the carrier dilution, when $V$ appears to decrease if no correction is applied for dilution of specific activity with the cold-labeled substrate. This observed velocity ($V_{app}$) is related to actual velocity ($V_{act}$) by

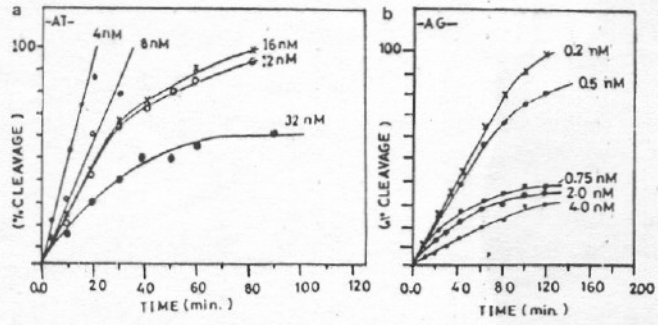


**FIG. 2.** Kinetics of cleavage of the two substrates containing the cognate and the mismatch *Bam*HI recognition sequence. (a) The amount of the product formed with time for the hexadecamer 5'-ATGGCGGATCCGCCAT at the following substrate concentrations: 4 (●), 8 (○), 12 (○), 16 (×), and 32 nmol (●). The substrate was incubated with 50 units of *Bam*HI in cleavage buffer at 37°C for different time intervals ranging from 0 to 100 min. (b) The amount of the product formed with time for the hexadecamer 5'-ATGGCGGAGCCGCCAT at the following substrate concentrations: 0.2 (×), 0.5 (●), 0.75 (■), 2 (○), and 4 nmol. (●). The reaction conditions were the same as those in (a) with time intervals ranging from 0 to 120 min.

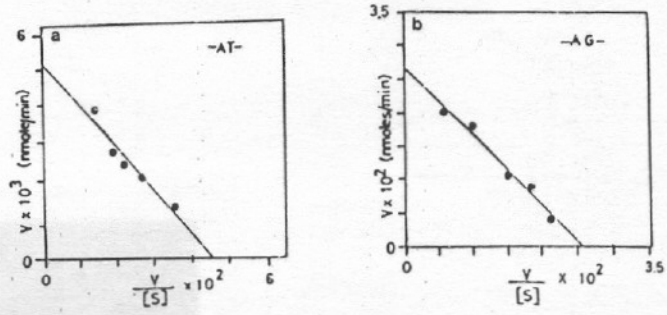


**FIG. 3.** Edie–Hoffstee plot for $K_m$, $K_{cat}$, and $V_{max}$ determination using $^{35}$S-labeled hexadecamers (a) 5'-ATGGCGGATCCGCCAT and (b) 5'-ATGGCGGAGCCGCCAT as substrates for *Bam*HI.

$$a = \frac{V_{app}}{V_{act}} = \frac{K_m + [S^*]}{K_m + [S] + [S^*]}, \qquad [1]$$

where $a$ is the competitive inhibition factor, $K_m$ is the Michaelis constant, and [S*] and [S] are the concentrations of the radiolabeled and cold-labeled substrates, respectively (15). Thus, without the value of $a$, $V_{act}$ and hence $K_m$ cannot be determined or vice versa. However, we observed that under our experimental conditions, the observed velocities ($V_{app}$) fall in correct order if multiplied by the dilution factor [S]/[S*]. It becomes possible then to analyze the data in terms of the Eadie–Hofstee equation

$$V_{act} = -K_m V_{act}/([S] + [S^*]) + V_{max}. \qquad [2]$$

Such linear regression plots are shown in Fig. 3, which gives a $K_m$ of 11.12 nmol ($V_{max} = 51.2 \times 10^2$ nmol/min) for the AT oligomer and 1.10 nmol ($V_{max} = 2.67 \times 10^2$) for the AG oligomer. We are aware that these are approximate values, but this empirical correction leads to values close to the precise values obtained by an iterative approach described below. The basis for this empirical correction factor is that the unlabeled substrate (S) competes with the labeled substrate (S*) for the same enzyme site and the rate of cleavage of the two are related as (20)

$$\frac{V_S^*}{V_S} = \frac{(K_{cat}/K_m)[S^*]}{(K_{cat}/K_m)[S]}$$

$$\text{or } V_S = V_S^* \cdot [S]/[S^*]. \qquad [3]$$

Thus, from the observed rate ($V_{app} = V_S^*$), $V_S$ can be calculated; since [S*] ≪ [S], the calculated velocities will

TABLE 1

| Substrate (16-mer) | $K_m$ (nmol) | $V_{max}$ ($\times 10^2$) (nmol/min) | $K_{cat}$ ($min^{-1}$) | $K_{cat}/K_m$ ($S^{-1}$ $M^{-1}$) | Remarks |
|---|---|---|---|---|---|
| 5′-ATGGCGGATCCGCCAT | 11.12 | 51.20 | 12.80 | $1.90 \times 10^7$ | Empirical |
| 5′-ATGGCGGATCCGCCAT | 11.08 | 44.17 | 11.04 | $1.66 \times 10^7$ | Iterated |
| 5′-ATGGCGGAGCCGCCAT | 1.10 | 2.67 | 0.57 | $8.50 \times 10^6$ | Empirical |
| 5′-ATGGCGGAGCCGCCAT | 1.16 | 2.49 | 0.62 | $8.95 \times 10^6$ | Iterated |
| pJC-80 (ccc) | 0.36 | — | — | | Ref. (22) |
| NTP-14 (ccc) | — | — | 1.60 | | Ref. (21) |
| SV40 DNA | 3.60 | — | 1.50 | $6.90 \times 10^6$ | Ref. (23) |
| pJC-80 (linear) | 0.90 | | — | | Ref. (22) |
| pJC-80 (ccc) | 0.30 | | 2.20 | $12.20 \times 10^7$ | Ref. (21) |

fall on a curve close to that based on $V_{act}$, and the slope of the Eadie–Hoftsee plot ($K_m$) will also be very close to the actual value.

Ideally, Eqs. [1] and [2] should be solved simultaneously. This suggested to us the possibility of an iterative approach to evaluating $K_m$ and $V_{max}$ from the observed $V_{app}$ data. The program for iteration was as follows:

Step 1: Input arbitrary $K_m$ value.

Step 2: Input observed $V_{app}$ values.

Step 3: Calculate $V_{act}$ from Eq. [1].

Step 4: Use $V_{act}$ and obtain $K_m$ using Eq. [3] via a linear regression.

Step 5: Repeat steps 1–3 until $K_m$ values converge.

About 300 cycles were needed for the $K_m$ value (or $V_{max}$) to converge irrespective of the initial choice of $K_m$ in Step 1. However, an examination of the approach showed that combination of Eqs. [1] and [3] yields a quadratic equation in $K_m$ as:

$$K_m^2 + K_m[(2 - V_{max}/V_{app})([S] + [S^*])] + [([S] + [S^*])^2 - (V_{max}/V_{app})[S^*]([S] + [S^*])] = 0.$$

Indeed, from the iterative approach we obtained two values for $K_m$, one of which was real and the other virtual (a negative value was obtained for the AT oligomer). The choice of the actual value for $K_m$ did not pose any problem, since the actual $K_m$ should fall within the substrate concentration range used. The $K_m$ values thus obtained were 11.08 nmol ($V_{max} = 44.17 \times 10^2$ nmol/min) for AT and 1.16 nmol ($V_{max} = 2.49 \times 10^2$ nmol/min) for the AG oligomer. Interestingly, the empirical approach described above gave values quite close to these iterative values and helped in selecting the actual $K_m$ from the set of two values obtained in the iterative approach. The latter approach yielded exact results subject to the validity of Eqs. [1] and [3], while the empirical approach yielded an approximate value and provided a quicker method of analyzing the experimental data.

Once the $K_m$ and $V_{max}$ are known, $K_{cat}$ can be evaluated from the equation $K_{cat} = V_{max}/E_0$, where $E_0$ is the total concentration of the enzyme. These results are given in Table 1. The $K_m$ values that we obtained were ~11 nM for the cognate AT sequence and ~10 times less for the mismatched AG oligomer. We cannot compare these values directly with any other study, as ours is the first report on oligonucleotide substrates. However, our values seem very reasonable considering that in studies with short deoxyoligonucleotides of 8–10 bp as substrates for a number of different restriction enzymes, $K_m$ values have always been higher than those in studies with DNA macromolecules (2,5,12). Usually, $K_m$ values with DNA macromolecules fall within the nanomolar range.

We want to point out here the interesting result that incorporation of G · A mispairs in the middle of the recognition sequence caused considerable reduction in both $K_m$ and $K_{cat}$, so that the specificity parameter, $K_{cat}/K_m$, is slightly altered. The significance of these results will be discussed in the context of results with other mismatch oligonucleotide substrates in a separate communication. We report here our method for determination of $K_m$ and $K_{cat}$ using small amounts of $^{35}S$-labeled substrates, which is simple, fast, inexpensive, and accurate.

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