Griffith, W. P., & Wickins, T. D. (1966) *J. Chem. Soc. A*, 1087.
Henderson, R. F., Henderson, T. R., & Woodfin, B. M. (1970) *J. Biol. Chem. 245*, 3733.
Hendrickson, W. A. (1981) in *Invertebrate Oxygen-Binding Proteins: Structure, Active Site, and Function* (Lamy, J., & Lamy, J., Eds.) p 503, Marcel Dekker, New York.
Hennessey, J. P., Jr., & Johnson, W. C., Jr. (1981) *Biochemistry 20*, 1085.
Hewkin, D. J., & Griffith, W. P. (1966) *J. Chem. Soc. A*, 472.
Hunt, H. R., & Taube, H. (1958) *J. Am. Chem. Soc. 80*, 2642.
King, R. M., & Callahan, K. P. (1969) *Inorg. Chem. 8*, 871.
Lankinen, H., Gräslund, A., & Thelander, L. (1982) *J. Virol.* (in press).
Loehr, J. S., & Loehr, T. M. (1979) *Adv. Inorg. Biochem. 1*, 235.
Loehr, J. S., Lammers, P. J., Brimhall, B., & Hermodson, M. A. (1978) *J. Biol. Chem. 253*, 5726.
Loehr, T. M., & Plane, R. A. (1968) *Inorg. Chem. 7*, 1708.
Loehr, T. M., Keyes, W. E., & Pincus, P. A. (1979) *Anal. Biochem. 96*, 456.
Murmann, R. K. (1980) *Inorg. Chem. 19*, 1765.
Murmann, R. K., & Shelton, M. E. (1980) *J. Am. Chem. Soc. 102*, 3984.
Murray, K. S. (1974) *Coord. Chem. Rev. 12*, 1.
Petersson, L., Gräslund, A., Ehrenberg, A., Sjöberg, B.-M., & Reichard, P. (1980) *J. Biol. Chem. 255*, 6706.
San Fillipo, J., Jr., Grayson, R. L., & Sniadoch, M. J. (1976) *Inorg. Chem. 15*, 269.
Sjöberg, B.-M., Gräslund, A., Loehr, J. S., & Loehr, T. M. (1980) *Biochem. Biophys. Res. Commun. 94*, 793.
Stenkamp, R. E., & Jensen, L. H. (1979) *Adv. Inorg. Biochem. 1*, 219.
Stenkamp, R. E., Sieker, L. C., Jensen, L. H., & Sanders-Loehr, J. (1981) *Nature (London) 291*, 263.
Swaminathan, K., & Busch, D. H. (1962) *Inorg. Chem. 1*, 256.
Swift, T. J., & Connick, R. E. (1962) *J. Chem. Phys. 37*, 307.
Thelander, L. (1973) *J. Biol. Chem. 248*, 4591.
Thelander, L., & Reichard, P. (1979) *Annu. Rev. Biochem. 48*, 133.
Wilkins, R. G., & Yelin, R. E. (1969) *Inorg. Chem. 8*, 1470.

Fluorometric Studies on the Binding of Gluconolactone, Glucose, and Glucosides to the Subsites of Glucoamylase†

Keitaro Hiromi,* Akiyoshi Tanaka, and Masatake Ohnishi

ABSTRACT: Static studies were made at pH 4.5 and 10 °C on the binding of *Rhizopus niveus* glucoamylase with four kinds of substrate analogues, i.e., gluconic acid 1,5-lactone (gluconolactone), glucose, methyl α-glucoside, and phenyl β-glucoside, by monitoring the enzyme fluorescence decrease caused by the ligand binding. The dissociation constant (K_d) and the percentage of maximum decrease of fluorescence intensity (ΔF_{max}) for the binary complex formation between these ligands and the enzyme were evaluated. Among these analogues, gluconolactone showed the smallest K_d value (1.1 mM) and the largest ΔF_{max} value (30%). From the fluorometric titration of the enzyme with gluconolactone in the presence of glucose or the glucosides, it was found that gluconolactone and glucose (or the glucosides) can bind to the enzyme simultaneously to form a ternary enzyme–gluconolactone–glucose (or -glucoside) complex. The percentage of maximum decrease of the fluorescence intensity caused by the ternary complex formation (about 30%) is almost equal to that observed for the binary enzyme–gluconolactone complex formation. The dissociation constant of gluconolactone from the ternary complex was considerably smaller (about a factor of 0.3) than that from the enzyme–gluconolactone complex. These results, together with the information obtained so far, lead to the following conclusions: (1) Gluconolactone binds to subsite 1 of the enzyme active site, where the nonreducing-end glucose residue of a substrate is bound in a productive binding mode, and glucose and the glucose moiety of the glucosides bind to subsite 2. (2) The decrease of the enzyme fluorescence arises from one tryptophan residue, which is supposed to be located at or close to subsite 1. (3) There is a positive interaction between subsite 1 and subsite 2 as to decrease the dissociation constant for the ligand binding at each subsite.

Glucoamylase[1] successively hydrolyzes glucosidic bonds from the nonreducing end of the substrate starch to produce glucose. By steady-state kinetic studies, the subsite structure (the arrangement of the affinities of subsites for glucose residues of linear substrates) of *Rhizopus* glucoamylase has been estimated by Hiromi et al. (Hiromi, 1970; Hiromi et al., 1973). The subsites are numbered from the nonreducing end of the substrate bound in the productive mode, as shown in Figure 1, and the catalytic site is considered to be located between subsite 1 and subsite 2 (Hiromi et al., 1973). Since subsite 2 has the highest affinity, the glucose or glucose moiety of glucosides is considered to bind predominantly to subsite 2 (Ohnishi et al., 1975, 1976). The apparent zero value of the affinity of subsite 1 (Hiromi et al., 1973) has been interpreted to indicate that the decrease in free energy due to the specific interaction between the nonreducing terminal glucose residue and this subsite is counterbalanced by the free

† From the Department of Food Science and Technology, Faculty of Agriculture, Kyoto University, Kyoto 606, Japan. *Received April 28, 1981.* This work was supported in part by a grant-in-aid for Scientific Research from the Ministry of Education, Science and Culture of Japan (No. 447094).

[1] Abbreviations used: E or glucoamylase, 1,4-α-D-glucan glucohydrolase from *Rhizopus niveus* (EC 3.2.1.3); L or gluconolactone, gluconic acid 1,5-lactone; G, glucose or glucose residue of glucosides.

0006-2960/82/0421-0102$01.25/0

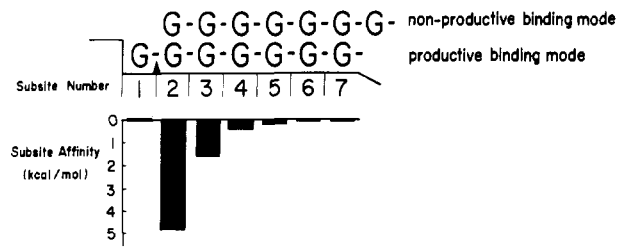

FIGURE 1: Subsite structure of glucoamylase and the two main binding modes of substrate maltodextrin. The histogram shows the subsite affinity of each subsite. The wedge indicates the position of the catalytic site of the enzyme at which the substrate glucosidic bond is cleaved. G indicates a glucose residue of a substrate. The nonreducing glucose residue is on the left side. The subsite affinities have been evaluated on the basis of Michaelis constants and the molecular activities of linear substrates of different degree of polymerization (Hiromi et al., 1973).

energy increase needed to bring the relevant glucose residue to a distorted form resembling the transition state. The high subsite affinity at the second subsite may help this situation (Hiromi, 1970, 1972; Hiromi et al., 1973; Phillips, 1966).

On the basis of this subsite structure, the binding of several kinds of ligands with the enzyme were studied statically and kinetically in terms of inhibition kinetics (Ohnishi et al., 1976), UV difference absorption spectrum (Ohnishi et al., 1975; Ohnishi & Hiromi, 1978), and fluorescence intensity change (Hiromi et al., 1974; Ohnishi et al., 1977).

Gluconolactone has a distorted half-chair conformation due to the partial double-bond character in the C_1–O_5 bond (Hackert & Jacobson, 1971) and is assumed to be a transition-state analogue for glucoamylase. Gluconolactone has been considered to bind to subsite 1 on the basis of information from inhibition kinetics and UV difference absorption spectrum studies as follows:

(1) Inhibition by gluconolactone of the hydrolysis of maltodextrin is of the mixed type (Dixon & Webb, 1979) whereas that by phenyl α-glucoside is purely competitive (Ohnishi et al., 1976). This difference in the inhibition type can well be explained by assuming that gluconolactone binds to subsite 1, inhibiting only the productive binding mode, and that phenyl α-glucoside predominantly binds to subsite 2 with its phenyl group on subsite 3, inhibiting both productive and nonproductive binding modes of a substrate (Ohnishi et al., 1976).

(2) Gluconolactone, like the substrates maltose and maltodextrin, produces a characteristic difference spectrum with a trough near 300 nm (Ohnishi et al., 1975). These substrates must occupy subsite 1 in their productive binding modes. On the contrary, the competitive inhibitor glucose does not produce the trough (Ohnishi et al., 1975).

In this paper, we set out to obtain detailed static information about the binding states of gluconolactone, glucose, methyl α-glucoside, and phenyl β-glucoside by monitoring the decrease in the enzyme fluorescence caused by their binding to the enzyme.

Experimental Procedures

Materials. Crystalline glucoamylase from *Rhizopus niveus* (pure grade) was purchased from Seikagaku Kogyo Co., Ltd., and used without further purification. The enzyme concentration was determined spectrophotometrically by using the absorption coefficient, $E^{1\%}_{280}$, of 16.3 cm^{-1} and a molecular weight of 58 000[2] (S. Takenishi and Y. Tsujisaka, personal communication). Glucose, gluconic acid 1,5-lactone (gluconolactone), methyl α-glucoside, and phenyl β-glucoside were of guaranteed grade from Nakarai Chemicals Co., Ltd. Gluconolactone was used within 10 min after its dissolution to minimize its hydrolysis into gluconic acid and conversion into gluconic acid 1,4-lactone (Shimahara & Takahashi, 1970). Hydrolysis of gluconolactone in 10 min was less than 2% of the initial concentration under the condition employed. For the completion of anomeric equilibrium, the glucose solution was kept at the experimental temperature for 5 h or more before use.

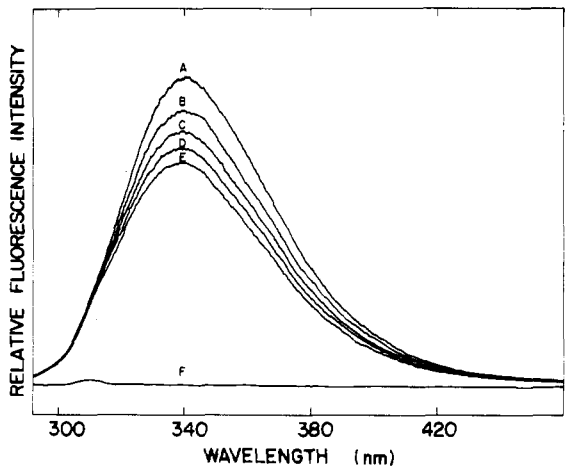

FIGURE 2: Fluorescence spectra of glucoamylase in the absence and presence of gluconolactone. λ_{ex} = 280 nm. $[Glucoamylase]_0$ = 0.9 (A–E) and 0 μM (F); $[gluconolactone]_0$ = 0 (A and F), 0.6 (B), 1.4 (C), 2.8 (D), and 5.0 mM (E); 0.02 M acetate buffer, pH 4.5, 10 °C.

Methods. The fluorescence spectra of the enzyme were measured with a Union Giken FS-401 fluorescence spectrophotometer. The excitation wavelength was fixed at 280 nm except for the system including phenyl β-glucoside. For this system, the excitation wavelength was fixed at 295 nm to avoid the possible effect arising from the absorption of phenyl β-glucoside at 280 nm. The fluorescence intensity decrease caused by the binding of ligands was observed at 340 nm as follows:

(1) Titration with Gluconolactone and Phenyl β-Glucoside. An appropriate volume of a concentrated ligand solution (2.5–10 μL) was added with a microsyringe to the enzyme solution (0.9 μM, 2.0 mL) in the quartz cell thermostated at 10 °C, and the fluorescence intensity was measured after each addition of the ligand solution. The observed fluorescence intensity was corrected for the dilution factor each time.

(2) Titration with Glucose and Methyl α-Glucoside. In this case, the enzyme–ligand mixture (25–100 μL) was added with a microsyringe or a micropipet to the enzyme solution (0.9 μM, 1.5 mL). Since the concentration of the enzyme was constant during the titration, no correction was made for the observed fluorescence intensity.

(3) Titration with Gluconolactone in the Presence of Glucose or Glucosides. The enzyme–glucose (or –glucoside) mixture (0.5 mL) was added to the enzyme solution (0.9 μM, 1.5 mL) with a pipet. After this, the gluconolactone solution was added, and the fluorescence intensity was measured in the same manner as described in (1).

All the fluorescence titrations were carried out in 0.02 M acetate buffer, pH 4.5, and at 10 °C.

Results and Discussion

Fluorometric Titration of Glucoamylase with Gluconolactone, Glucose, and the Glucosides. Figure 2 shows the

[2] This value was determined by the sedimentation equilibrium method using a Hitachi 282 analytical ultracentrifuge with an absorption scanner.

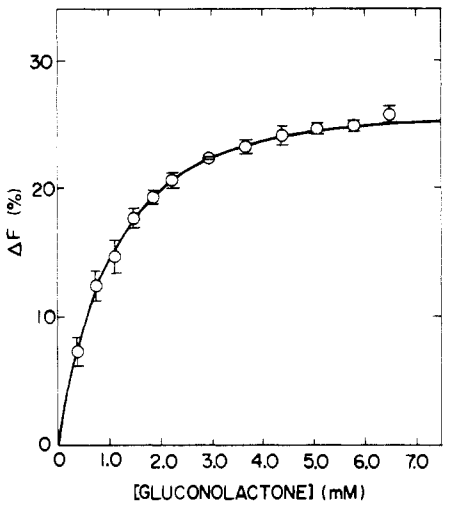

FIGURE 3: Dependence of the percentage of the fluorescence intensity decrease, ΔF, at 340 nm on the initial concentration of gluconolactone. $[\text{Glucoamylase}]_0 = 0.9\ \mu M$; pH 4.5, 10 °C, $\lambda_{ex} = 280$ nm. The solid line is a theoretical curve obtained according to eq 10 with $K_L = 1.1$ mM and $\Delta F_{max}{}^L = 30\%$.

fluorescence spectra of the enzyme with and without the transition-state analogue gluconolactone. It was found that the emission spectra of the enzyme have a peak around 340 nm at the excitation wavelength (λ_{ex}) of 280 nm, and its fluorescence intensity was decreased by the addition of gluconolactone. Figure 3 shows the dependence of the degree of fluorescence intensity decrease at 340 nm, ΔF, on the initial concentration of gluconolactone.

The value of the dissociation constant K_L of the enzyme–gluconolactone complex, EL, was determined by assuming a simple bimolecular binding equilibrium between the enzyme, E, and gluconolactone, L[3]:

$$E + L \rightleftarrows EL \tag{1}$$

$$K_L = \frac{[E][L]}{[EL]} \tag{2}$$

Under the conditions employed, the following equations are valid:

$$[E]_0 = [E] + [EL] \tag{3}$$

$$[L]_0 = [L] + [EL] \simeq [L] \tag{4}$$

where $[E]_0$ and $[L]_0$ are the initial concentrations of the enzyme and gluconolactone, respectively; [E], [L], and [EL] are the concentrations of free enzyme, free gluconolactone, and the enzyme–gluconolactone complex, respectively.

From eq 2, 3, and 4, we have

$$\frac{[EL]}{[E]_0} = \frac{[L]_0}{K_L + [L]_0} \tag{5}$$

The total fluorescence intensity F_t in the presence of gluconolactone is expressed as

$$F_t = f_E[E] + f_{EL}[EL] \tag{6}$$

[3] In the subsequent paper, (Tanaka et al., 1981), it will be suggested that gluconolactone can bind not only at subsite 1 but also transiently at subsite 2. Hence the possibility that two gluconolactone molecules bind with the enzyme cannot be excluded. However, since the dissociation constants for the two subsites are largely different (the probability of binding at subsite 2 is about one-sixteenth as large as that at subsite 1), at least as an approximation, it can be reasonably treated as a simple 1:1 binding as will be described here.

Table I: Dissociation Constant (K_d) and Percentage of Maximum Fluorescence Intensity Decrease (ΔF_{max}) of the Enzyme–Ligand Complexes (pH 4.5, 10 °C)

ligand	λ_{ex} (nm)	K_d (mM)	ΔF_{max} (%)
gluconolactone	280	1.08 ± 0.04	30.1 ± 0.3
gluconolactone	295	1.09 ± 0.09	32.0 ± 1.0
glucose	280	127 ± 9	20.0 ± 0.6
methyl α-glucoside	280	540 ± 60	24.1 ± 1.5
phenyl β-glucoside	295	10.9 ± 0.5	18.4 ± 0.3

where f_E and f_{EL} are the proportionality coefficients (proportional to the quantum yields) of the fluorescence intensity for the enzyme and the complex, respectively. From eq 3 and 6, we have

$$F_t = f_E[E]_0 + (f_{EL} - f_E)[EL] \tag{7}$$

ΔF is defined as the percentage of the fluorescence intensity decrease relative to the fluorescence intensity of the free enzyme, i.e.

$$\Delta F = \frac{f_E[E]_0 - F_t}{f_E[E]_0} \times 100(\%) \tag{8}$$

From eq 7 and 8

$$\Delta F = \left(\frac{f_E - f_{EL}}{f_E} \times 100\right)\frac{[EL]}{[E]_0} \equiv \Delta F_{max}{}^L\frac{[EL]}{[E]_0} \tag{9}$$

where $\Delta F_{max}{}^L$ is the percentage of fluorescence intensity decrease which would be observed when the enzyme is saturated with gluconolactone, as specified in eq 9. From eq 5 and 9, we have

$$\Delta F = \Delta F_{max}{}^L\left(\frac{[L]_0}{K_L + [L]_0}\right) \tag{10}$$

By the nonlinear least-squares method using Taylor expansion (Sakoda & Hiromi, 1976), the values of $\Delta F_{max}{}^L$ and K_L were determined to be 30.1 ± 0.3% and 1.08 ± 0.04 mM, respectively. The values are means ± the standard deviation. The solid line in Figure 3 shows the theoretical curve drawn with these values according to eq 10.

Glucose, methyl α-glucoside, and phenyl β-glucoside were also found to decrease the enzyme fluorescence, and the fluorometric titration of the enzyme with these ligands was performed in a manner similar to that described above. The percentage of maximum fluorescence intensity decreases, ΔF's, and the dissociation constants, K_d's, were evaluated by the same procedure as in the case of gluconolactone. The results are summarized in Table I.

Fluorometric Titration with Gluconolactone in the Presence of Glucose. Titration of the enzyme with gluconolactone was done in the presence of glucose. A typical plot of ΔF vs. $[L]_0$, in the presence of 100 mM glucose, is shown in Figure 4 (open circles). For interpretation of the results, the following two cases were considered.

(*Case 1*) *Competitive Type.* Gluconolactone (L) and glucose (G) are assumed to bind competitively to the same subsite, as shown schematically in Figure 5a, without formation of a ternary complex, EGL. In this case, there are three enzyme species, i.e., E, EG, and EL. As in the case of the enzyme and gluconolactone, the following equation is derived:

$$\Delta F = \frac{\Delta F_{max}{}^G[G]_0/K_G + \Delta F_{max}{}^L[L]_0/K_L}{1 + [G]_0/K_G + [L]_0/K_L} \tag{11}$$

where $[G]_0$ represents the initial concentration of glucose, $\Delta F_{max}{}^G$ the percentage of maximum fluorescence intensity

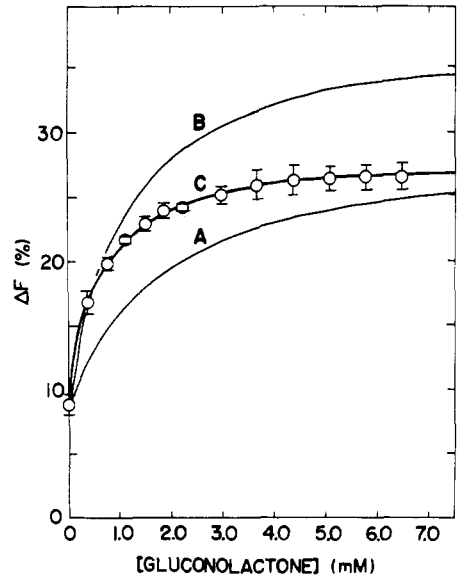

FIGURE 4: Dependence of ΔF on the initial concentration of gluconolactone in the presence of 100 mM glucose (open circles). [Glucoamylase]$_0$ = 0.9 μM; 0.02 M acetate buffer, pH 4.5, 10 °C, λ_{ex} = 280 nm. The solid curve A represents a theoretical curve for the competitive-type binding of gluconolactone and glucose according to eq 11. The solid curve B is obtained for the noncompetitive-type binding by assuming that $K_L' = K_L$ = 1.1 mM and $\Delta F_{max}^{GL} = \Delta F_{max}^{G} + \Delta F_{max}^{L}$ = 50% according to eq 12. The solid curve C is another theoretical curve for noncompetitive-type binding obtained from eq 12 using K_L' = 0.33 mM and ΔF_{max}^{GL} = 28%.

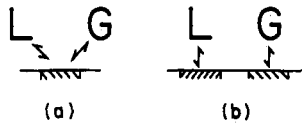

FIGURE 5: Schematic models for the binding of gluconolactone (L) and glucose (G) to the enzyme. The competitive model (a) and the noncompetitive model (b).

decrease which would be caused by glucose, and K_G the dissociation constant of the enzyme–glucose complex. Since all the values of the right-hand side of eq 11 are known, we can obtain the theoretical values of ΔF at given concentrations of gluconolactone and glucose.

(Case 2) Noncompetitive Type. Under the assumption that gluconolactone and glucose bind noncompetitively at different subsites, as shown in Figure 5b, to form a ternary complex EGL, there are four enzyme species: E, EG, EL, and EGL. In this case, ΔF is given as

$$\Delta F = \{\Delta F_{max}^{G}[G]_0/K_G + \Delta F_{max}^{L}[L]_0/K_L + \Delta F_{max}^{GL}[L]_0[G]_0/(K_G K_L')\}/\{1 + [G]_0/K_G + [L]_0/K_G + [L]_0[G]_0/(K_G K_L')\} \quad (12)$$

where K_L' is the dissociation constant of gluconolactone from the EGL complex, defined as $K_L' = [EG][L]_0/[EGL]$, and ΔF_{max}^{GL} is the percentage of maximum fluorescence intensity decrease that would be observed when the enzyme is saturated with both gluconolactone and glucose. in eq 12, there are two adjustable parameters, K_L' and ΔF_{max}^{GL}, which could be determined experimentally. The dissociation constant of glucose from the ternary enzyme–glucose–gluconolactone complex, K_G', can be calculated with the following equation by using the values of K_L, K_L', and K_G':

$$K_G' = K_G \frac{K_L'}{K_L} \quad (13)$$

The solid curve A in Figure 4 represents a theoretical curve drawn by assuming that gluconolactone and glucose bind competitively to the same subsite (case 1). Obviously curve A does not agree with the experimental results (open circles). Curve B is one of the theoretical curves for case 2 (the noncompetitive type), which is obtainable from eq 12 with two assumptions in relation to K_L' and ΔF_{max}^{GL}: (1) $K_L' = K_L$ and (2) $\Delta F_{max}^{GL} = \Delta F_{max}^{G} + \Delta F_{max}^{L}$. Curve B also deviates significantly from the experimental data.

Table II: Dissociation Constants of Gluconolactone (K_L') and Glucose (K_G') from the Ternary Enzyme–Gluconolactone–Glucose Complex and the Percentage of Maximum Fluorescence Intensity Decrease of the Ternary Complex (ΔF_{max}^{GL})[a]

$[G]_0$ (mM)	K_L' (mM)	K_G' (mM)	ΔF_{max}^{GL} (%)
100	0.33	39	28
200	0.67	79	29
250	0.48	56	30
av	0.49 ± 0.14	58 ± 20	29 ± 1

[a] ΔF_{max}^{GL} obtained by the fluorometric titration with gluconolactone in the presence of various concentrations of glucose (pH 4.5, 10 °C, λ_{ex} = 280 nm).

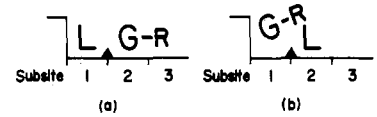

FIGURE 6: Schematic models of the binding states of gluconolactone (L) and glucoside (G-R, G is the glucose moiety and R is aglycon of methyl α-glucoside or phenyl β-glucoside). The noncompetitive type (a) and competitive type (b).

The experimental data are consistent with curve C, which is another theoretical curve for case 2, with the following modifications: (1) Glucose and gluconolactone bind to different subsites with some interaction, i.e., $K_L' \neq K_L$. (2) The maximum fluorescence decrease of the EGL complex is less than the sum of the fluorescence decreases of the EL complex and EG complex, i.e., $\Delta F_{max}^{GL} < \Delta F_{max}^{G} + \Delta F_{max}^{L}$. Curve C was drawn according to eq 12 with ΔF_{max}^{GL} = 28% (cf. $\Delta F_{max}^{G} + \Delta F_{max}^{L} \simeq$ 50%) and K_L' = 0.33 mM (cf. K_L = 1.1 mM). The values were determined by the least-squares method to obtain the best fit. The values of K_L' and ΔF_{max}^{GL} obtained when $[G]_0$ = 200 and 250 mM are also listed in Table II, together with the values of K_G' calculated according to eq 13. As seen from Table II, K_L', ΔF_{max}^{GL}, and K_G' are almost independent of the initial concentration of glucose, indicating that the experimental data can be explained satisfactorily by case 2. Thus it is quite reasonable to conclude that gluconolactone and glucose bind to two different subsites with such a positive interaction as to decrease the dissociation constants (K_G and K_L) to K_G' and K_L' respectively, of the enzyme–ligand complex.

Fluorometric Titration with Gluconolactone in the Presence of Glucosides: Effect of Aglycon on the Binding of Gluconolactone. The above-mentioned conclusion has supported the validity of the previous assumption that gluconolactone binds to subsite 1 and glucose predominantly binds to subsite 2 (Ohnishi et al., 1975). If this is the case, it may be expected that gluconolactone and glucosides, such as methyl α-glucoside and phenyl β-glucoside, will be able to bind to the enzyme simultaneously to form a ternary enzyme–gluconolactone–glucoside complex as in the case of gluconolactone and glucose mentioned above. In this case, the glucose moiety of the glucoside most probably binds to subsite 2 with its aglycon on subsite 3 (Ohnishi et al., 1976) as shown in Figure 6a (the noncompetitive model). On the contrary, if the glucose moiety of the glucoside binds to subsite 1 with its aglycon on subsite 2, as shown in Figure 6b, these two ligands cannot bind to the

Table III: Dissociation Constants of Gluconolactone (K_L') and the Glucosides (K_G') from the Ternary Enzyme–Gluconolactone–Glucoside Complex and the Percentage of Maximum Fluorescence Intensity Decrease of the Ternary Complex (ΔF_{max}^{GL})[a]

glucoside	λ_{ex} (nm)	K_L' (mM)	K_G' (mM)	ΔF_{max}^{GL} (%)
methyl α-glucoside	280	0.30	150	31
phenyl β-glucoside	295	0.41	4.1	28

[a] ΔF_{max}^{GL} obtained by the fluorometric titration with gluconolactone in the presence of methyl α-glucoside and phenyl β-glucoside (pH 4.5, 10 °C).

enzyme simultaneously, since the aglycon of the glucoside would compete with gluconolactone for subsite 2 (the competitive model). To discriminate between these two models, we carried out fluorometric titrations with gluconolactone in the presence of methyl α-glucoside or phenyl β-glucoside. Parts a and b of Figure 7 show the dependence of ΔF on the initial concentration of gluconolactone in the presence of methyl α-glucoside and phenyl β-glucoside, respectively (open circles). The solid curve B's in Figure 7 are the theoretical curves for the competitive model, which would be expected when there is competition for the binding between gluconolactone and the glucosides. Evidently the experimental results are not consistent with the competitive model. Next, on the basis of the noncompetitive model, the dissociation constant of gluconolactone, K_L', and of glucoside, K_G', from the ternary enzyme–gluconolactone–glucoside complex, and the percentage of maximum fluorescence intensity decrease of the complex, ΔF_{max}^{GL}, were determined to obtain the best fit with the least-squares method in the same way as in the case of gluconolactone and glucose. The values obtained are summarized in Table III. Curve A's in Figure 7 show the theoretical curves obtained by using the values listed in Table III. The curves are in good agreement with the experimental data, indicating that the noncompetitive model is adequate for the enzyme–gluconolactone–glucoside system. We can conclude, therefore, that gluconolactone binds to subsite 1 and the glucosides bind to subsite 2 with their aglycons on subsite 3.

From Tables II and III, the following points are noted concerning the percentage of maximum fluorescence intensity decrease ΔF_{max} of each complex. First, ΔF_{max} of the enzyme–gluconolactone (EL) complex (about 30% both for λ_{ex} = 280 and 295 nm) is greater than that of any other binary complex (about 20%). Second, ΔF_{max} of the EGL complex (G is either glucose or glucoside) is almost equal to that of the EL complex, which means that no additional fluorescence intensity decrease is caused by the binding of glucose or glucosides to the binary EL complex. It can be speculated from these findings that the fluorescence intensity decrease by the binding of gluconolactone, glucose, or the glucosides is attributable to a single tryptophan residue located at the subsite where gluconolactone binds, namely, subsite 1 (Ohnishi et al., 1975, 1976). The tryptophan residue is most likely one of the two tryptophan residues which have been considered to be located at the active-site region (Ohnishi & Hiromi, 1976).

Moreover, from Tables II and III, it should be noted that K_L' is considerably smaller than K_L for each case. This indicates that glucose and the glucosides stabilize the enzyme–gluconolactone complex. In other words, subsites 1 and 2 show positive cooperativity in reducing the dissociation constants for ligand binding.

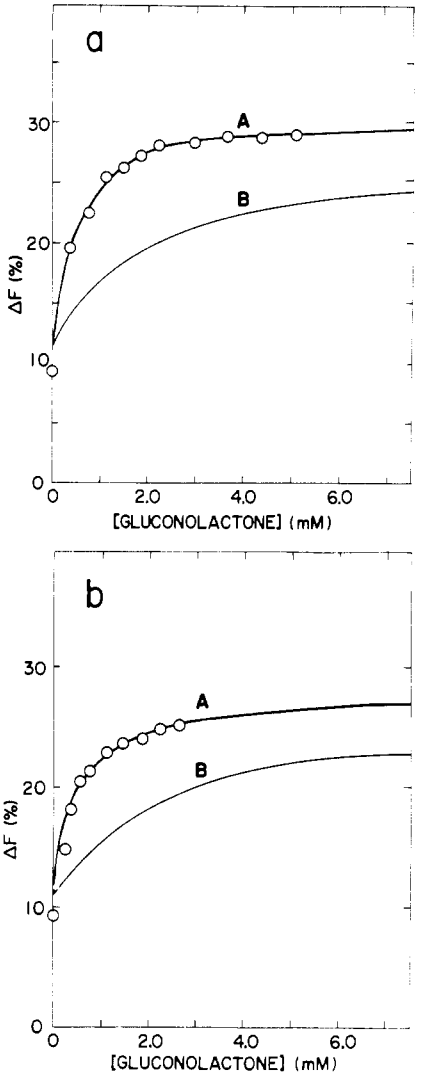

FIGURE 7: Fluorometric titrations with gluconolactone in the presence of methyl α-glucoside (500 mM) (a) and phenyl β-glucoside (18 mM) (b). $[Glucoamylase]_0$ = 0.9 μM; pH 4.5, 10 °C. λ_{ex} = 280 (a) and 295 nm (b). The solid curve A's represent theoretical curves based on the noncompetitive model (Figure 6a) using the parameters obtained from the least-squares method. The solid curve B's represent theoretical curves from the competitive model (Figure 6b).

References

Dixon, M., & Webb, E. C. (1979) *Enzymes*, 3rd ed., pp 332–381, Longman, London.

Hackert, M. L., & Jacobson, R. A. (1971) *Acta Crystallogr., Sect. B B27*, 203–209.

Hiromi, K. (1970) *Biochem. Biophys. Res. Commun. 40*, 1–6.

Hiromi, K. (1972) in *Proteins: Structure and Function* (Funatsu, M., Hiromi, K., Imahori, K., Murachi, T., & Narita, K., Eds.) Vol. 2, pp 1–46, Kodansha, Ltd., Halsted Press, Tokyo, New York.

Hiromi, K., Nitta, Y., Numata, C., & Ono, S. (1973) *Biochim. Biophys. Acta 302*, 362–375.

Hiromi, K., Ohnishi, M., & Yamashita, T. (1974) *J. Biochem. (Tokyo) 76*, 1365–1367.

Ohnishi, M., & Hiromi, K. (1976) *J. Biochem. (Tokyo) 79*, 11–16.

Ohnishi, M., & Hiromi, K. (1978) *Carbohydr. Res. 61*, 335–344.

Ohnishi, M., Kegai, H., & Hiromi, K. (1975) *J. Biochem. (Tokyo) 77*, 695–703.

Ohnishi, M., Yamashita, T., & Hiromi, K. (1976) *J. Biochem.*

(Tokyo) 79, 1007–1012.
Ohnishi, M., Yamashita, T., & Hiromi, K. (1977) J. Biochem. (Tokyo) 81, 99–105.
Phillips, D. C. (1966) Sci. Am. 215 (Nov), 78.
Sakoda, M., & Hiromi, K. (1976) J. Biochem. (Tokyo) 80, 547–555.
Shimahara, K., & Takahashi, T. (1970) Biochim. Biophys. Acta 201, 410–415.
Tanaka, A., Ohnishi, M., & Hiromi, K. (1981) Biochemistry (following paper in this issue).

Stopped-Flow Kinetic Studies on the Binding of Gluconolactone and Maltose to Glucoamylase†

Akiyoshi Tanaka, Masatake Ohnishi, and Keitaro Hiromi*

ABSTRACT: The binding of the transition-state analogue gluconolactone and a substrate maltose to *Rhizopus niveus* glucoamylase was studied kinetically by using the stopped-flow method by monitoring the decrease in the enzyme fluorescence, in relation to the subsite structure of the enzyme [Hiromi, K. (1970) *Biochem. Biophys. Res. Commun. 40*, 1–6]. The binding kinetics of both ligands (represented by L) agreed well with a two-step mechanism which consists of a fast bimolecular process followed by a slow unimolecular process:

$$E + L \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} EL \underset{k_{-2}}{\overset{k_{+2}}{\rightleftharpoons}} EL^*$$

where EL and EL* are the enzyme–ligand complexes loosely bound and tightly bound, respectively. In each case, the observed fluorescence intensity decrease of the enzyme protein was confirmed to be accompanied solely with the slow unimolecular process. A competitive inhibitor glucose, which is considered to bind predominantly to subsite 2 (the second subsite counted from the terminal subsite to which the nonreducing-end glucose residue of substrate is bound in a productive mode), inhibits only the fast bimolecular process for the maltose binding. On the other hand, for the gluconolactone binding, glucose inhibits the fast bimolecular process and the k_{-2} step, but not the k_{+2} step, in the unimolecular process. These results are most reasonably accounted for by the following mechanism: In the fast bimolecular process of the binding, gluconolactone transiently binds to subsite 2 and maltose to subsites 2 and 3, and in the unimolecular process, they relocate to subsite 1 and subsites 1 and 2, respectively, accompanied by the decrease in fluorescence intensity. The fluorescence change may be caused by the microenvironmental change of a tryptophan residue located at subsite 1.

Glucoamylase[1] hydrolyzes the nonreducing-end glucosidic bond of starch. To this enzyme the subsite theory was successfully applied quantitatively (Hiromi, 1970; Hiromi et al., 1973).

The fluorescence intensity of the enzyme protein is decreased by the binding of substrates or analogues, and the binding of ligands with the enzyme was investigated by the static and the kinetic method with the decrease in the enzyme fluorescence as a probe (Hiromi et al., 1974; Ohnishi et al., 1977).

In the preceding paper (Hiromi et al., 1981), we investigated the binding states of gluconic acid 1,5-lactone (gluconolactone) and glucose by the fluorometric titration at equilibrium. It would be reasonably concluded that gluconolactone binds to subsite 1 of the enzyme (the terminal subsite at which the nonreducing-end glucose residue of substrate is situated in the productive binding mode) and that glucose predominantly binds to subsite 2.

The static method, however, cannot provide any information about elementary processes of the ligand binding. To obtain such mechanistic information, it is necessary to use the transient kinetics with fast reaction techniques. In this paper, we aimed to elucidate kinetically the elementary processes in the binding of two ligands, a transition state analogue gluconolactone and a substrate maltose, to the enzyme by using the fluorescence stopped-flow method.

Experimental Procedures

Materials. Glucoamylase, gluconic acid 1,5-lactone (simply referred to as gluconolactone), and glucose were the same as those described in the preceding paper (Hiromi et al., 1981). Maltose was purchased from Nakarai Chemicals Co., Ltd., and was confirmed to be pure by the paper chromatographic method (Robyt & French, 1963). Gluconolactone was used within 5 min after its dissolution to minimize its hydrolysis into gluconic acid and conversion into gluconic acid 1,4-lactone (Shimahara & Takahashi, 1970). Maltose and glucose solutions were kept at the experimental temperature for 5 h or more before use to complete anomeric equilibration.

Methods. Binding kinetics of ligands to the enzyme was studied by monitoring the fluorescence intensity decrease of the enzyme protein by the stopped-flow method. The time course of the binding reactions was observed with a gas pressure driven type stopped-flow apparatus (Union Giken RA-401) (Hiromi, 1979) with a 200-W D_2 lamp as the light source. The change in the fluorescence intensity (excited at 280 nm throughout the experiment) was observed through a cutoff filter (Toshiba Kasei Kogyo, UV-31; 50% transmittance at 310 nm) from the right angle to the incident beam. With the quartz cell used (2-mm-inner diameter), the dead time of the apparatus was determined to be 0.9 ms under the operating condition (6 kg/cm^2 nitrogen gas pressure for driving) according to the method with ascorbic acid and 2,6-dichloro-

† From the Department of Food Science and Technology, Faculty of Agriculture, Kyoto University, Kyoto 606, Japan. *Received April 28, 1981.* This work was supported in part by a grant-in-aid for Scientific Research from the Ministry of Education, Science and Culture of Japan (No. 447094).

[1] Abbreviations used: E or glucoamylase, 1,4-α-D-glucan glucohydrolase from *Rhizopus niveus* (EC 3.2.1.3); L or gluconolactone, gluconic acid 1,5-lactone; G, glucose; M, maltose.

0006-2960/82/0421-0107$01.25/0