Kinetic Assay of Fluorescein Mono-β-D-galactoside Hydrolysis by β-Galactosidase: A Front-Face Measurement for Strongly Absorbing Fluorogenic Substrates†

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ABSTRACT: A novel enzymatic assay method was developed for fluorogenic substrates that have significant intrinsic absorbance and fluorescence under the assay conditions. Fluorescein mono-β-D-galactoside (FMG) was chosen as the substrate for the fluorescence enzymatic assay because of the high fluorescence of its hydrolytic product (fluorescein) and suitability of being hydrolyzed by β-galactosidase. The fluorescence–concentration relationships for fluorescein and for FMG in both the right-angle detection mode of a fluorometer and the front-face detection mode of a fluorescence plate reader were exactly established and used to determine the kinetics of the enzyme assay. The results show that only front-face detection in the fluorescence plate reader can overcome the fluorescence concentration quenching that inevitably results from high absorbance by the intrinsically absorbing substrate in the conventional fluorometer, which utilizes right-angle detection. Only with front-face detection was the fluorescent assay of FMG hydrolysis under conditions of high optical density possible. The enzymatic measurements on the fluorescence plate reader were particularly efficient for determination of the enzyme kinetics because of the high rate of data collection. In this assay system, Michaelis–Menten constant K_m and enzymatic catalysis rate k_2 of FMG were determined as 117.6 μM and 22.7 μmol·(min·mg)$^{-1}$, respectively. The results and methods described in this paper can be generalized for any assay using a fluorogenic substrate whether or not it has a high background absorbance.

Synthetic enzyme substrates that release chromophoric or fluorescent products are commonly used to measure the activity of hydrolytic enzymes, including several different enzymes that hydrolyze glycosides. Fluorogenic substrates offer advantages of high sensitivity and practical convenience in many enzymatic assays (Guilbault, 1990). In favorable cases, the enzymatic hydrolysis results in formation of a chromophoric product that absorbs and fluoresces at wavelengths significantly longer than does the substrate. High intrinsic absorbance (or fluorescence) of the substrate at the wavelength where the product formation is measured makes the measurement of enzymatic activity technically difficult or impossible, especially when the Michaelis–Menten constant K_m for the substrate is high. In many assays, however, the substrate concentration used is made higher than its K_m for the enzyme so that the optical changes can be directly related to the enzyme concentration and activity. In addition, measurements of the enzyme reaction rates at substrate concentrations higher than K_m are essential for obtaining kinetic parameters of the enzymatic reaction.

The success of measuring the hydrolysis rate under conditions of high intrinsic absorbance or background depends on the equipment and technique used. Absorption measurements are made "in line" with the light path by diminution of the lamp intensity across the light path. It is technically difficult to measure small changes in absorbance over a large background. Fluorescence intensity measurements are typically made at an angle to the light path. The fluorescence emission for a dilute sample is isotropic. In fluorometers, the intensity measurement is commonly made at a right angle to the light path in a 1-cm cuvette in a narrow band centered at half of the path length of the cuvette. At high absorbance, however, the intensity of light reaching the center of the cuvette, and therefore the fluorescence intensity is diminished by the absorbance in the path of the cuvette (Figure 3). In concentrated solutions this "inner filter effect" can result in almost total loss of detectable fluorescence. Consequently, at high substrate absorbance, the fluorescence development after enzyme action when measured in a cuvette does not correspond exactly to the enzymatic turnover rate. Low absorbance and fluorescence background of the substrate are thus essential requirements for making right-angle fluorometric measurements in a cuvette.

Fluorescein mono-β-D-galactoside (FMG)[1] is a hydrolyzable substrate of β-galactosidase. FMG is also an intermediate in the hydrolysis of fluorescein di-β-D-galactoside (FDG). Although it is a good substrate for the enzyme, FMG has quite high absorption [extinction coefficient ϵ_{452nm} = 0.045 (cm·μM)$^{-1}$ in the unit defined below] and is intrinsically fluorescent at the wavelength used for the detection of its hydrolytic product, fluorescein. Consequently Hofmann and Sernetz (1983) failed to determine the Michaelis–Menten constant K_m and the enzymatic catalysis rate k_2 for FMG fluorometrically (i.e., in a right-angle manner using a fluorometer) because of its high absorbance and fluorescence background at the high substrate concentration.

It is useful to be able to utilize the high sensitivity in enzyme analysis offered by a fluorescent substrate, even though the fluorescent substrate already has significant absorbance. To overcome high intrinsic absorbance in concentrated samples and solids has usually required detection of the fluorescence near the point of illumination at which the absorbance is still low and the fluorescence can still be related to the amount of fluorophore (Blumberg et al., 1977; Lamola et al., 1977). In this paper, quantitative fluorescence–concentration relationships of fluorescein and FMG in right-angle and front-face measurement geometries were established and successfully applied to the kinetic assay of FMG hydrolysis by β-galactosidase.

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[1] Abbreviations: FMG, fluorescein mono-β-D-galactoside; FDG, fluorescein di-β-D-galactoside; PETG, phenylethyl β-D-thiogalactoside.

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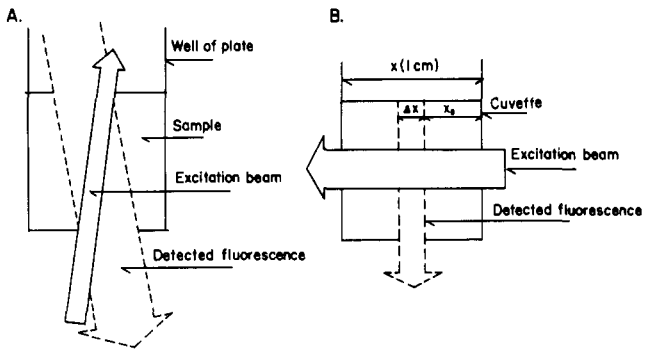

FIGURE 1: Illustrations of (A) the side view of the front-face measurement in the CytoFluor 2300 and (B) the top view of the right-angle measurement in a fluorometer.

Experimental Procedures

Materials and Instrumentation. Fluorescein, FMG, and PETG were from Molecular Probes, Inc. (Eugene, OR). *Escherichia coli* β-galactosidase (molecular weight 540 000) was purchased from Scripps Laboratories (San Diego, CA). Fluorescein, FMG, and PETG were dissolved in DMSO to make 15 mg/mL stock solutions that were then diluted with buffer (specified below) to the desired concentrations. All fluorescence measurements and enzyme reactions of β-galactosidase were run in 100 mM sodium phosphate buffer, pH 7.5, containing 110 mM 2-mercaptoethanol and 1 mM $MgCl_2$ at room temperature (about 22 °C). Right-angle fluorescence detection in a 1-cm cuvette was done with a Model LS50 fluorometer made by Perkin-Elmer Ltd. (Buckinghamshire, England). Front-face fluorescence detection was run with a CytoFluor 2300 multiwell fluorescence plate reader from Millipore (Bedford, MA), using Corning 96 round-bottom well plates from Corning Glass Works (Corning, NY). Unless specified otherwise, the total sample volume in each well for any CytoFluor measurement was 150 μL.

Fluorescence and Enzymatic Turnover in the CytoFluor (Front-Face Manner). The detected fluorescence of a fluorophore depends on the absorbance of the excitation beam. For a very small path dX in an excitation path X, a small intensity change of the excitation beam, dI, in a sample that contains multiple fluorophores can be expressed as

$$dI = \sum dI_i = -(\sum \epsilon_i C_i) I\, dX \quad (1)$$

where ϵ_i is the extinction coefficient of each fluorophore and C_i is the concentration of each fluorophore; I is the intensity of the excitation beam. For an excitation beam path using the front-face manner of the CytoFluor as in Figure 1A, the integrated form of eq 1 is

$$I = I_0 \exp[(-\sum \epsilon_i C_i)X] \quad (2)$$

where I_0 is the initial intensity of excitation beam. ϵ used in this paper is defined and spectrophotometrically determined as eq 2 in a form of a single fluorophore. The absorbance of each fluorophore is easily derived as

$$\Delta I_i = \int -dI_i = I_0[\epsilon_i C_i/(\sum \epsilon_i C_i)]\{1 - \exp[(-\sum \epsilon_i C_i)X]\} \quad (3)$$

Assuming there is no reabsorption of the fluorescence emission by the fluorophore, the observed fluorescence should be proportional to the fluorophore absorbance and the fluorescence emission efficiency. Considering the fluorescein (indicated 1) and FMG (indicated 2) system using the CytoFluor front-face measurement manner, the detected fluorescence F should be

$$F = \sum F_i = \sum(\Delta I_i E_i) = I_0[(E_1\epsilon_1 C_1 + E_2\epsilon_2 C_2)/(\epsilon_1 C_1 + \epsilon_2 C_2)]\{1 - \exp[-(\epsilon_1 C_1 + \epsilon_2 C_2)X]\} \quad (4)$$

where E_1 and E_2 are the fluorescence emission efficiencies (which actually are the products of dye quantum yield and an instrument factor) for fluorescein and FMG, respectively. Of course, in the special case of a single fluorescein or FMG species, the fluorescence response simplifies to

$$F_i = I_0 E_i[1 - \exp(-\epsilon_i C_i X)] \quad (4')$$

Equation 4 must be used to account for the fluorescence variation from the substrate consumption or the product formation for the substrates such as FMG where an appreciably fluorescent substrate is converted to an appreciably fluorescent product. At the point where β-galactosidase is added to an FMG sample with concentration C, there must be a relationship

$$(dC/dt) = (dC_1/dt) = -(dC_2/dt) \quad (5)$$

where dC/dt, dC_1/dt, and dC_2/dt are the system conversion rate (enzymatic turnover rate), fluorescein producing rate, and FMG consumption rate, respectively. From the derivative of eq 4 against time (t) and the use of the relationship in eq 5 as well as $(C_1)_{t=0} = 0$ and $(C_2)_{t=0} = C$, the initial rate for fluorescence change that results from the FMG hydrolysis will be

$$(dF/dt)_{t=0} = I_0\{(E_1 - E_2)\epsilon_1[1 - \exp(-\epsilon_2 CX)]/(\epsilon_2 C) + E_2 X(\epsilon_1 - \epsilon_2)\exp(-\epsilon_2 CX)\}(dC/dt)_{t=0} \quad (6)$$

Fluorescence and Enzymatic Turnover in the Fluorometer (Right-Angle Manner). Figure 1B shows the excitation path through a fluorometer cuvette in a conventional fluorometer. The detected fluorescence is from the center of the cuvette with the small path ΔX. Again, there is presumably no reabsorption of fluorescence emission by the fluorophore. The fluorescence of a fluorophore measured at a right angle to the illumination should be

$$F_i = I_0 E_i\{\exp(-\epsilon_i C_i X_0) - \exp[-\epsilon_i C_i (X_0 + \Delta X)]\} \quad (7)$$

or

$$F_i = I_0 E_i[\exp(-\epsilon_i C_i X_0)][1 - \exp(-\epsilon_i C_i \Delta X)] \quad (7')$$

Obviously F_i has a linear response at low fluorophore concentrations and becomes zero at high concentrations. The term $\exp(-\epsilon_i C_i X)$ can be treated as the inner filter effect caused by the fluorophore itself in the fluorometer. By differentiating eq 7′, a concentration at which the fluorescence reaches a maximum (C_m) can be calculated and approximated with $\Delta X \ll X_0$:

$$(C_m)_i = [\ln(1 + \Delta X/X_0)]/(\Delta X \epsilon_i) \approx 1/(\epsilon_i X_0) \quad (8)$$

The total fluorescence in a cuvette containing both fluorescein and FMG measured in the fluorometer may also be calculated at any time t by replacing $\{1 - \exp[-(\epsilon_1 C_1 + \epsilon_2 C_2)X]\}$ in eq 4 with $\{\exp[-(\epsilon_1 C_1 + \epsilon_2 C_2)X_0] - \exp[-(\epsilon_1 C_1 + \epsilon_2 C_2)(X_0 + \Delta X)\}$, and the initial rate of the change of the fluorescence after adding β-galactosidase to FMG is then given by

$$(dF/dt)_{t=0} = (I_0 \Delta X)[\exp(-\epsilon_2 CX_0)][E_1\epsilon_1 - E_2\epsilon_2 + E_2\epsilon_2(\epsilon_2 - \epsilon_1)CX](dC/dt)_{t=0} \quad (9)$$

Results

Fluorescence Response in the CytoFluor Measurement (Front-Face Manner). As predicted by eq 4′, the experimental measurement of the fluorescence of fluorescein or FMG follows an exponential saturation curve for the fluorescence versus fluorophore concentration (Figure 2). According to eq 4′, the fluorophore's concentration exhibiting half-maximum

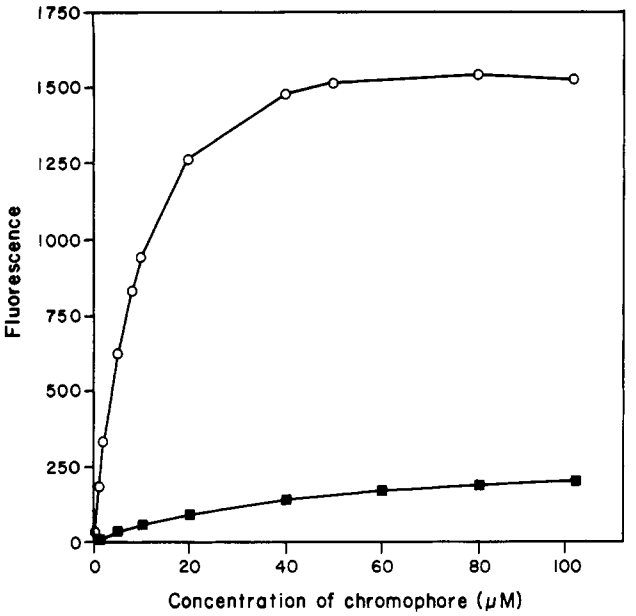

FIGURE 2: Fluorescence of fluorescein (O) and FMG (■) as determined in the CytoFluor using the front-face manner. Excitation was set at 485 nm (EX filter B) and emission was at 530 nm (EM filter B), with the instrument sensitivity setting = 1 (low sensitivity).

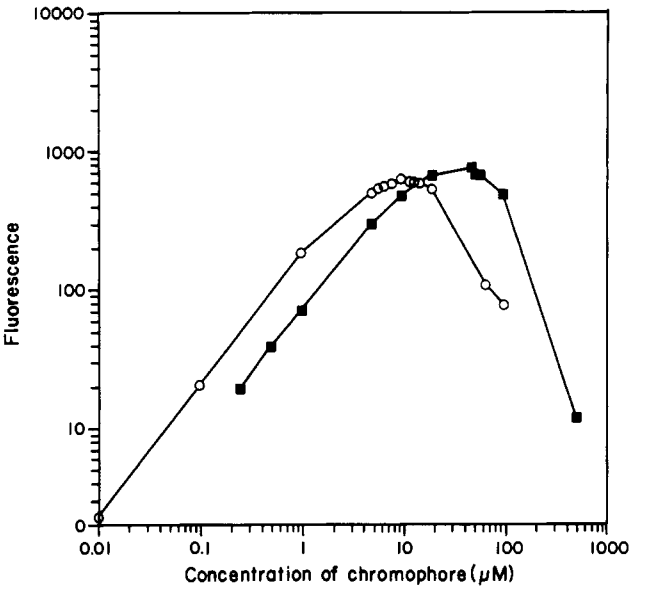

FIGURE 3: Fluorescence of fluorescein and FMG detected in the right-angle manner on the Perkin-Elmer fluorometer. Fluorescein (O) was excited at 488 nm, ΔEX 2.5 nm, with the fluorescein emission measured at 540 nm, ΔEM 2.5 nm. FMG (■) was excited at 452 nm, ΔEX 10 nm, and the FMG emission was measured at 518 nm with ΔEM 5 nm.

fluorescence, C_H, should be proportional to the reciprocal of the path length X. However, the path is linearly determined by the sample volume (V). Thus, a linear relationship between C_H and $1/V$ should be seen. Indeed, such linearity was observed in both fluorescein and FMG (data not shown). Because of the effect of sample volume on the detected fluorescence, the sample volume used for the CytoFluor measurement must be carefully controlled. Therefore, the 150-μL volume for the enzymatic assay was kept constant in all of the other experiments performed in the CytoFluor. With this volume, the maximum fluorescence (in counts on the CytoFluor instrument) of fluorescein and of FMG, respectively, were experimentally determined as $(F_m)_1 = I_0E_1 = 1520$, $(F_m)_2 = I_0E_2 = 220$; ϵX of fluorescein and FMG were respectively determined as $\epsilon_1 X = (\ln 2)/(C_H)_1 = 0.102\ \mu M^{-1}$, $\epsilon_2 X = (\ln 2)/(C_H)_2 = 0.024\ \mu M^{-1}$; ϵ_1/ϵ_2 should therefore be equal to $(C_H)_2/(C_H)_1 = 4.26$, which is consistent with the absorbance measurements for fluorescein and FMG at 485 nm in a spectrophotometer.

Fluorescence Response in Fluorometer (Right-Angle) Measurements. Figure 3 shows the fluorescence of fluorescein and of FMG measured in a fluorometer. Both curves are linear at concentrations below 1 μM and tend toward zero at high concentrations. For fluorescein, the maximum fluorescence obtainable is at a concentration of about 10 μM, which is very consistent with the calculated value of 10.1 μM determined by use of eq 8 with the fluorescein extinction coefficient $\epsilon_{488nm} = 0.198\ (\mu M \cdot cm)^{-1}$ (defined as in eq 2 and determined in a spectrophotometer) and $X_0 = 0.5$ cm. The FMG concentration exhibiting maximum fluorescence was determined to be 45 μM, which is very close to the calculated value of 44.4 μM using $\epsilon_{452nm} = 0.045\ (\mu M \cdot cm)^{-1}$ (defined as in eq 2 and determined in a spectrophotometer) and $X_0 = 0.5$ cm.

FMG Hydrolysis Detected in the CytoFluor (Front-Face Manner). The fluorescence development after enzyme addition to FMG at several typical concentrations was detected in the CytoFluor and is illustrated in Figure 4. There is a steady linear fluorescence increase up to 15 min so that the initial fluorescence rate $(dF/dt)_{t=0}$ may be easily measured. Replacing the parameters in eq 6 with the determined data given

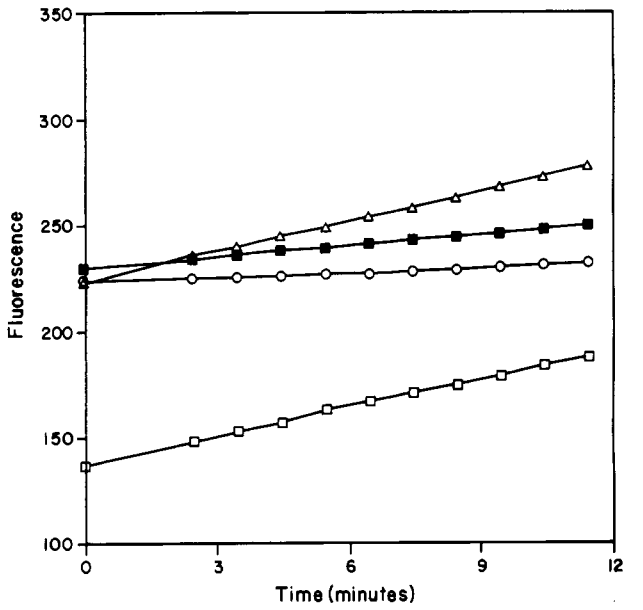

FIGURE 4: Fluorescence development after the addition of β-galactosidase to FMG detected with use of the CytoFluor front-face manner. FMG concentrations are indicated as follows (O) 800 μM; (■) 500 μM; (Δ) 160 μM; (□) 40 μM. The enzyme reaction was initiated by the addition of β-galactosidase (final concentration 8.33 μg/L) to FMG and monitored in a time sequence with use of the same instrument parameters as Figure 2.

in Figure 2, the initial turnover time $[C/(dC/dt)]_{t=0}$ is obtained as

$$[C/(dC/dt)]_{t=0} = \{5544 \times [1 - (1 - 0.0031C)\exp(-0.024C)]\}/(dF/dt)_{t=0} \quad (10)$$

On the other hand, the Michaelis–Menten hypothesis may be written as

$$[C/(dC/dt)]_{t=0} = K_m/\{[E]k_2\} + C/\{[E]k_2\} \quad (11)$$

where K_m is the Michaelis–Menten constant, k_2 is the enzyme turnover rate, and [E] is the enzyme concentration. Figure

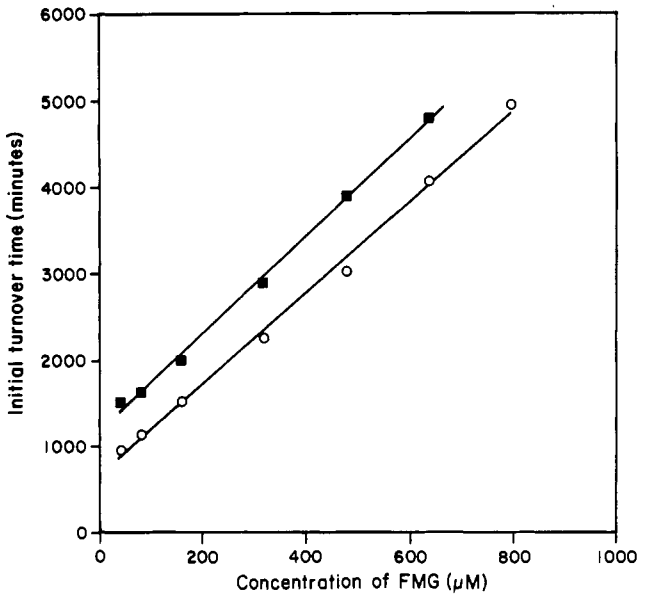

FIGURE 5: Plots of β-galactosidase initial turnover time versus FMG concentration based on eqs 10 and 11. With use of methods similar to those specified in Figure 4, the initial fluorescence rates were determined in the CytoFluor with β-galactosidase at 8.33 μg/L (final concentration) and a series of FMG concentrations in the absence (○) or presence (■) of 0.67 μM PETG. Instrument parameters were the same as in Figure 2.

5 gives linear plots of $[C/(dC/dt)]_{t=0}$ versus C based on eq 10 and the initial rates of development due to β-galactosidase actions on a series of FMG concentrations (Figure 4). K_m was obtained as 117.6 μM, and k_2 was 22.7 μmol·(min·mg)$^{-1}$. In the presence of 0.67 μM PETG, a competitive inhibitor of β-galactosidase (DeBruyne & Yde, 1977), K_m and k_2 were found to be 201.0 μM and 21.2 μmol·(min·mg)$^{-1}$, respectively.

The dependence of the FMG hydrolysis rate on the β-galactosidase activity or concentration was also determined. The initial fluorescence development by enzyme action within the initial 15 min was linearly related to the enzyme concentrations up to 120 μg/L (data not shown), as described shown in eq 6.

FMG Hydrolysis Detected in the Fluorometer (Right-Angle Manner). Figure 6 shows the fluorescence response detected in the fluorometer after the addition of relatively high amounts of β-galactosidase to concentrations of FMG higher than 200 μM. The intensity of fluorescence was very low (<40 relative units) because of the inner filter effect caused by the high FMG absorbance. No fluorescence development can be seen at any enzyme level when the concentration of FMG was higher than 500 μM (data not shown). As discussed below, the fluorescence development in this situation cannot be used to calculate the enzymatic conversion rate.

Discussion

Fluorescence Measurement Geometry and Fluorescence Response versus Fluorophore Concentration. While applications of fluorescence analysis are widespread, special caution should be paid to the many variables involved in measurements that include geometry of the system (this geometry governs the fluorescence–concentration relationship), fluorophore–fluorophore interaction (Förster energy transfer), physical or chemical quenching factors, etc. (Lakowicz, 1983; Holland et al., 1973, 1977; Christmann et al., 1980). For either fluorescein or FMG measured in a conventional fluorometer (i.e., with use of the right-angle manner), the major complication in signal response versus fluorophore concentration is considered as the inner filter effect, since the requirements for

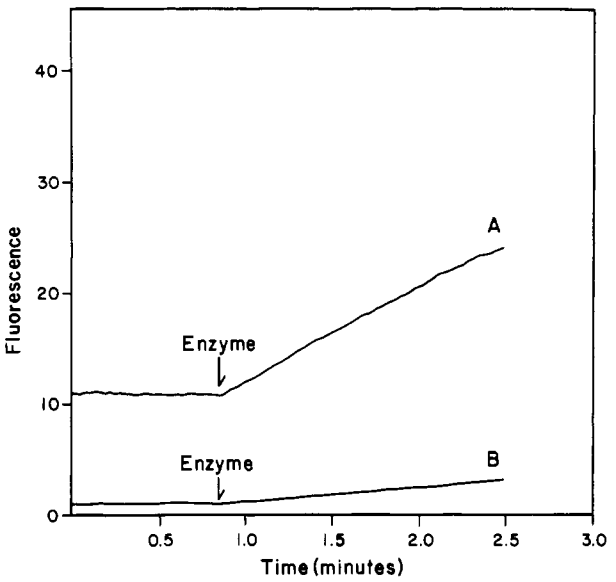

FIGURE 6: Fluorescence development after the addition of β-galactosidase to FMG detected in the fluorometer right-angle manner. β-Galactosidase (final concentration 375 μg/L) was added to (A) 200 μM and (B) 500 μM FMG solutions. Fluorescence was excited at 480 nm, ΔEX 10 nm, and the emission was monitored at 520 nm, with ΔEM 2.5 nm.

Förster energy transfer are not well fulfilled in terms of the distance between free fluorophores when the concentrations are less than 1000 μM, even with extensive spectral overlap of the excitation and emission. There are two ways to deal with the inner filter effect. One is to calibrate fluorometrically measured fluorescence with the sample absorbance in order to establish the linearity between the calibrated fluorescence and the fluorophore concentration (Holland et al., 1973, 1977; Christmann et al., 1981).

The other approach to reliable fluorescence measurements at high absorbance is to utilize front-face geometry to eliminate the interference of background absorbance and to directly obtain the linearity between the measured fluorescence signal and the fluorophore concentration (Eisinger & Flores, 1979; Blumberg et al., 1977; Lamola et al., 1977). This measurement is facilitated by use of a fluorescence plate reader that also has high throughput and reproducibility. In view of the accuracy and reproducibility of the experimental data in this paper, these results indicate that the front-face geometry produces reliable results. The calibration method for fluorometer right-angle measurements is susceptible to poor data quality, especially when the fluorescence is very weak or measured in the presence of high absorbance.

In this work, particular attention was paid to the inner filter effect caused exclusively by the fluorophore. Therefore, regardless of the non-fluorophore absorbance, the quantitative relationships of fluorescence versus fluorophore concentration were established in both the right-angle manner (fluorometer) and the front-face manner (CytoFluor fluorescence plate reader), and the theoretical predictions were firmly confirmed by the experimental data. Comparison between eq 4′, Figure 2, eq 7′, and Figure 3 leads to the conclusion that the front-face measurement gives a larger detectable fluorophore concentration range than does the right-angle one. Obviously the fluorescence reading at high fluorophore concentrations measured in the front-face manner is much more reliable than that from right-angle measurements.

Assay of Fluorescence Substrate Hydrolysis by β-Galactosidase. The accurate interpretation of the fluorescence of fluorescein and FMG implies the basis for enzymatic quan-

titation, as indicated in eq 10 or eq 9. The successful kinetic assay of FMG hydrolysis by β-galactosidase in terms of having good linear curves using the CytoFluor front-face manner is shown in Figure 5. A limitation of the instrument for collection of kinetic data is that it is not readily temperature regulated. Using the fluorescence plate reader, data can be collected on up to 96 variables in about 1 min so that the internal consistency and intraplate reproducibility are excellent. This assay method is also very sensitive. A very low enzyme concentration (8.33 μg/L) was sufficient for the kinetic assay, while one can enhance the hydrolysis signal by at least 10-fold by adding more enzyme since the detected fluorescence development linearly responds to the enzyme concentration up to 120 μg/mL. The significant change of K_m and slight change k_2 in the presence of PETG indicate that PETG is a competitive inhibitor of β-galactosidase. K_i, the dissociation constant of PETG for the enzyme, was found to be 0.943 μM, which is very close to the value of 0.969 μM given by DeBruyne and Yde (1977).

In the case of high FMG concentrations (>100 μM), a condition that is needed for FMG kinetic measurements, eq 6 concludes that the initial rate of change of the fluorescence should be proportional to the reciprocal of the FMG concentration in the CytoFluor front-face manner, whereas eq 9 suggests that the initial rate decreases exponentially with FMG concentration in the right-angle manner in the fluorometer. Undoubtedly the detectable initial fluorescence rate attenuates more dramatically in the fluorometer right-angle manner than in the CytoFluor front-face manner when a high FMG concentration is employed. On the other hand, the signal of initial fluorescence rate from high FMG concentration detected in the fluorometer is so weak that the rate of conversion based on the detected fluorescence is experimentally not reliable.

In accordance with the conclusion above, the actual fluorescence rate detected in the fluorometer right-angle manner (Figure 6) cannot be kinetically interpreted. Setting α to the hydrolysis rate or fluorescence rate of 500 μM FMG sample and β to that of 200 μM FMG sample, the following ratio of the initial fluorescence rate can be read from Figure 6:

$$[(dF/dt)_\alpha]/[(dF/dt)_\beta] = 0.12 \qquad (12)$$

From consideration of eq 9 with $\epsilon X = 0.015\ \mu M^{-1}$ (the low estimate of the FMG extinction coefficient at 480 nm), the ratio of the initial enzymatic turnover rate for two samples is given by

$$[(dC/dt)_\alpha/(dC/dt)_\beta] > [(dF/dt)_\alpha/(dF/dt)_\beta] \exp[\epsilon X(C_\alpha - C_\beta)] = 10.8 \qquad (13)$$

This result is apparently not acceptable since the turnover rate when the substrate concentration is 500 μM should not be 2.5-fold greater than that when the concentration is 200 μM. The rate of fluorescence development at high FMG concentrations in the sample cannot quantitatively be interpreted by eq 9, probably because in this case a portion of the collected fluorescence was from the random reflection of the dense fluorophore emission off the cuvette surface.

In conclusion, the front-face measurement using the CytoFluor plate reader has provided a practical and sensitive method for the kinetic assay of FMG hydrolysis and, in principle, for the assay of any fluorogenic substrate that has strong absorbance and that, because of the absorbance, cannot be utilized in the right-angle manner in a fluorometer. As completely nonabsorbing substrates cannot always be found in the search for fluorescent substrates (e.g., FMG), the assay method in this paper actually makes it possible that any compound that exhibits a change of fluorescence after enzyme action could work as a useful fluorogenic substrate even in the presence of strong background absorbance or fluorescence. Moreover, the hydrolysis parameters of FMG by β-galactosidase obtained in this paper have been used as reliable data for studies of the stepwise hydrolysis mechanism of fluorescein di-β-D-galactoside (FDG), another fluorogenic substrate, by β-galactosidase (Huang, 1991).

Finally, using the multiwell plate system of the CytoFluor, many enzymatic variables (up to 96) can be measured at the same time. The data generation and manipulation rate is dramatically increased versus measurements in a cuvette with use of a fluorometer. This is very useful for obtaining consistent data for enzymology studies.

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Registry No. FMG, 102286-67-9; β-galactosidase, 9031-11-2.

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