Static and Kinetic Studies by Fluorometry on the Interaction between Gluconolactone and Glucoamylase\textsuperscript{1} from \textit{Rh. niveus}\textsuperscript{2}

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A transition-state analog, gluconolactone, was found to partially quench the protein fluorescence of glucoamylase [EC 3.2.1.3] from \textit{Rhizopus niveus}. The interaction between gluconolactone and the enzyme was studied statically and kinetically at pH 4.5 in terms of fluorescence change. The dissociation constant $K_d$ of the enzyme-analog complex determined by fluorometric titration at 25° ($K_d = 1.6$ mm) was in good agreement with that obtained by difference spectrophotometric titration (Ohnishi, M. \textit{et al.} (1975) \textit{J. Biochem.} 77, 695–703) and with the inhibitor constant determined for the hydrolysis of maltodextrin (Ohnishi, M. \textit{et al.} (1976) \textit{J. Biochem.} 79, 1007–1012).

The kinetics of the interaction were studied by the fluorescence stopped-flow method. The dependence of the apparent first-order rate constant, $k_{pp}$, on gluconolactone concentration showed a saturation curve, consistent with a two-step mechanism involving a rapid bimolecular association followed by a slow unimolecular isomerization process. The dissociation constant, $K_t$, for the rapid bimolecular process and the forward and backward rate constants for the isomerization were obtained at 25° and 5°, and the activation parameters were evaluated.

It was found that the isomerization process, but not the bimolecular association, is accompanied by fluorescence intensity change, indicating that the former process involves a micro-environmental change of a tryptophan residue(s) of the enzyme.

Maltose was found to decrease the rate of interaction of gluconolactone with the enzyme by competing with the analog for the active site.

\textit{Rhizopus} glucoamylase is the first enzyme to which the subsite theory was successfully applied to determine the subsite affinities from the dependence of the rate parameters on the degree of polymerization of linear substrates (1–3). Knowledge of the subsite structure of the enzyme permits us to...
predict the binding mode of substrates and analogs. Gluconolactone has been considered to be a transition-state analog for lysozyme-catalyzed reactions, because of its distorted half-chair conformation \( (4, 5) \). It is likely that this analog also acts as a transition-state analog for glucoamylase. If this is the case, it will bind at the first subsite adjacent to the catalytic site. Evidence for this prediction has been obtained from two independent approaches: first, gluconolactone was found to show a characteristic trough at around 300 nm in the difference spectrum upon binding with glucoamylase \( (6) \). The trough has been observed with the substrates maltose \( (6) \) and maltodextrin (Ohnishi, M. et al. in preparation), which certainly occupy the first subsite in their productive binding mode, whereas it was not observed with a competitive inhibitor, glucose.\(^6\) Second, in contrast with glucose, gluconolactone shows a mixed-type inhibition of the hydrolysis of maltodextrin with an inhibitor constant \( K_i = 1.5 \) mM \( (8) \).

Maltodextrin has been found to decrease the protein fluorescence intensity upon binding with the enzyme, and this property was used for a kinetic study of the interaction between the substrate and the enzyme \( (9) \).

Prior to systematic studies of the transient kinetics with a variety of substrates, static and kinetic investigations were undertaken, as described in this paper, to elucidate the mechanism of the interaction between gluconolactone and the enzyme, using the protein fluorescence as a probe.

**EXPERIMENTAL**

*MATERIALS*—Glucoamylase from *Rhizopus niveus*, maltose and gluconolactone were the same materials as those described in the previous paper \( (6) \). Gluconolactone solution was used within 15 min after dissolving to minimize its hydrolysis \( (8) \) and conversion to glucono-1,4-lactone \( (10) \). The fraction lost in 15 min was less than 4%.

*METHODS*—Static measurements of fluorescence spectra and titration of the enzyme with gluconolactone were carried out with a spectrofluorometer (Union Giken) at 25°, the excitation wavelength being fixed at 280 nm.

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\(^6\) Glucose is considered to bind at the second subsite of the enzyme with an inhibitor constant, \( K_i \), of 0.1 mM \( (7) \).

Kinetic studies of the interaction between the enzyme and gluconolactone was carried out using a fluorescence stopped-flow apparatus (Union Giken SF-70) \( (9, 11) \) at 25°, 15°, and 5°. The excitation beam at 280 nm was obtained using a 200W D\(_2\) lamp and a monochromator, and the fluorescence was observed at right angles through a cut-off filter with a half-transmission wavelength of 296 nm. The dead time of the apparatus with a 2 mm cylindrical cell was about 1 msec under the operating conditions. To study the effect of maltose on the binding of gluconolactone, a solution containing maltose and gluconolactone was mixed with enzyme solution in the apparatus.

All the experiments were conducted at pH 4.5 in 0.02 M acetate buffer.

**RESULTS AND DISCUSSION**

*Fluorometric Titration of the Enzyme with Gluconolactone*—The protein fluorescence of the enzyme, with a peak around 340 nm, was found to be partially quenched upon the addition of gluconolactone, as shown in Fig. 1.

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Figure 2 shows the dependence of the decrement of fluorescence intensity at 340 nm, $\Delta F$, on the gluconolactone concentration. The hyperbolic dependence of the plot indicates the equilibrium:

$$ E + I \rightleftharpoons EI $$

(1)

where $E$, $I$, and $EI$ represent the enzyme, the analog, and the enzyme-analog complex, respectively. Under the conditions employed, the total concentration of the enzyme, $[E]_0$, is negligible in comparison with the analog concentration $[I]$, so that we can write

$$ [EI] = \frac{[E]_0[I]}{K_d + [I]} $$

(2)

where $K_d$ is the dissociation constant of $EI$. Since $\Delta F$ is proportional to $[EI]$, Eq. 2 can be rewritten in a linear form:

$$ [I]/\Delta F = K_d/\Delta F_{\text{max}} + [I]/\Delta F_{\text{max}} $$

(3)

where $\Delta F_{\text{max}}$ is the maximum fluorescence decrement observed when the enzyme is saturated by gluconolactone. The validity of Eq. 3 was confirmed by the linearity of the plot between $[I]/\Delta F$ and $[I]$ (Fig. 3), from which the values of $K_d$ and $\Delta F_{\text{max}}$ can be obtained. The $K_d$ value was determined to be 1.6 mM using the least-squares method. This value is in good agreement with those obtained by difference spectrophotometric titration ($K_d = 1.5$ mM) (8) and from an inhibitory study ($K_i = 1.5$ mM) (8), indicating that these three phenomena have the same origin. The binding of gluconolactone to the enzyme probably involves some change in the microenvironment of a tryptophan residue, which is considered to be situated at the first subsite (6, 8).

The data included in Figs. 1–3 show that the ratio of the maximum fluorescence decrement, $\Delta F_{\text{max}}$, to the fluorescence intensity of the free enzyme, $F_E$, i.e., $\Delta F_{\text{max}}/F_E$, amounts to 0.28.

Kinetics of the Interaction—Figure 4 shows a typical example of the stopped-flow traces of the fluorescence change due to the interaction between the enzyme and the analog. A single relaxation was observed, obeying first-order kinetics. The dependence of the apparent first-order rate constant, $k_{\text{app}}$, obtained from a Guggenheim plot (12) on gluconolactone concentration is shown in Figs. 5 and 6 at 25° and 5°, respectively.

The hyperbolic form of the plots is consistent with a two-step mechanism consisting of a rapid bimolecular association followed by a slow unimolecular isomerization process (13):

$$ E + I \rightleftharpoons EI \rightleftharpoons EI^* $$

(4)

where EI and EI* are conformational isomers of the enzyme-gluconolactone complex. The apparent rate constant, $k_{\text{app}}$, is considered to be the...
Fig. 4. An example of the time course of the decrease in enzyme fluorescence intensity upon binding of gluconolactone. [Glucoamylase] = 6.9 μM. [Gluconolactone] = 5.0 mM. 0.02 M acetate buffer, pH 4.5, 15.0°. Vertical scale: 0.02 volt per major division. Horizontal scale: 5 msec per major division (SF-803, 33B).

Fig. 5. Dependency of $k_{app}$ upon the concentration of gluconolactone at 25°. [Glucoamylase] = 6.9 μM. 0.02 M acetate buffer, pH 4.5. The solid line represents a theoretical curve according to Eq. 5 with $k_3 = 900$ sec$^{-1}$, $k_4 = 60$ sec$^{-1}$, and $K_1 = 8$ mM.

$\frac{1}{\tau_2} = k_4 + \frac{k_3}{K_1 + [I]}$ (5)

where $K_1 = k_2/k_1$, the dissociation constant of EI, and the approximation $[I] \gg [E]_0$ is employed.

Fig. 6. Dependency of $k_{app}$ upon the concentration of gluconolactone at 5°. [Glucoamylase] = 6.9 μM. 0.02 M acetate buffer, pH 4.5. The solid line represents a theoretical curve according to Eq. 5 with $k_3 = 150$ sec$^{-1}$, $k_4 = 10$ sec$^{-1}$, and $K_1 = 7.2$ mM.

TABLE I. Values of kinetic parameters for the interaction between glucoamylase and gluconolactone at pH 4.5.

<table>
<thead>
<tr>
<th>Temperature (°)</th>
<th>$K_1$ (m)</th>
<th>$k_3$ (sec$^{-1}$)</th>
<th>$k_4$ (sec$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>25</td>
<td>$8 \times 10^{-3}$</td>
<td>900</td>
<td>60</td>
</tr>
<tr>
<td>5</td>
<td>$7 \times 10^{-5}$</td>
<td>150</td>
<td>10</td>
</tr>
</tbody>
</table>

The values of $K_1$, $k_3$, and $k_4$ obtained from Figs. 5 and 6 are summarized in Table I. The temperature dependences of $k_3$ and $k_4$ permit us to evaluate the activation parameters at 25° as follows: Δ$G^\dagger_3 = 13$ kcal/mole, Δ$H^\dagger_3 = 14$ kcal/mole, Δ$S^\dagger_3 = 2$ e.u., and Δ$G^\dagger_4 = 15$ kcal/mole, Δ$H^\dagger_4 = 14$ kcal/mole, Δ$S^\dagger_4 = -3$ e.u., where Δ$G^\dagger$, Δ$H^\dagger$, and Δ$S^\dagger$ are the free energy, enthalpy, and entropy of activation, respectively, and the subscripts 3 and 4 refer to the $k_3$ and $k_4$ processes, respectively.

From the $K_1$ values in Table I, the standard free energy change, Δ$G$, the standard enthalpy change, Δ$H$, and the standard entropy change, Δ$S$, of the overall reaction can be calculated. The overall dissociation constant, $K_d$, obtained statically is related to $K_1$ and $k_3/k_4$ by the following equation:

$K_d = K_1/(1 + k_3/k_4)$ (6)

The $K_d$ value at 25° calculated from Eq. 6 (0.5 mM) is somewhat smaller than that obtained by titration (1.6 mM). The discrepancy, however, will not be discussed here because of possible error involved in the evaluation of the rate parameters, although the possibility could not be excluded that an additional step may be included in the mechanism shown in Eq. 4.
GLUCOAMYLASE-GLUCONOLACTONE INTERACTION

TABLE II. Comparison between the fractional fluorescence intensity changes observed by the static method and the stopped-flow method at 25°.

<table>
<thead>
<tr>
<th>Gluconolactone (mm)</th>
<th>((\Delta F/F_E)_{\text{static}})</th>
<th>((\Delta F/F_E)_{\text{SF}})</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0</td>
<td>0.10</td>
<td>0.12</td>
</tr>
<tr>
<td>2.0</td>
<td>0.15</td>
<td>0.15</td>
</tr>
<tr>
<td>3.0</td>
<td>0.19</td>
<td>0.19</td>
</tr>
<tr>
<td>5.0</td>
<td>0.24</td>
<td>0.24</td>
</tr>
</tbody>
</table>

\(a\) Corrected for the dead time according to Eq. 7.

change, \(\Delta H\), and the standard entropy change, \(\Delta S\), for the less specific binding \(E + I \rightleftharpoons EI\) can be evaluated as follows: \(\Delta G = -2.9\) kcal/mole, \(\Delta H = -3\) kcal/mole, and \(\Delta S = 6\) e.u.

It is worthwhile to compare the statically observed fluorescence change, \(\Delta F/F_E\), with that observed by the stopped-flow method. Let \(\Delta F_{\text{obs}}\) be the fluorescence change actually observed by the stopped-flow apparatus with dead time \(t_d\), and \(\Delta F\) be the change which should be observed when \(t_d = 0\). The value of \(\Delta G\) can be obtained from the equation:

\[
\ln(\Delta F/F_{\text{obs}}) = \frac{\Delta G}{R} t_d
\]

The fluorescence intensity of the free enzyme, \(F_E\), can be obtained with the stopped-flow apparatus by mixing the enzyme solution with the buffer instead of gluconolactone solution. Values of \((\Delta F/F_E)_{\text{static}}\) and \((\Delta F/F_E)_{\text{SF}}\) are compared in Table II, where SF refers to values obtained by the stopped-flow method. The excellent agreement between the two ratios indicates that all the fluorescence change occurs in the slower isomerization process in Eq. 4. The unimolecular process is considered to involve some environmental change of tryptophan, probably of the residue located in the first subsite of the enzyme (6, 8).

The Effect of Maltose on the Rate of Interaction—In preliminary experiments, the interaction between the enzyme and substrate maltose was also accompanied by a decrease in the protein fluorescence, but the rate at 25° was too large to be measured using the stopped-flow apparatus. When maltose was added to the gluconolactone-enzyme system, it was found that the rate of interaction of the analog with the enzyme was appreciably decreased. The results are summarized in Table III, which shows the effect of maltose concentration upon the apparent rate constant, \(k_{\text{app}}\), at a gluconolactone concentration of 2 mm. If we assume that maltose competes for the enzyme active site with gluconolactone, and that rapid equilibria are established for the maltose binding and the bimolecular association step of gluconolactone in Eq. 4, the apparent rate constant, \(k_{\text{app}}\), in the presence of maltose can be expressed as follows (see "APPENDIX"):

\[
k_{\text{app}} = k_4 + \frac{k_3[I]}{K_M + [I]}
\]

where \(K_M\) is the dissociation constant of the enzyme-maltose complex ES and [S] is the maltose concentration. If we use the value of \(K_M\) obtained by spectrophotometric titration, \(K_M = 1.2\) mm (6), we can calculate the \(k_{\text{app}}\) value according to Eq. 8. The values thus obtained are included in the last column of Table III. It is apparent that the observed effect of maltose in retarding the gluconolactone interaction is more than would be predicted from Eq. 8. The value of \(k_{\text{app}}\) (obs.) (22 sec\(^{-1}\)) at [S] = 6 mm, even lower than \(k_4\) (= 60 sec\(^{-1}\)), clearly indicates over-simplification of the mechanism (cf. Eqs. A-1 and A-2 in "APPENDIX"), in which no ternary complex ESI is considered. Presumably maltose can also bind with EI complex and inhibits the \(k_4\) process through which gluconolactone can dissociate.

\(a\) Calculated from Eq. 8 using the values; \(k_4 = 8\) mm, \(k_3 = 900\) sec\(^{-1}\), \(k_4 = 60\) sec\(^{-1}\), \(K_M = 1.2\) mm.

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REFERENCES


APPENDIX

Relaxation time for glucoamylase-gluconolactone interaction in the presence of maltose—The derivation of the equation for the relaxation time (or the apparent rate constant, $k_{app}$) for the glucoamylase-gluconolactone interaction in the presence of maltose will be given here.

The scheme assumed is as follows:

$$E + I \underset{k_1}{\overset{K_1}{\rightleftharpoons}} EI \underset{k_2}{\overset{E}{\rightarrow}} EI^*$$

where $S$ represents maltose, $K_M$ is the dissociation constant of $ES$, the maltose-enzyme complex, and the other symbols are the same as those used in the text. As mentioned in the text, the binding of maltose is too fast to be measured by the stopped-flow method, and the pre-equilibrium $E + I \rightarrow EI$ is also sufficiently fast compared with the isomerization process $ES \rightleftharpoons ES^*$, so that these two processes can be regarded as rapid equilibria whose time course may be omitted from the rate equation.

Let $c_i$ and $\dot{c}_i$ be the concentrations of species $i$ at time $t$ and at equilibrium, respectively, and then let $\Delta c_i$ be $\dot{c}_i - c_i$. Then material balance requires:

$$\Delta c_I + \Delta c_{EI} + \Delta c_{EI^*} + \Delta c_{ES} = 0$$
$$\Delta c_I + \Delta c_{EI} + \Delta c_{EI^*} = 0$$
$$\Delta c_E + \Delta c_{ES} = 0$$

For the two rapid equilibria, the dissociation constants, $K_1$ and $K_M$, are defined as:

$$K_1 \dot{c}_{EI} = \dot{c}_E \dot{c}_I$$
$$K_M \dot{c}_{ES} = \dot{c}_E \dot{c}_S$$

By differentiating with respect to time, we have:

$$K_1 \dot{c}_{EI} = \dot{c}_E \dot{c}_I + \dot{c}_E \dot{c}_E$$
$$K_M \dot{c}_{ES} = \dot{c}_E \dot{c}_S + \dot{c}_E \dot{c}_S$$

or

$$K_1 \Delta c_{EI} = \dot{c}_E \Delta c_{EI} + \dot{c}_E \Delta c_I$$
$$K_M \Delta c_{ES} = \dot{c}_E \Delta c_{EI} + \dot{c}_E \Delta c_S$$

where the $\Delta$ symbols indicate concentration change in a small time interval.

The rate equation for $EI^*$ is as follows:

$$\frac{d c_{EI^*}}{dt} = k_3 c_{EI} - k_4 c_{EI^*}$$

which can be rewritten as

$$\frac{d \Delta c_{EI^*}}{dt} = -k_3 \Delta c_{EI} + k_4 \Delta c_{EI^*}$$

since at equilibrium $k_3 \dot{c}_{EI} = k_4 \dot{c}_{EI^*}$.

By using Eqs. A-3 through A-5, A-8, and A-9, $\Delta c_{EI}$ can be expressed in terms of $\Delta c_{EI^*}$ by eliminating four other variables, $\Delta c_i$'s, and substituted into Eq. A-11. Thus we have:

$$\frac{\Delta c_{EI}}{\Delta c_{EI^*}} \equiv \alpha = \frac{\dot{c}_I + \dot{c}_E \left(1 + \frac{\dot{c}_S}{K_M + \dot{c}_E}\right)}{\dot{c}_I + (K_1 + \dot{c}_E) \left(1 + \frac{\dot{c}_S}{K_M + \dot{c}_E}\right)}$$

and the reciprocal relaxation time, $1/\tau$ or $k_{app}$, is given by:

$$1/\tau = k_{app} = \alpha k_3 + k_4$$

where $\alpha$ is defined by Eq. A-12.

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When $\tilde{c}_M \ll K_1$, $K_M$ (as is usually the case), and $\tilde{c}_E/\tilde{c}_1 \ll \tilde{c}_S/K_M$ (which is also usually valid), Eq. A-12 reduces to:

$$a = \frac{\tilde{c}_1}{(1 + \tilde{c}_S/K_M) + \tilde{c}_1} \quad (A-14)$$

Thus we have

$$1/\tau = k_{\text{app}} = k_4 + \frac{k_2 \tilde{c}_1}{K_1 (1 + \tilde{c}_S/K_M) + \tilde{c}_1} \quad (A-15)$$

which is identical with Eq. 8 in the text, since $[I]=\tilde{c}_1$ and $[S]=\tilde{c}_S$.

The above treatment is also valid even when the substrate binding involves a two-step mechanism,

$$E + S \rightleftharpoons ES \rightleftharpoons ES^*,$$

insofar as these steps can be regarded rapid equilibria. In this case, $K_M$ should be replaced by $K'_M (= K_M/(1 + k_3/k_4))$, where $k_3$ and $k_4$ are the rate constants for the forward and backward processes in $ES \rightleftharpoons ES^*$.