Kinetic Fluorescence Measurement of Fluorescein Di-β-D-galactoside Hydrolysis by β-Galactosidase: Intermediate Channeling in Stepwise Catalysis by a Free Single Enzyme†

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ABSTRACT: Kinetic fluorescence measurements were employed to quantitative to stepwise hydrolysis of fluorescein di-β-D-galactoside (FDG) by β-galactosidase and the intermediate fluorescein mono-β-D-galactoside (FMG) channeling. The kinetic parameters, Michaelis–Menten constant K_m and enzymatic catalysis rate k_2, for FDG hydrolysis to FMG by β-galactosidase were obtained as 18.0 μM and 1.9 μmol·(min·mg)$^{-1}$, respectively. The FMG intermediate is hydrolyzed via two modes: (1) FMG that is in free solution binding to the enzyme substrate binding site in competition with FDG and then being hydrolyzed (binding mode); (2) FMG being directly hydrolyzed into the final products of fluorescein and galactose before the FMG can diffuse away from the enzyme active site (channeling mode). The extent of the FMG channeling mode was found to depend on the FDG hydrolysis rate but to be independent of the free enzyme concentration. A channeling factor, defined as the ratio of the real FMG hydrolysis rate with both binding and channeling modes over that which would be observed with an exclusive binding mode, was used to quantitate the effect of the intermediate channeling. The FMG channeling factor was determined to be close to 1 at low FDG concentration (about 5.1 μM), where the slow FDG hydrolysis rate gives an ineffective channeling and where the FMG is then hydrolyzed mainly via the binding mode. However, the channeling factor dramatically increases at higher FDG concentrations ($>K_m$), strongly indicating that the effective FMG channeling mode, resulting from the considerable FDG hydrolysis rate at high FDG concentrations, becomes a primary pathway to channel a steady system hydrolysis with a high rate. Under these conditions, FMG hydrolysis via the binding mode becomes insignificant compared to hydrolysis via the channeling mode as the substrate binding site of the enzyme is mostly occupied by FDG with high concentrations. The calculated FMG concentration formed as an intermediate in free solution in the stepwise hydrolysis is fairly low as a result of the effective FMG channeling at high FDG concentrations or, in the opposite case, relatively high in the case of weak FMG channeling at low FDG concentrations.

Intermediate distribution and lifetime are of a great interest for understanding the kinetics of a multistep enzymatic reactions (Friedrich, 1984). In the immobilized or organized multienzyme systems, the concentrations of an intermediate in the immediate vicinity of the component enzymes were found to be greater than that in the bulk solution and the overall system reaction rates were always higher than those expected by the overall available concentrations of intermediate (Mosbach, 1976; Rugh, 1982; Gaertner, 1978; Matchett, 1974; Vitto et al., 1980; Akiyama & Hammes, 1981; Miziorko et al., 1990; Dunn et al., 1990; Sumegi et al., 1990). Recently, bifunctional complexes of β-galactosidase–galactokinase (Bülow et al., 1985; Bülow, 1987) and β-galactosidase–galactose dehydrogenase (Ljungcrantz et al., 1989) were prepared by means of an artificial gene fusion. These unique complexes provided direct evidence that an intermediate (galactose) can accumulate around the next component enzyme instead of diffuse into the medium and then be more efficiently hydrolyzed into a final product through an enzyme proximity effect. The efficient flux of biological metabolites mediated by enzyme(s) systems has been termed "channeling". However, free enzymes or separate multienzyme systems seemed to take no advantage of such intermediate accumulation or channeling since their intermediates have to diffuse through the bulk medium to reach their next component enzymes (Goldman & Katchalski, 1971; Mosbach & Mattiasson, 1978).

Compared to immobilized or organized enzyme systems, there are relatively few reports of the channeling mechanism in a stepwise catalysis of an oligomeric substrate by a single free enzyme, although an intermediate formed in this stepwise reaction could accumulate around the enzyme and then be catalyzed by the same enzyme if it does not diffuse rapidly into the bulk medium. Such direct conversion of intermediate has been demonstrated in porcine pancreatic α-amylase, whose polysaccharide substrates may be gradually hydrolyzed in the active site (Chan et al., 1984). However, it is not clear whether the polysaccharide intermediates can still diffuse into the bulk medium or which factors determine the kinetics of the intermediate hydrolysis via the channeling or the diffusion.

Hofmann and Sernetz (1983) observed that fluorescein di-β-D-galactoside (FDG)[1] is hydrolyzed by free β-galactosidase by such a two-step hydrolysis process. They proposed that the intermediate, fluorescein mono-β-D-galactoside (FMG), may accumulate in the vicinity of the enzyme and then be hydrolyzed through its enhanced competition with FDG. However, they ignored a possibility that FMG is continuously hydrolyzed into the final products of fluorescein and galactose at the same enzyme active site where the FMG is formed as the initial FDG hydrolysis product. In addition,

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

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their interpretation of the FMG accumulation is not adequate in that arbitrary kinetic parameters were used to support the FMG accumulation. There was even a disagreement on the existence of FMG as an intermediate in the hydrolysis of FDG (Sontag, 1977; Hofmann & Sernetz, 1983). It could be possible that FMG existence is, at least partially, determined by the accumulation or channeling mechanism.

FDG has been widely used as a sensitive fluorogenic substrate for detecting β-galactosidase activity from various sources both in vitro or in vivo (Rotman, 1961; Rotman et al., 1963; Yashphe & Halvorson, 1976; Jongkind et al., 1986; Nolan et al., 1988). An accurate determination of the enzyme activity should be made by considering the two-step hydrolysis mechanism, the FMG channeling effect, and the FDG concentration used. The fluorescence that results from fluorescein and FMG released from FDG by β-galactosidase is kinetically informative and therefore has been used here as a unique quantitative means for determining the FDG stepwise hydrolysis and FMG channeling under conditions of either high or low FDG concentration.

Experimental Procedures

Materials. Fluorescein, FMG, FDG, and PETG were from Molecular Probes, Inc. (Eugene, OR). These chemicals were dissolved in DMSO to make a 15 mg/mL stock solution. The stock solutions were then diluted to the desired concentration with the buffer indicated below. β-Galactosidase from *Escherichia coli* (molecular weight 540000) was purchased from Scripps Laboratories (San Diego, CA). All fluorescence measurements and enzyme reactions were performed at room temperature (about 22 °C) in 100 mM sodium phosphate buffer, pH 7.5, containing 110 mM 2-mercaptoethanol and 1 mM $MgCl_2$.

Enzyme Reactions and Fluorescence Measurements. The enzyme reaction was initiated either by adding constant amounts of β-galactosidase to different concentrations of the FDG substrate solution or by adding the FDG substrate to solutions of the enzyme prepared at different concentrations. The fluorescence increase was then measured by use of the front-face method previously described (Huang, 1991) with time-sequential data collected in a CytoFluor 2300 multiwell fluorescence plate reader made by Millipore (Bedford, MA), using Corning 96-well plates purchased from Corning Glass Works (Corning, NY). In this system, the fluorescence was determined to be proportional to the fluorophore concentration for FMG concentrations <10 μM and for fluorescein concentrations <2 μM. This is consistent with the calculated maximal useful concentrations for these two dyes, which are based on the measurement geometry and signal response of the CytoFluor instrument and on the absorbance of the two dyes. The sample volume in each well for any enzyme reaction and fluorescence measurement was set at 150 μL, at which the fluorescence proportionality constants were determined as $\alpha_M = 5.3\ \mu M^{-1}$ for FMG and $\alpha_P = 150\ \mu M^{-1}$ for fluorescein with 485-nm excitation and 530-nm emission wavelengths as well as a sensitivity of 1 in the CytoFluor instrument.

Kinetic Models for Fluorescence Time Courses. Figure 1 gives the kinetic elements for FDG hydrolysis. There are two separated β-galactosidase cycles in which free FDG and FMG compete mutually for the enzyme. As shown in the bottom FMG cycle, FMG in free solution binds to the enzyme substrate binding site in competition with FDG and then is hydrolyzed. This FMG hydrolysis mechanism is named as the binding mode. The top FDG cycle represents an alternative cycle in which a portion of the intermediate (E·FMG)*, the initial hydrolysis product of FDG still staying around the

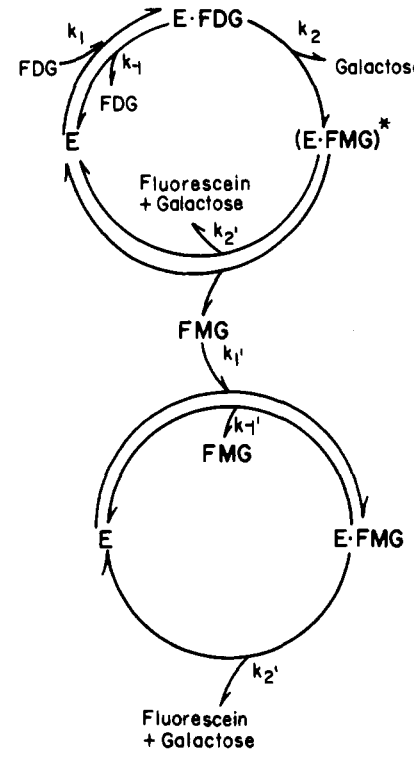

FIGURE 1: β-Galactosidase cycles including the FDG cycle (top) and the FMG cycle (bottom). $K_m = (k_{-1} + k_2)/k_1$; $K_m' = (k_{-1}' + k_2')/k_1'$. The FMG channeling mode for FMG hydrolysis is present in the FDG cycle; the FMG binding mode for FMG hydrolysis is present in the FMG cycle.

enzyme active site, can be directly converted into final products, fluorescein and galactose, without affecting the FDG cycling rate as well as the FDG hydrolysis rate since k_2 is smaller than k_2' (following data; Huang, 1991). The mechanism of the direct hydrolysis of intermediate (E·FMG)* is named as the channeling mode.

The first hydrolysis conditions considered will be those of high FDG concentration and low β-galactosidase concentration. Since under these conditions the intermediate FMG concentration in free solution is quite low (this point will be supported by latter results) and its K_m' of 117.6 μM (Huang, 1991) is relative high, the effect of FMG competition on FDG binding can be practically ignored. The FMG hydrolysis via the binding mode follows the kinetics of two competitive substrates and is therefore related to the concentrations of both free FMG and enzyme. FMG hydrolysis via the channeling mode is dependent on the residual (E·FMG)* concentration at the enzyme active site. (E·FMG)* is proportional to the concentrations of both FMG and enzyme too, as a result of diffusion exchange between free FMG in bulk medium and the enzyme. The total FMG hydrolysis rate based on the above two modes must therefore be proportional to the concentrations of both free FMG and enzyme. Thus, to demonstrate the role of channeling, the real overall FMG hydrolysis rate can be empirically forced to be equal to a hydrolysis rate via an exclusive binding mode as well as an enhancement factor. Therefore the system reaction described in Figure 1 can be quantitated by the equations

$$(dS/dt) = -k_2ES/(K_m + S) \quad (1)$$

$$(dP/dt) = Ak_2'EM/[K_m'(1 + S/K_m)] \quad (2)$$

$$S_0 = S + M + P \quad (3)$$

where S is FDG and its concentration; S_0 is the initial FDG concentration; M is FMG; P is fluorescein; K_m and K_m' are

the Michaelis–Menten constants for FDG and FMG respectively; k_2 and k_2' are the enzymatic turnover rates for FDG and FMG respectively; and A is the enhancement factor compensating for the real FMG hydrolysis rate, which is, however, expected by an exclusive FMG binding mode. A can be named as the channeling factor since it actually describes the effect of the FMG channeling. Because of the low enzyme concentration and the high FDG concentration, the FDG concentration actually changes very slightly (i.e., $S \approx S_0$) during the observation period. Thus, following resolution, eq 1 to eq 3 can give

$$M = \{k_2E\cdot S_0/[(K_m + S_0)R]\}[1 - \exp(-Rt)] \quad (4)$$

$$P = [k_2ES_0/(K_m + S_0)]\{t - [1 - \exp(-Rt)]/R\} \quad (5)$$

where $R = Ak_2'E/[K_m'(1 + S_0/K_m)]$ can be termed the relaxation constant. Since FDG is nonfluorescent, the system fluorescence (F) should be

$$F = \alpha_P P + \alpha_M M \quad (6)$$

Referring to eqs 4 and 5, the fluorescence rate can then be given as

$$(dF/dt) = [k_2ES_0/(K_m + S_0)][\alpha_P + (\alpha_M - \alpha_P)\exp(-Rt)] \quad (7)$$

At low FDG concentrations around 5 μM, it is impossible to observe the above whole hydrolysis process featured by a system hydrolysis rate transition from a lower value to a steady and higher value. However, in a system chosen to represent the opposite condition of a relatively high amount of β-galactosidase and a low concentration of FDG, the hydrolysis will be governed by the equations

$$(dS/dt) = -k_2ES/K_m \quad (8)$$

$$(dP/dt) = Ak_2'E\cdot M/K_m' \quad (9)$$

$$S_0 = S + M + P \quad (10)$$

where A is the channeling factor at low FDG concentration, where A is substantially small (i.e. close to 1) that it is assumedly treated as a constant. Thus, M behaves as a typical intermediate that has a maximum concentration $(M)_m$ at a certain time t_m (Freifelder, 1982) given by

$$(M)_m = S_0 \exp[(-k_2'EA/K_M')t_m] \quad (11)$$

Since α_P is much larger than α_M, we may treat the fluorescence of fluorescein as the total fluorescence. As an approximation, the maximum rate of fluorescence increase should therefore be

$$(dF/dt)_m = [(\alpha_P S_0 k_2'A/K_m')E]\exp[(-k_2'A/K_m')(Et_m)] \quad (12)$$

It should be noted that in the presence of a competitive inhibitor, phenylethyl β-D-thiogalactoside (PETG), all of the above equations are still valid by adding $K_m(I/K_i)$ to form the relevant apparent Michaelis–Menten constant. Of course, I and K_i are the inhibitor concentration and dissociation constant, respectively.

Results

FDG Hydrolysis with High Substrate Concentration and Small Amounts of β-Galactosidase. FDG is a β-galactosidase substrate that has no fluorescence unless hydrolyzed by the enzyme. The product fluorescence of FMG and fluorescein can be used to monitor the hydrolysis process. Figure 2 shows two typical examples of fluorescence development by the action

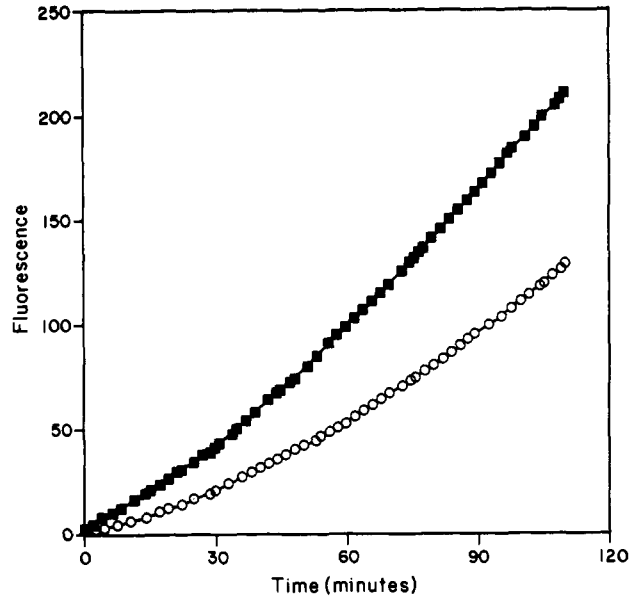

FIGURE 2: Fluorescence time courses developed by adding 8.33 μg/L (final concentration) β-galactosidase to FDG (○) 40 μM and (■) 200 μM (final concentration) in a total volume of 150 μL 0.1 M sodium phosphate buffer, pH 7.5, containing 0.11 M 2-mercaptoethanol and 1 mM $MgCl_2$. The fluorescence in the Corning 96-well plate was measured in time sequence in the CytoFluor with excitation filter B (485 nm), emission filter B (530 nm), and sensitivity 1.

of very small amounts of β-galactosidase (8.33 μg/L) at a relatively high FDG concentration (>40 μM). The fluorescence rate increased gradually and then reached a maximum, indicating a transition of the intermediate hydrolysis rate from zero to the system steady rate (i.e., FDG hydrolysis rate at a steady state). The steady maximum rate can last for the total observation period (120 min). This fluorescence time course may be exactly interpreted by eq 7. According to eq 7, there are two parameters to characterize the fluorescence time course like Figure 2, i.e., the maximum fluorescence rate $(dF/dt)_m$ and the relaxation constant R. $(dF/dt)_m$ can be read from the saturated fluorescence rate after a sufficient time. The relaxation constant can be measured as $(\ln 2)/T$, where T is the time that the fluorescence rate reaches $[(\alpha_P + \alpha_M)/2\alpha_P](dF/dt)_m = 0.534(dF/dt)_m$.

The steady fluorescence development rate $(dF/dt)_m$ versus enzyme concentration E was measured under conditions where the FDG concentration was fixed at 200 μM and the β-galactosidase concentration was changed. Under these conditions the steady-state hydrolysis process as in Figure 2 was surely kept, and the steady rate was found to be linearly proportional to the enzyme concentration (data not shown). This proportionality is consistent with the following relation derived from eq 7

$$(dF/dt)_m = \alpha_P k_2 ES_0/(K_m + S_0) \quad (13)$$

With the use of eq 13, a double-reciprocal plot of $[(dF/dt)_m/\alpha_P]$ versus S_0 (from 40 to 200 μM) yielded a linear curve (data in circles in Figure 3) and gave K_m = 18.0 μM and k_2 = 1.9 μmol·(min·mg)$^{-1}$ (17.1 s^{-1}). With 0.67 μM PETG in the FDG hydrolysis system, the dissociation constant of PETG K_i was determined as 0.83 μM (data in solid squares in Figure 3), which is close to the 0.943 μM measured in the FMG hydrolysis system at the same experimental time (Huang, 1991). In this paper K_i was taken as 0.943 μM because it is closer to the data of other workers (DeBruyne & Yde, 1977).

Figure 4 gives the relationship between the relaxation constant (R) and the enzyme concentration (E). As predicted in eq 2, a linear relation between the relaxation constant and

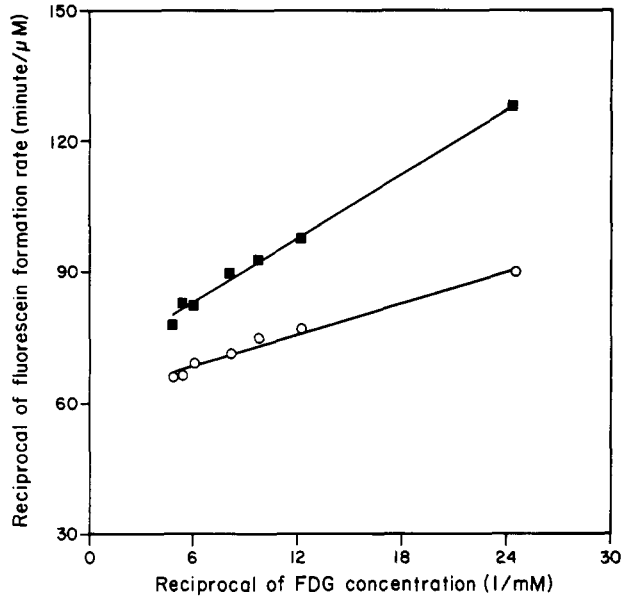

FIGURE 3: Double-reciprocal plots of the steady-state fluorescein formation rate versus the FDG concentration ranging from 40 to 200 μM in the presence of (○) 0 μM and (■) 0.67 μM PETG. Fluorescence development was initiated by adding 8.33 μg/L (final concentration) β-galactosidase and was detected with the instrument conditions in Figure 2.

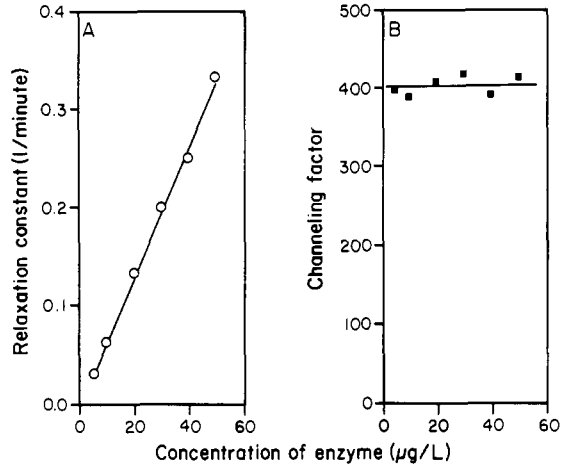

FIGURE 4: Relationship between the relaxation constant (○, curve A) and the enzyme concentration. The enzymatic reactions were inititated by adding 200 μM (final concentration) FDG to solutions of various β-galactosidase concentrations. The conditions for fluorescence measurement were the same as in Figure 2. Curve B (■) is the intermediate FMG accumulation factor versus the enzyme concentration calculated from the relaxation constant (curve A) using eq 14.

the enzyme concentration was experimentally obtained as shown as curve A of Figure 4.

Once the relaxation constants are read from the fluorescence time course, the channeling factor of the corresponding reaction system can be calculated by the equation

$$A = RK_m'(1 + S_0/K_m + I/K_i)/(k_2'E) \qquad (14)$$

where the inhibition of PETG is considered; K_m and K_i have already been determined as 18.0 μM and 0.943 μM, respectively; and K_m' and k_2' were determined as 117.6 μM and 22.7 μmol·(min·mg)$^{-1}$ (Huang, 1991). Transferring the relaxation constant of curve A in Figure 4 to the channeling factor using eq 14 results in the channeling factor versus enzyme concentration shown as curve B of Figure 4. The flatness of curve B suggests that the enzyme concentration is not likely to be related to the intermediate channeling.

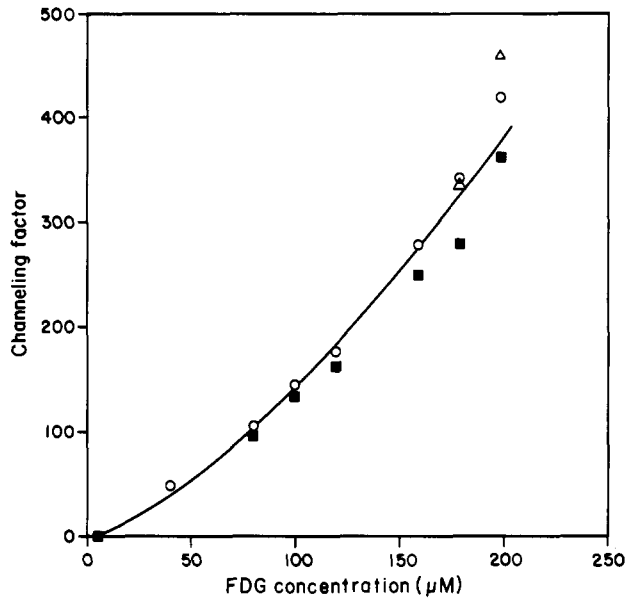

FIGURE 5: The intermediate channeling factor versus the initial FDG substrate concentration. A series of enzymatic reactions was initiated by adding 8.33 μg/L (final concentration) β-galactosidase to the FDG solution with concentrations ranging from 40 to 200 μM in the presence of (○) 0 μM, (■) 0.67 μM, and (△) 6.7 μM PETG. The reaction buffer and fluorescence instrument conditions were the same as in Figure 2. The FMG accumulation factors were calculated from the relaxation constants of the corresponding fluorescence time courses by eq 14. The channeling factor at 5.1 μM FDG (●) was calculated as specified in the text.

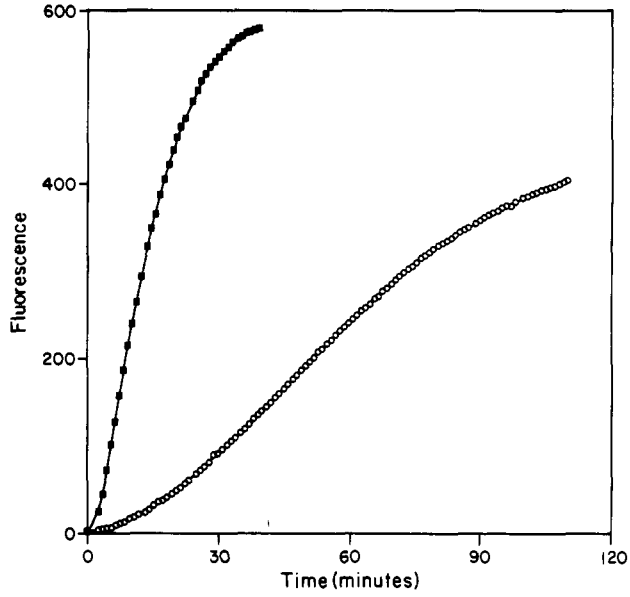

FIGURE 6: The fluorescence time courses developed by adding 5.1 μM (final concentration) FDG to (○) 0.125 mg/L and (■) 1.0 mg/L (final concentrations) β-galactosidase. The enzymatic reaction buffer and the fluorescence instrument conditions were the same as in Figure 2.

The channeling factors measured at FDG concentrations ranging from 40 to 200 μM with or without PETG are plotted in Figure 5 (except for the point at 5.1 μM, which will be specified later). These channeling factors were calculated by use of eq 14 and the measured relaxation constant from the fluorescence time courses like Figure 2. Fitting the curve in Figure 5 gives the relationship

$$\text{channeling factor} = 0.22(\text{FDG concn})^{1.4} + 1 \qquad (15)$$

FDG Hydrolysis at High Enzyme Concentrations and Low Substrate Concentrations. In this condition the FDG substrate

is hydrolyzed completely to the final products of fluorescein and galactose in the observation time (120 min). Figure 6 shows the FDG fluorescence time courses generated by two typical enzyme concentrations. The fluorescence rates increased gradually from zero to a maximum and then back to zero following hydrolysis of all the FDG. There are two parameters to characterize this fluorescence process, i.e., the maximum time t_m and the maximum fluorescence rate $(dF/dt)_m$, both of which may be graphically read from the fluorescence time courses like that in Figure 6.

In fact, t_m can be proven to be

$$t_m = [\ln (k_2K_m')/(k_2K_mA)]/[E(k_2/K_m - k_2'A/K_m')] \quad (16)$$

To verify eq 16, a series of enzymatic reactions at a fixed FDG concentration of 5.1 μM and various enzyme concentrations were carried out and the corresponding t_m values were read. Plotting the t_m versus E^{-1} according to eq 16 gives a linear curve that has a slope of 5.50 (min·mg)/L. This slope actually represents the experimentally determined value of the parameter (Et_m), a constant not related to the enzyme concentration.

Equation 12 can be transferred into the form

$$[(dF/dt)_m/E] = (\alpha_P k_2'S_0A/K_m') \exp[-(k_2'A/K_m')(Et_m)] \quad (17)$$

Since (Et_m) is a constant, the left hand term of eq 17 $[(dF/dt)_m/E]$ should also be a constant too. Plotting $(dF/dt)_m$ from the same FDG hydrolysis series described above, versus the enzyme concentration, indeed gives a linear curve with a slope of 52.5 L/(min·mg). This slope is, of course, the experimentally determined value of the constant $[(dF/dt)_m/E]$.

Substituting the experimental values for (Et_m) and $(dF/dt)_m/E$ and other known parameters for the relevant terms into eq 17 results in

$$2.81A = \exp(1.06A) \quad (18)$$

Resolving eq 18 gives $A \approx 1.06$, which apparently means that the channeling effect is nearly insignificant when the concentration of FDG is 5.1 μM or lower. This channeling factor (1.06) was then added into Figure 5 to complete the 5.1 μM point.

Discussion

The fluorescence kinetic models including the β-galactosidase cycles in Figure 1 and the equations described under Experimental Procedures successfully predicted the experimental results. Indeed, many channeling effects were found in organized multienzyme complexes or multifunctional enzyme complexes (Friedrich, 1984). But two independent FDG and FMG binding or catalysis sites in the β-galactosidase molecule can be ruled out since both FDG and FMG hydrolysis may be identically inhibited by a common competitive inhibitor PETG. So an FMG channeling effect of such stepwise catalysis by a single free enzyme, if any, cannot be explained by the spatial proximity effect of two separated binding sites in the enzyme molecule.

As shown in Figure 1, (E·FMG)* should have a fair chance to be directly converted into the final products before diffusing into the free solution since it is the immediate product of the FDG·E complex and is close to the enzyme active (binding and catalysis) site. In other words, by a small adjustment of the spatial orientation of FMG or the enzyme, a portion of (E·FMG)* could form an E·FMG complex that is identical with the complex of FMG and enzyme in the FMG cycle of Figure 1 and thus be converted into the final products by action of the enzyme. However, such direct FMG channeling does not slow down the FDG cycling rate as it participates in the cycle with a velocity constant k_2' [22.7 μmol·(min·mg)$^{-1}$], which is much greater than k_2 [1.9 μmol·(min·mg)$^{-1}$]. The FMG channeling also does not affect the FMG cycle in Figure 1.

It should be noted that (E·FMG)* represents a distribution of FMG near the enzyme active site rather than some kind of bound complex of FMG and the enzyme. The FMG channeling is obviously determined by the residual (E·FMG)* concentration at the enzyme active site. As the residual (E·FMG)* concentration is the result of FMG accumulation at the enzyme active site, it is then dependent on its formation rate and, therefore, the FDG hydrolysis rate. Of course, besides the FMG channeling mechanism, as an intermediate FMG can also be hydrolyzed by β-galactosidase via the binding mode shown as the FMG cycle in Figure 1.

The notion that the FMG channeling is critically determined by the FDG hydrolysis rate is strongly confirmed by the experimental results shown in Figure 5. The FMG channeling factor at FDG concentrations below 5 μM is likely close to 1 where the FDG hydrolysis rate is lower than 20% of the saturated rate. The FMG channeling factor then dramatically increases when the FDG concentration is above the K_m (18 μM), where the FDG hydrolysis precedes at a rate greater than 50% of the saturated rate. As the factor represents the extent of the channeling pathway, the high FMG channeling factor (for instance greater than 100) at high FDG concentrations is a clear indication that the FMG channeling already becomes a major pathway for the intermediate hydrolysis, and the hydrolysis by the binding mode is meanwhile very ineffective since a high FDG concentration precludes FMG binding to the enzyme via the binding mode.

Hofmann and Sernetz (1983) omitted the possibility of the above FMG channeling mechanism. Instead they only took into account the FMG binding mode (as shown in Figure 1) for the intermediate FMG hydrolysis. However, their argument cannot account for an experimental observation in this work that high concentrations of FDG (around 200 μM) do not inhibit the fluorescein formation, i.e., inhibit the rate of intermediate FMG hydrolysis. But this observation can be well explained by the observation of effective FMG channeling described in this work. According to the hypothesis of Hofmann and Sernetz, however, at FDG concentrations much higher than K_m, the FDG binding and hydrolysis as well as the effect of local FMG accumulation should be saturated and cannot increase as the FDG concentration increases. But the FMG hydrolysis should be inhibited as a result of increased FDG competition with the increased concentration. Furthermore, they obtained a significant FMG accumulation (same effect as the channeling defined in this work) when they simulated a high initial FMG hydrolysis rate at a low FDG concentration (10 μM) with arbitrary settings of kinetic parameters (which were not experimentally available in their work). It should be noted that by using the accurately experimentally determined K_m' (117.6 μM) and k_2' [22.7 μmol·(min·mg)$^{-1}$, an equivalent of 204.3 S^{-1}] (Huang, 1991) one can compensate for the high initial FMG hydrolysis rate in their work without introducing an FMG accumulation effect. In addition, the close correlation between kinetic parameters in Hofmann and Sernetz's curve simulation already leads to a conclusion that the hydrolysis process should be described as eqs 8–10 in this work, where the parameter correlations are indeed present. In conclusion, the FMG channeling mechanism described in Figure 1 should be taken into account for the intermediate FMG hydrolysis in such a

stepwise hydrolysis system and the interpretation of Hofmann and Sernetz's data should be based on this work.

It is very interesting to establish the intermediate concentrations needed to channel a stepwise catalysis with the channeling effect. In the case of 200 μM FDG and small amounts of β-galactosidase, the steady-state concentration fraction of intermediate FMG over FDG concentration can be calculated, according to eq 4, as $(k_2K_m')/(Ak_2'K_m) \approx 0.0014$. It appears that only very small amounts of FMG are needed to channel the flux of stepwise hydrolysis in the presence of an effective intermediate channeling. Similarly, a FMG fraction of 0.014 is needed to channel 40 μM FDG hydrolysis. At low FDG concentrations like 5.1 μM or less, the maximum concentration fraction of FMG can be calculated, according to eq 11, as $\exp[-(k_2'A/K_m')(Et_m)] \approx 0.33$. This large fraction is consistent with the experimental data obtained by Hofmann and Sernetz (1983) using 10 μM FDG and large amounts of β-galactosidase ($\approx$16.7 mg/L). Therefore, the results in this work suggest that the concentration fraction of an intermediate in a stepwise catalysis could be reduced as the intermediate channeling is enhanced. The small fraction of FMG present at high FDG concentrations as the result of effective channeling is supported by the observation of Sontag (1977) that no evident fluorescein acetate, a fluorogenic substrate similar to FMG that is hydrolyzed by nonspecific esterase, was found as an intermediate in the hydrolysis of high fluorescein diacetate concentration.

The unique FMG channeling mechanism described in Figure 1 is different from the intermediate channeling in many organized multienzyme or multifunction enzyme systems (Friedrich, 1984); however, it can be quite efficient since the spatial distance for FMG to reach the next hydrolysis step is very short (within an enzyme molecule). Actually, porcine pancreatic α-amylase may utilize amylose and its hydrolysis intermediate by a mode of the consecutive moving of glucose residues within the active site of the enzyme (Chan et al., 1984). It appears that the specific recognition and binding between the glucose residues and the enzyme subsites result in such residue moving with a striking order. However, if the interaction between the residues and the enzyme is not strong enough, the amylose intermediate might diffuse into the bulk medium as FMG does at low FDG concentrations. Obviously the amount of the amylose intermediate in solution can be very low as a result of the efficient channeling effect. It is, therefore, of great interest to investigate whether many multistep catalysis enzymes in biological systems such as proteinase, nuclease, or other hydrolyases utilize the similar intermediate channeling mechanism to achieve an efficient metabolic flux with a small fraction of their intermediates.

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Registry No. FDG, 17817-20-8; β-galactosidase, 9031-11-2.

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