Characteristics of the Inhibition of Potato (Solanum tuberosum) Invertase by an Endogenous Proteinaceous Inhibitor in Potatoes

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ABSTRACT

Effect of several parameters on inhibition of potato (Solanum tuberosum) invertase by its endogenous proteinaceous inhibitor was determined using homogeneous preparations of both proteins. The inhibitor and invertase formed an inactive complex with an observed association rate constant at pH 4.70 and 37°C of $8.82 \times 10^3$ per molar per second and a dissociation rate constant of $3.3 \times 10^{-6}$ per minute. The inhibitor appeared to bind to invertase in more than one step. Initial interaction (measured by loss of invertase activity) was rapid, relatively weak, readily reversible (K of $2 \times 10^{-4}$ molar) and noncompetitive with substrate at pH 4.70. Initial interaction was probably followed by isomerization to a tighter (K of $6.23 \times 10^{-4}$ molar) complex, which dissociated slowly with a half-time of 3.5 hour. Interaction between enzyme and inhibitor appeared to be of ionic character and essentially pH independent between pH 3.5 and 7.4.

We have investigated further the complex formation of potato invertase with its endogenous inhibitor using homogeneous preparations of both proteins. We determined the effect of sucrose concentration, ionic strength, pH, and dielectric constant of the buffer on the inhibition (or binding) rate of enzyme by inhibitor. Experiments involving enzyme, inhibitor, and sucrose together in the reaction from zero time are referred to as inhibition experiments. Experiments in which enzyme and inhibitor were incubated together prior to removal of an aliquot into sucrose are referred to as binding experiments. Evidence is presented to support a minimum two-step binding process.

MATERIALS AND METHODS

Homogeneous potato (Solanum tuberosum) invertase and invertase inhibitor, prepared as described previously (4), were used in all inhibition and binding experiments. All other chemicals were of reagent grade. The water was doubly deionized.

Invertase Activity

Invertase activity was determined by the Somogyi method as modified by Nelson (13) and as described previously by Bracho and Whitaker (4). In rate or equilibrium studies involving invertase and inhibitor combinations, aliquots of the reaction were removed into 0.30 mL of 143 mM sucrose in 80 mM acetate buffer (pH 4.70 and 37.0°C), the total reaction volume was made to 0.50 mL, and the amount of invertase activity left was determined. The difference between the velocity of the reaction at zero time and that at any time $t$ measured the amount of invertase complexed with inhibitor.

The initial velocity ($v_0$) was determined from the initial slope of a plot of reducing sugars formed versus time of reaction. Initial velocity determinations were based on a minimum of samples taken at nine time intervals. Controls were run in all cases in which invertase alone was incubated for the desired times to measure its stability.

Effect of pH on Initial Rate of Binding of Inhibitor with Invertase

The optimal pH for binding of potato invertase to potato invertase inhibitor was determined by measuring the rate of inhibition of invertase by inhibitor at various pH values and 37.0°C. The reactions contained 6.83 nM invertase, 565 nM...
inhibitor, and 0.11 mg/mL BSA (to stabilize the enzyme) in buffers of various pHs containing 10 mM each of sodium citrate, sodium phosphate, and sodium borate and enough sodium chloride to adjust the ionic strength to 0.10. After equilibration at 37°C, the reaction was initiated by addition of enzyme. Aliquots (0.20 mL; total of nine) were removed from the reaction at intervals up to 15 min to determine invertase activity left as described above.

**Rate of Binding of Inhibitor with Invertase in Absence of Substrate**

These studies were conducted by addition of enzyme (5.12 nM) to several tubes containing varying concentrations (140 to 1690 nM) of inhibitor in 80 mM acetate buffer (pH 4.70 at 37.0°C). At timed intervals, aliquots were removed, and the remaining invertase activity was determined as described above.

The effect of inhibitor and invertase concentrations on rate of binding was also determined by using two reactions in which both the enzyme and inhibitor concentrations differed by a factor of two. The first reaction mixture contained 6.83 nM invertase and 13.7 nM inhibitor in 80 mM acetate buffer (pH 4.70 at 37°C), and the second reaction mixture contained 13.7 nM invertase and 27.4 nM inhibitor in the same buffer at the same temperature. At various times, aliquots were removed to determine the remaining invertase activity as described above.

**Inhibition of Invertase by Inhibitor in Presence of Substrate: Determination of Ki**

The experiments were performed at pH 4.70 and 37.0°C in 80 mM acetate buffer containing 0.11 mg/mL of BSA, 8.54 nM invertase (added last), variable concentrations of substrate (sucrose concentration from 7 to 215 mM), and variable concentrations of inhibitor (0 to 1690 nM). Aliquots were removed from the reaction at nine time intervals up to 15 min, and the amount of reducing groups was determined. The data were analyzed by the Lineweaver-Burk method (10) for \( V_{max} \), \( K_m \) and \( K_i \).

**Binding of Inhibitor with Invertase in Absence of Substrate: Determination of Ki**

The experiments were performed at pH 4.70 in 80 mM acetate buffer and at 37.0°C. The invertase concentration was held constant at 2.05 nM while the inhibitor concentration was varied from 56.4 to 705 nM. After 1.0 h of incubation of inhibitor with enzyme, the remaining invertase activity was determined as described above.

The \( K_i \) was determined from the invertase activity remaining as described by Bieth (3). The data were plotted as \( L_0/(1-a) \) versus \( 1/a \), where \( a \) is the fraction of total active enzyme (not bound to the inhibitor) and \( L_0 \) is the total inhibitor concentration, which was varied as indicated above. The slope of the plot is \( K_i \).

**Effect of Ionic Strength and Organic Solvent on Initial Rate of Binding of Inhibitor with Invertase**

Reactions were performed at 37.0°C in 80 mM acetate buffer, pH 4.70, containing 0.11 mg/mL BSA, 5.13 nM invertase and 282 nM inhibitor. The reactions contained sodium sulfate (0 to 0.50 M) to increase the ionic strength of the solution or isopropanol (0 to 1.3 M) to decrease the dielectric constant of the solution. The pH of each buffer containing sodium sulfate or isopropanol was readjusted to 4.70. Aliquots were removed at various reaction times and the remaining invertase activity determined. Stability of invertase and inhibitor in sodium sulfate and isopropanol was determined in separate experiments.

**RESULTS**

**Effect of pH on Initial Rate of Binding of Inhibitor to Invertase**

The effect of pH on the initial rate of binding of the inhibitor to invertase is shown in Table I. Between pH 3.5 and 7.4, there was very little effect. The initial rate of binding, as measured by loss of invertase activity, ranged from \( 14.4 \times 10^{-3} \) min\(^{-1} \) at pH 3.5 and pH 7.4 to \( 15.8 \times 10^{-3} \) min\(^{-1} \) at pH 5.5, a change of 8.9%. While the values are barely statistically significant, the trend of the data indicates this small difference is real. One can certainly rule out ionization of one or more groups with pK\( a \) values within the range of 3.5 to 7.4 as being important in complex formation.

**Rate of Binding of Inhibitor to Enzyme**

Figure 1 shows the rate of binding of inhibitor to enzyme in the absence of substrate. The data fit pseudo-first-order kinetics for loss of activity on incubation of enzyme (5.12 nM) with varying concentrations of inhibitor (140–1690 nM). From the values of \( k_{on} \) for rates of inactivation at various concentrations of inhibitor used in Figure 1 (slopes of lines), the second-order rate constant \( k_i \) was determined from the slope of the plot (Fig. 1 insert) to be \( 8.86 \times 10^2 \) m\(^{-3}\)s\(^{-1}\). If the reaction were diffusion controlled, this rate constant should be about \( 10^6 \) m\(^{-3}\)s\(^{-1}\) for the two proteins (8).

**Table I. Effect of pH on Initial Rate of Binding of Potato Invertase with Inhibitor**

<table>
<thead>
<tr>
<th>pH Value</th>
<th>( k_{on} \times 10^3 ) (min(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.5</td>
<td>14.4 ± 1.1</td>
</tr>
<tr>
<td>4.1</td>
<td>15.2 ± 0.8</td>
</tr>
<tr>
<td>4.5</td>
<td>15.3 ± 0.3</td>
</tr>
<tr>
<td>5.1</td>
<td>15.6 ± 0.5</td>
</tr>
<tr>
<td>5.5</td>
<td>15.8 ± 0.6</td>
</tr>
<tr>
<td>6.0</td>
<td>14.3 ± 0.7</td>
</tr>
<tr>
<td>7.4</td>
<td>14.4 ± 0.5</td>
</tr>
</tbody>
</table>

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If binding between enzyme and inhibitor were truly diffusion controlled and followed second-order kinetics, then a simultaneous doubling of the concentrations of enzyme and inhibitor should increase the binding rate four times and reduce the half-time to one-fourth. The experiment, done with 6.83 nM enzyme and 13.7 nM inhibitor and then with double concentrations of each, at pH 4.70 and 37.0°C, gave $k_{on}$ values of 20.9 $\times$ 10$^{-3}$ min$^{-1}$ and 39.6 $\times$ 10$^{-3}$ min$^{-1}$, respectively. The calculated half-times for the two reactions were 33 min and 18 min, respectively. Inhibition of invertase activity was followed by the rate assay until 90% of the reaction was completed. These data indicate that binding between potato invertase and its endogenous inhibitor does not follow true second-order reaction kinetics. The mechanism of binding seems to be more complex.

Inhibition of Enzyme by Inhibitor in Presence of Sucrose

Initial velocities, at pH 4.70 and 37.0°C, of inhibition of invertase by inhibitor in presence of sucrose were determined by addition of enzyme (8.54 nM) to solutions containing varying concentrations of sucrose (7–215 mM) at several fixed concentrations of inhibitor (0, 210, 420, 850, and 1690 nM). Analysis of the data by the Lineweaver-Burk method (10) indicated simple linear noncompetitive inhibition of the enzyme since the five lines converged on the $x$-axis (Fig. 2); $K_m$ was constant for all five sets of data. This plot allows determination of $V_{max}$, $V_{max}$ (and $K_m$), and $K_v$. $V_{max}$ was 9.61 $\times$ 10$^{-6}$ M min$^{-1}$ with a $K_v$ ($V_{max}/E_0$) value of 1125 min$^{-1}$. The $K_m$ value of invertase for sucrose was 16 mM. As expected for simple linear noncompetitive inhibition, this same value (15.78 ± 0.34 mM) was obtained in the presence of the various concentrations of inhibitor.

$K_v$ was determined from a replot of the $y$-intercept of each line in Figure 2 versus the concentration of inhibitor (insert A) and by a replot of the slopes of those lines versus inhibitor concentration (insert B). Data from insert A and insert B gave identical values of $1.88 \times 10^{-6}$ M for $K_v$, as expected for simple linear noncompetitive inhibition.

Binding of Inhibitor to Enzyme in Absence of Substrate: Determination of $K_v$

The enzyme (2.05 nM) was incubated for 1 h with inhibitor (50–700 nM) at pH 4.70 and 37.0°C and the remaining invertase activity determined. A plot of remaining invertase activity versus inhibitor concentration is shown in Figure 3. Data points below 400 nM inhibitor are not shown on this plot; they were linear to 100% original invertase activity. The data were replotted by the method of by Bieth (3) as shown in the insert. The $K_v$ was 6.23 $\times$ 10$^{-8}$ M from the slope of this plot.

Effect of Ionic Strength and Organic Solvent on the Rate of Binding of Inhibitor to Invertase

The rate of binding of invertase (5.13 nM) with inhibitor (282 nM) decreased moderately as the ionic strength was increased at pH 4.70 (Table II). At 1.56 ionic strength, the binding rate was 56% lower than the rate in buffer alone. Decrease in rate of binding of invertase with inhibitor with increase in ionic strength of the solution is indicative of ionic interaction being of importance in complex formation. Other salts had similar effects as Na$_2$SO$_4$.

The rate of binding of invertase (1.02 nM) with inhibitor (846 nM) did not show a consistent trend as the isopropanol concentration was increased at pH 4.70 (Table III). We conclude that the rate of binding was not affected by a change in the dielectric constant of the solution.
According to the fraction of dissociation, the amounts of invertase indicated by Na$_2$SO$_4$ was firm.

The reactions, at 37.0°C, contained 1.02 nm invertase and 846 nm inhibitor in 80 mM acetate buffer containing BSA (0.11 mg/mL) plus indicated isopropanol concentration (pH 4.70). Remaining invertase activity was determined at intervals for 15 min. Dielectric constants used were: buffer, 78.5; isopropanol, 18.3.

<table>
<thead>
<tr>
<th>[Isopropanol] (m)</th>
<th>Dielectric Constant</th>
<th>$k_{max} \times 10^3$ (min$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>78.5</td>
<td>33.1 ± 0.7</td>
</tr>
<tr>
<td>0.325</td>
<td>77.3</td>
<td>34.3 ± 0.9</td>
</tr>
<tr>
<td>0.625</td>
<td>76.3</td>
<td>31.8 ± 1.2</td>
</tr>
<tr>
<td>0.975</td>
<td>75.0</td>
<td>37.5 ± 0.3</td>
</tr>
<tr>
<td>1.30</td>
<td>73.8</td>
<td>34.4 ± 1.8</td>
</tr>
</tbody>
</table>

Complex formation between potato invertase and invertase inhibitor seems to be of ionic character. The rate of binding between the two proteins decreased as the ionic strength of the buffer increased at pH 4.70 (Table I). Changing the dielectric constant of the solution had no effect on rate of binding.

binding of invertase inhibitor to invertase is slow, requiring a prior incubation for inhibition to be maximally expressed. The second-order rate constant, $k_1$, was $8.82 \times 10^2$ M$^{-1}$s$^{-1}$ (Fig. 1). Typically, formation of the initial enzyme-inhibitor complex is rapid, with a biomolecular rate constant of $10^5$ to $10^7$ M$^{-1}$s$^{-1}$ for complex formation (8). For example, Cha et al. (5) reported a value of $2 \times 10^6$ M$^{-1}$s$^{-1}$ for coformycin and adenosine deaminase association. The low value of $8.82 \times 10^2$ M$^{-1}$s$^{-1}$ for invertase and invertase inhibitor association suggests that another rate-determining step controls rate of the association. Slow association of enzymes with large inhibitors has been reported for other systems (5, 7, 14, 18).

Simultaneous doubling of the concentrations of invertase and invertase inhibitor increased the binding rate two-fold and reduced the half-time by 50%. A four-fold increase in the binding rate is expected if binding between enzyme and inhibitor follows second-order kinetics. These data further indicate that complex formation between the enzyme and inhibitor is complex.

Another evidence for the complexity of binding between invertase and invertase inhibitor is the different values obtained for the dissociation constant ($K_i$) of the complex by the Lineweaver-Burk method (10) (in presence of sucrose) and the Bieth method (3) (in absence of substrate). By the Lineweaver-Burk method, the dissociation constant, $K_i$, was $2 \times 10^{-6}$ M; by the Bieth method the value of $K_i$ was $6.23 \times 10^{-8}$ M, about 33 times smaller. The Lineweaver-Burk method is a kinetic method which is influenced by slower rate steps prior to the equilibrium complex. Its measurement also requires the presence of substrate. The Bieth method provides information only on the final equilibrium constant. It is well

**DISCUSSION**

The rate of inhibition of enzyme and of binding between potato invertase and potato invertase inhibitor did not vary much with pH at 37.0°C. Pressey (15) reported that potato tuber invertase activity is highest at pH 4.75, which is confirmed by our data (4). Our data do not show a double pH optimum for binding of inhibitor to invertase; Anderson et al. (1) also did not find a double pH optimum. Based on our data and those of Anderson et al. (1), we conclude that there is no ionizable group on either the enzyme or the inhibitor with a pK$_a$ between 3.5 and 7.4 that is important for complex formation between the two.

Figure 3. Equilibrium binding of invertase-invertase inhibitor. Remaining invertase activity was measured after 1 h preincubation of varying amounts of invertase inhibitor (0 to 710 nm) with 2.05 nm potato invertase at 37.0°C and pH 4.70. Insert: A replot of data according to Bieth (12). $I_p$ is the initial inhibitor concentration and $a$ is the fraction of enzyme not bound to inhibitor. The slope of the line is the dissociation constant.

**Table II. Effect of Sodium Sulfate Concentration on Rate of Binding of Potato Invertase with Inhibitor**

The reactions, at 37°C, contained 5.13 nm invertase and 282 nm inhibitor in 80 mM acetate buffer containing BSA (0.11 mg/mL) plus indicated Na$_2$SO$_4$ concentration (pH 4.70). Remaining invertase activity was determined at intervals for 15 min.

<table>
<thead>
<tr>
<th>[Na$_2$SO$_4$] (m)</th>
<th>Ionic Strength*</th>
<th>$k_{max} \times 10^9$ (min$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.065</td>
<td>16.5 ± 1.2</td>
</tr>
<tr>
<td>0.040</td>
<td>0.18</td>
<td>14.3 ± 0.9</td>
</tr>
<tr>
<td>0.075</td>
<td>0.29</td>
<td>13.1 ± 0.9</td>
</tr>
<tr>
<td>0.150</td>
<td>0.510</td>
<td>10.1 ± 0.2</td>
</tr>
<tr>
<td>0.300</td>
<td>0.960</td>
<td>9.5 ± 0.5</td>
</tr>
<tr>
<td>0.500</td>
<td>1.56</td>
<td>9.3 ± 0.4</td>
</tr>
</tbody>
</table>

* Includes ionic strength of the buffer.

**Table III. Effect of Isopropanol Concentration on Rate of Binding of Potato Invertase with Inhibitor**

The reactions, at 37.0°C, contained 1.02 nm invertase and 846 nm inhibitor in 80 mM acetate buffer containing BSA (0.11 mg/mL) plus indicated isopropanol concentration (pH 4.70). Remaining invertase activity was determined at intervals for 15 min. Dielectric constants used were: buffer, 78.5; isopropanol, 18.3.
known that the Lineweaver-Burk method is not a useful analysis for slow tight-binding inhibitors. However, for a $K_i$ of $2 \times 10^{-6}$ M, it is an appropriate analysis.

Based on the dissociation constant for the invertase-invertase inhibitor complex ($2 \times 10^{-6}$ M [Lineweaver-Burk]) or $6.23 \times 10^{-8}$ M [Bieth] at pH 4.70 and 37.0°C, inhibition of potato invertase by its endogenous proteinaceous inhibitor seems to be an example of slow, non-tight binding inhibition, or simply, slow-binding inhibition (12, 21). Inhibition of isocitrate lyase by nitropropionate (18) with a dissociation constant, $K_i$ of $1.7 \times 10^{-3}$ M and alanine racemase by (1-aminoethyl)phosphonate (2) with a $K_i$ of $1.2 \times 10^{-6}$ M are other examples of slow, non-tight binding inhibition. Wilcox and Whitaker (20) determined that red kidney bean $\alpha$-amylase inhibitor and porcine pancreatic $\alpha$-amylase form an initial active complex rapidly, with $K_d$ of $3.1 \times 10^{-5}$ M followed by a slow isomerization to a tight, low activity complex with $K_d$ of $3 \times 10^{-11}$ M.

In conclusion, invertase inhibitor exhibits slow-binding inhibition of potato invertase with an association rate constant ($k_1$) at pH 4.70 and 37.0°C of $8.82 \times 10^{2}$ M$^{-1}$ s$^{-1}$ and a dissociation rate ($k_2 = K_i \times k_0$) of $3.3 \times 10^{-3}$ min$^{-1}$. One possible explanation for our observed data for complex formation is that the invertase inhibitor binds to invertase in a minimum two-step fashion, forming an initial, rapid, reversible noncompetitive complex with an apparent $K_i$ of $2 \times 10^{-6}$ M at pH 4.70, followed by isomerization to a tighter complex ($K_i$ of $6.23 \times 10^{-8}$ M) which in turn dissociates slowly, with a half-time of about 3.5 h.

LITERATURE CITED