Interaction of Sweet Potato β-Amylase with Its Reaction Product, Maltose

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To identify amino acid residues present in the active site of sweet potato β-amylase, the interaction of the reaction product of the enzyme, maltose, has been studied. Addition of maltose to β-amylase solution produced a difference spectrum, which indicates that four out of forty tryptophan residues in the tetrameric enzyme have specific interactions with maltose. The sugar also affects the chemical modification of sulfhydryl groups in β-amylase with p-mercuribenzoate (pMB). Modification of four out of ten sulfhydryl groups which are reactive in the native state is prevented almost completely and that of two is retarded by maltose. Inactivation of β-amylase is accompanied by the modification of the two slowly reactive residues. The interaction between maltose and the enzyme is further manifested by the formation of crystals of β-amylase from a buffer solution containing maltose. A method we have developed shows that the crystals are those of a complex of four maltoses with one enzyme molecule. These results strongly suggest that four maltoses bind to four substrate binding sites, in which four tryptophan and six cysteinyl residues are located. During the course of the study very unusual results were obtained, namely, restoration of β-amylase activity and release of mercury from β-amylase simply by dialysis of the enzyme in which ten sulfhydryl groups had been modified with pMB to produce an almost inactive protein.

The difference spectrophotometric method is one of the useful tools for the study of specific binding of substrates or products to an enzyme. Hayashi et al. (1) reported that the addition of substrate analogs to lysozyme produced difference spectra due to changes in the environment around one tryptophan residue in the active site. Later this technique was applied to investigate the interactions of various amylases with their substrates or products, i.e., α-amylases from Bacillus subtilis (2, 3), from porcine pancreas (4), and a glucoamylase from Rhizopus niveus (5). In these studies tryptophan residue(s) were shown to be located in the binding sites of the enzymes without exception. However, no information has been obtained for β-amylase, another glycosidic enzyme. The purpose of this study was to use this technique to determine whether sweet potato β-amylase, a tetrameric enzyme with a monomer molecular weight of 50,000, has a specific interaction with its reaction product, maltose, and whether tryptophan residue(s) are also involved in the interaction. Although Thoma et al. (6) and Spradlin and Thoma...
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(7) proposed, on the basis of chemical modification studies with alkylating agents, that sulfhydryl groups were not essential, we studied the effect of maltose on the modification of sulfhydryl groups of β-amylase with ρMB.

The appearance of the difference spectrum obtained when maltose was added to β-amylase solution suggested that four tryptophan residues might be located in the binding sites of the enzyme. Crystallization of the enzyme-maltose complex, and determination of the number of bound maltose molecules per enzyme are described in the second part of this paper.

MATERIALS AND METHODS

β-Amylase was purified from sweet potato (Kokei No. 14) according to the method of Balls et al. (8), except that the enzyme fraction before the crystallization step was purified by Sephadex G-100 gel filtration and crystallized twice. Before use, the crystals were dissolved in 50 mM phosphate buffer, pH 7.0, and desalted by passing the enzyme solution through Sephadex G-50. Maltose monohydrate was obtained from Wako Pure Chemicals (Osaka) and recrystallized from ethanol-water, mp 125–126°, [α]D +128.6°. Other sugars were commercial preparations. Sodium p-hydroxymercuribenzoate was purchased from Sigma Chemical Co. (U.S.A.), and dithiothreitol from Seikagaku Kogyo Co. (Tokyo).

The method for β-amylase assay was described in the previous paper (9). After incubation of the starch solution with β-amylase at 35°C for 10 min, the amount of maltose liberated was determined by the hypoiodide method of Willsatter and Schudel (10). The concentration of β-amylase was calculated from the observed absorbance at 280 nm and the absorption coefficient of the enzyme as estimated by us (A1% at 280 nm = 17.5). The percent concentration was converted to the molar concentration using a molecular weight of 200,000 (7, 11). The difference spectra were recorded on a Shimadzu UV-200S spectrophotometer equipped with a recorder and a thermostatted cell holder. Experiments were carried out at 19–20°C. The protein concentration was kept at around 4 μM, which gave an absorbance at 280 nm of 1.4. The number of ρMB moieties bound to sulfhydryl groups was determined by the spectroscopic method of Boyer (12) by measuring the increment in absorbance at 250 nm. The mercury content of β-amylase modified with ρMB was determined on a Rigaku Denki flameless atomic absorption mercury analyzer. Maltose concentration was determined by the phenol-sulfuric acid method of Dubois et al. (13). The thin layer chromatography of sugars was carried out with the following combination of plates and solvent systems (14): (a), Kieselguhr G and n-butanol–pyridine–water = 75 : 15 : 10, (b), Silica gel G-0.02 N sodium acetate and chloroform–methanol = 6 : 4, (c), Kieselguhr G-0.1 N sodium acetate and ethyl acetate–isopropanol–water = 32 : 12 : 6.

RESULTS

Difference Spectrum of β-Amylase with Maltose—To determine whether β-amylase interacts with maltose the difference spectrum produced by the addition of maltose to amylase solution was recorded at pH 3.8. Most of the experiments were carried out at pH 3.8 because β-amylase had a specific interaction with maltose at this pH. As shown in Fig. 1, three positive peaks appeared at 292, 285, and 275 nm and a trough at around

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Fig. 1. Difference spectrum of β-amylase induced by maltose. The difference spectrum of a solution containing β-amylase (4 μM) and maltose (117 mM) in a mixture of 20 mM glycine-HCl buffer, pH 3.0 and 5 mM potassium phosphate buffer, pH 7.3 (final pH 3.8) was recorded against the reference without maltose. The temperature was 19°C.

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302 nm. Since tyrosine has no contribution at around 290 nm, the highest peak at 292 nm was assigned to tryptophan residue(s) in β-amylase. The observed molar difference absorption coefficients ($\Delta \varepsilon_{obs}$) at 292 nm of the enzyme were dependent on maltose concentration (Fig. 2). Since $\Delta \varepsilon_{obs}$ gradually increased even at high maltose concentrations, the dissociation constant of the enzyme for maltose appeared to be fairly large, so allowance for the nonspecific perturbation by maltose of the tryptophan residues became necessary. As shown in Fig. 3, the difference spectrum of β-amylase produced in the presence of 10% ethylene glycol had the highest peak at around 284 nm, perhaps because of the contribution of many tyrosine residues at the surface of the enzyme. The increase in $\Delta \varepsilon_{obs}$ per 10% ethylene glycol at 292 nm due to tryptophan residues was estimated to be 1,900 from a plot of $\Delta \varepsilon_{obs}$ against the concentration of ethylene glycol. Although the reported molar absorption difference of N-acetyl-L-tryptophan ethyl ester per 10% ethylene glycol at 292 nm varies from 155 to 199 (2, 3, 5), the results indicate that about 10.8 out of 40 tryptophan residues (11) per β-amylase were accessible to ethylene glycol. Elődi et al. (4) reported that the molar absorption change of N-acetyltryptophan ethyl ester produced by 10% maltose was 100 at 292 nm. Using this value, the nonspecific solvent perturbation of maltose on 10.8 exposed tryptophan residues was calculated at each concentration of maltose and subtracted from the $\Delta \varepsilon_{obs}$ values of Fig. 2. The corrected $\Delta \varepsilon_{obs}$ values are written as $\Delta \varepsilon_{obs}'$. Since the saturation curve thus obtained obeyed the Michaelis-Menten law (15), the equilibrium equation can be written as $K_d = (\Delta \varepsilon_{max} - \Delta \varepsilon_{obs}) / [\varepsilon_{obs}]$, where $K_d$ is the dissociation constant of the enzyme-maltose complex, [M] is the maltose concentration, and $\Delta \varepsilon_{max}$ is the maximum molar absorption difference obtained when all of the enzyme forms the complex with maltose. Thus if we plot [M]/$\Delta \varepsilon_{obs}'$ against [M] according to Lineweaver and Burk (16), $-K_d$ and $\Delta \varepsilon_{max}$ are obtained from the intercept on the [M] axis and the reciprocal of the slope, respectively. Figure 4 shows the results. $K_d$ and $\Delta \varepsilon_{max}$ thus obtained were 24 mM and 3,930, respectively. Since β-amylase is a tetrameric enzyme which has four subunits of the same molecular weight (50,000)
Fig. 4. Plot of [maltose]/$\Delta \varepsilon_{\text{obs}}$ versus maltose concentration. Maltose concentration is expressed in mol per liter. $\beta$-Amylase, 4.5 $\mu$m. Other conditions were as in Fig. 1. The nonspecific perturbation effect of maltose (see the text) was subtracted from the $\Delta \varepsilon_{\text{obs}}$ values in Fig. 2 and the corrected values are replotted on this figure. The maximum molar difference ($\Delta \varepsilon_{\max}$) at 292 nm and the dissociation constant ($K_d$) were calculated from the slope and the intercept on the abscissa, respectively.

The presence of maltose also show a trough at around 302 nm. Hence we concluded that one tryptophan residue per monomer was affected by the binding of maltose to $\beta$-amylase.

Other sugars tested were lactose, sucrose, D-glucose, D-galactose, and D-fructose. Glucose was slightly effective and produced the same type of spectrum as maltose. The difference spectrum with fructose was different from that with maltose. Other sugars were without effect. Thus, maltose may have a specific interaction with $\beta$-amylase.

Effect of Maltose on the Modification of $\beta$-Amylase Sulfhydryl Groups with pMB—Sweet potato $\beta$-amylase has twenty sulfhydryl groups. We modified these sulfhydryl groups with pMB. In the range of pH 3 to 8 about ten sulfhydryl groups of the enzyme were titrated in the native state. $\beta$-Amylase thus modified had an enzyme activity of 0.006% of the original, but it was quite stable and recovered full activity on incubation with dithiothreitol. These ten sulfhydryl groups showed different types of reactivity toward pMB. At pH 7 four sulfhydryl groups reacted very rapidly and were preferentially modified upon addition of four equivalents of pMB. However, about 90% of the original activity was retained. Maltose had no effect on the modification. These sulfhydryl groups may therefore not be involved in the enzyme action or the interaction with maltose. Maltose affected the modification of the remaining six sulfhydryl groups with pMB at pH 3.8 (glycine-HCl buffer). In the absence of maltose those six groups were modified rapidly. However, in the presence of 1% maltose, the modification of four groups was strongly retarded, and that of two groups was also depressed but to a lesser extent (Fig. 5). $\beta$-Amylase lost enzymatic activity either by the modification of the six sulfhydryl groups or of the two slowly reactive groups. This indicates that at least the two groups are important for enzymatic activity. Therefore, we may conclude that these six sulfhydryl groups are in the region of the active sites, where the four
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Fig. 6. Maltose-induced difference spectrum of β-amylase modified with pMB. Twelve equivalents of pMB was added to β-amylase in 10 mM potassium phosphate, pH 7.3, and the mixture was allowed to stand at room temperature for about 2 h. The solution in which 10 sulfhydryl groups of the enzyme were fully modified was then mixed with one-half volume of 80 mM glycine-HCl, pH 3.0 (final pH, 3.8). To this, one-fourth volume of 20% maltose or water was added and the difference spectrum of the modified amylase containing maltose versus that without maltose was recorded. Final concentrations of components were: β-amylase, 4 μM; maltose, 117 mM.

The six reactive sulfhydryl groups and four tryptophan residues in β-amylase are located in the active sites, but that the sulfhydryl groups are in the interior of the sites relative to the position of the tryptophan residues.

This view was further supported by the following observation. As mentioned above, ten sulfhydryl groups per enzyme are modified by pMB at pH 5. We have found that the modified enzyme, which was almost inactive, recovered its activity simply by dialysis against glycine-HCl buffer, pH 3.8, concomitantly with a decrease in the mercury (pMB) content of the modified enzyme. The results are shown in Fig. 7. After dialysis for 72 h about 60% of the original activity was restored, and the number of pMB moieties in the enzyme decreased from ten to three.

Maltose prevented the restoration of the enzyme activity. The activity was only 15% even after dialysis for 72 h. Removal of mercury was also retarded, and 5 atoms per enzyme molecule were retained. Incubation of the modified enzymes dialyzed in the presence and absence of maltose with excess dithiothreitol restored the enzyme activity further. The enzyme solution dialyzed against the buffer recovered 77% of the original activity, and that dialyzed against maltose solution recovered 93% of the original activity. Thus, it was confirmed that maltose did not inactivate the modified enzyme. Maltose had a protective effect against inactivation of β-amylase during dialysis. Other sugars did not show such an effect. These results suggest that the binding of maltose to the outside of the active sites retards the removal of pMB groups from sulfhydryl groups at some internal position in the active sites.

Crystallization of β-Amylase in the Presence of Maltose—These results indicate that the reaction product, maltose, had a specific interaction with the substrate binding sites of β-amylase. We therefore decided to attempt the crystallization of β-amylase-maltose (E-P) complex. To avoid the effect of high salt concentration on the mode of binding of maltose to β-amylase we placed β-amylase in 50 mM buffers containing maltose. The concentrations of β-amylase and maltose were 10 μM and 14.6 mM, respectively. On allowing solutions of various pH values to stand at room temperature for a few hours crystals began to appear from acetate buffer, pH 4.0 to 4.4. The
Fig. 7. Effect of maltose on the restoration of the enzyme activity and removal of mercury from β-amylase modified with pMB by dialysis. Ten sulfhydryl groups of β-amylase were modified with pMB in a mixture of 20 mM acetate buffer, pH 5.0, and 5 mM potassium phosphate, pH 7.3. The enzyme activity was about 0.006% of that of the native enzyme. The modified amylase solution was diluted with 7 volumes of the same buffer mixture as in Fig. 1 (20 mM glycine-HCl, pH 3.0-5 mM phosphate, pH 7.3; final pH, 3.8), and dialyzed against the same buffer mixture with or without 1% maltose. The dialysis temperature was 6°C and the outer solution was stirred. The concentration of β-amylase during dialysis was 0.48 μM. At intervals aliquots were withdrawn from the dialysis bags and subjected to enzyme assay and the determination of mercury (pMB) content using a flameless atomic absorption mercury analyzer. To determine the full activity of the dialyzed enzyme the dialysate was incubated before assay with 1 mM dithiothreitol in 10 mM phosphate buffer, pH 7.3, for 45 min at 35°C. Activity in the absence (Δ) and presence (▲) of maltose; □, ■, after reactivation with dithiothreitol; mercury content in the absence (○) and presence (●) of maltose.

optimal pH for the crystallization was found to be 4.2. The crystals are illustrated in Fig. 8. At this pH and buffer concentration β-amylase alone does not crystallize. Hehre et al. reported that sweet potato β-amylase catalyzed the rapid condensation of β-maltose to maltotetraose (17). However, we could not detect maltotetraose when the crystalline suspension or crystals of β-amylase-maltose complex were subjected to thin layer chromatography (14). This indicated that maltotetraose was not the ligand of β-amylase.

Various sugars were used instead of maltose, but failed to form crystals with β-amylase. These sugars were D-fructose, D-xylose, D-arabinose, D-ribose, D-mannose, D-galactose, lactose, sucrose, D-sorbitol, D-mannitol, and L-rhamnose. D-Glucose was the sole exception. In the presence of glucose a very small amount of crystals of the same shape appeared.

Number of Bound Maltose Molecules in β-Amylase Crystallized in the Presence of Maltose—To determine whether or not β-amylase complexed with maltose and crystallized, the maltose content in the crystals was determined. First, the crystal suspension was centrifuged and the supernatant solution was discarded. Then, a known amount of 20 μM HCl (pH 4.5), in which the crystals were hardly soluble, was added and the suspension was stirred for a few minutes in an ice bath. After each washing the suspension was centrifuged and the amount of maltose in the wash was determined by the phenol-sulfuric acid method of Dubois et al. (13). The ratio of maltose in the wash to β-amylase originally present in the crystals was calculated. Taking the volume of the first precipitate obtained by centrifugation of the crystalline suspension as A, and the volume of each wash
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Fig. 9. Changes in the amount of maltose washed out from the \( \beta \)-amylase-maltose complex crystals. \( \beta \)-Amylase was crystallized at room temperature with 2% maltose in 50 mM acetate buffer, pH 4.5. After crystallization was complete, the crystals were collected by centrifugation. The amount of \( \beta \)-amylase in the crystals was determined from the amylase concentration of the crystalline suspension and that of the supernatant solution. A known amount of 20 mM HCl (pH 4.5), in which the crystals were hardly soluble, was added to the precipitate and stirred with a glass rod for a few minutes in an ice bath, then the crystalline suspension was centrifuged to separate the crystals from the supernatant solution. This process was repeated about ten times. The maltose content in the washes was determined by the phenol-sulfuric acid method of Dubois et al. (13) using maltose-H_2O as a standard. Exp. 1 (△), \( \beta \)-amylase was 22.9 mg and the volume per wash was 1.0 ml; Exp. 2 (○), amylase was 65.8 mg and the wash volume was 2.0 ml. After thorough washing no maltose remained in the crystal fraction.

as B, the concentration of maltose in the first wash can be expressed as \( C_0 \times (A/A+B) \), where \( C_0 \) is the concentration of maltose in the crystallization medium. After \( n \) washings the maltose concentration of the wash will fall to \( C_0 \times (A/A+B)^n \) because the crystals were hardly soluble and therefore the volume A will remain almost constant. For the same reason the concentration of \( \beta \)-amylase remains unchanged during washings and is expressed as \( E_0 \times (A/A+B) \) where \( E_0 \) is the enzyme concentration at the first precipitate. So the ratio of maltose to \( \beta \)-amylase after \( n \) washings (R) can be written as follows,

\[
R = \frac{C_0 \times (A/A+B)^n}{E_0 \times (A/A+B)} = \frac{C_0}{E_0} \times \frac{(A/A+B)^n}{(A/A+B)} = \frac{C_0}{E_0} \times (A/A+B)^{n-1}
\]

This may be converted to the form

\[
\log R = \log \left( \frac{C_0}{E_0} \right) + (n-1) \log \left( \frac{A}{A+B} \right) = n \log \left( \frac{A}{A+B} \right) + \log \left( \frac{C_0}{E_0} \right) (A+B/A).
\]

Therefore, there should be a linear relationship between the log ratio and the number of washings \( n \). As indicated in Fig. 9, however, two straight lines were obtained and there was a clear inflection point. We thought, therefore, that the rapid decrease in maltose in the early stages of washing might result from simple dilution of maltose in the solvent outside and inside the crystals in the first precipitate, and that the slowly eluted maltose might correspond to the bound maltose. The sum of the maltose eluted slowly during the first to tenth washings reached 4.4 molecules per \( \beta \)-amylase molecule in both experiments 1 and 2. Therefore, we concluded that four mol of maltose was bound per mol of \( \beta \)-amylase in the crystals.

DISCUSSION

The difference spectrum of \( \beta \)-amylase produced by maltose was quite similar to those of glucoamylase-maltose (substrate) complex (5) and porcine pancreas \( \alpha \)-amylase-maltose (product) complex (4). This suggests that the conformations of the substrate-binding sites of glucosidic enzymes are similar. The maximum molar absorption difference (\( \Delta \varepsilon_{\text{max}} \)) at 292 nm produced by maltose (1,000) was much smaller than the reported value for the denaturation blue shift of a tryptophan residue in a protein (−1,500 to −1,600) (18). This may result from the summation of the positive difference at 292 nm and the broad negative difference (trough), as has been discussed by Elddi et al. (4) and by Ohnishi et al. (5). After correction for the negative contribution \( \Delta \varepsilon_{\text{max}} \) appears to approach the normal value, so that four tryptophan residues per enzyme (one per monomer) may be masked by the binding of maltose.

Modification of sulfhydryl groups with pMB revealed that ten out of twenty sulfhydryl groups
Fig. 10. Schematic hypothesis for the modification of sulfhydryl groups of \( \beta \)-amylase in the presence of maltose at pH 3.8. A, Maltose added before the addition of pMB; B, pMB added first, followed by maltose.

per \( \beta \)-amylase were reactive toward pMB. Four of these ten reactive sulfhydryl groups, which were most reactive at pH 7, were probably not involved in the enzyme action or in the interaction with maltose and, therefore, may not be in the vicinity of the active site. Misra and French (19) reported that maltose was a competitive inhibitor of \( \beta \)-amylase, while Thoma et al. (20) claimed that the inhibition was noncompetitive. However, they agreed that maltose was bound to the active site. Our results that amylase activity was strongly inhibited by the modification of two sulfhydryl groups with pMB and that the modification of these two and four other sulfhydryl groups was affected by maltose suggest that these cysteine and tryptophan residues are in the vicinity of the active sites of \( \beta \)-amylase. If we assume that the relative positions of these groups in the active site of the enzyme are as shown in Fig. 10, the results obtained can be explained. When maltose is added to the \( \beta \)-amylase, it binds in the close vicinity of the tryptophan residue and one sulfhydryl group (SH 1) is covered by the bound maltose. The other sulfhydryl group (SH 2), however, is not covered completely (Fig. 10A). Thus, the modification of SH 1 is strongly protected but that of SH 2 proceeds slowly, resulting in inactivation of the enzyme (Fig. 5). On the other hand, when the sulfhydryl groups (SH 1 and 2) are first modified with pMB and then maltose is added, maltose still binds close to the tryptophan residues (Fig. 6), but in a different manner (Fig. 10B). Thus the removal of pMB from SH 1 by dialysis is possible, but that from SH 2 is retarded. The finding that the difference in mercury content between pMB-\( \beta \)-amylases dialyzed in the presence and absence of maltose was only two mol although the difference in the amylase activity restored was about 45\% (Fig. 7) supports this assumption. If four SH 1 sulfhydryl groups are essential for the enzymatic activity, we may explain the results just in terms of Fig. 10A, i.e., some SH 1 and two SH 2 sulfhydryl groups are released by dialysis, but the enzyme activity hardly recovers.

\( \beta \)-Amylase in which ten sulfhydryl groups were modified with pMB and which had almost no activity (0.006\%) recovered its enzyme activity simply on dialysis. Since pMB has a very high affinity for sulfhydryl groups in proteins, removal of pMB by dialysis is usually almost impossible. If the modified enzyme was dialyzed at a suitable protein concentration, more than 90\% of the original activity could be restored. At pH 5.0 and in acetate buffer, no activity was restored. It is not known at present whether or not the dissociation of pMB from sulfhydryl groups is specific to \( \beta \)-amylase or results from the acidic pH or the presence of a weak chelator, glycine. Spontaneous reactivation of homoserine dehydrogenase activity accompanied by self-dissociation of pMB from sulfhydryl groups in the interior of the enzyme was recently reported (21).

Crystalline enzyme-substrate (product) complexes of some enzymes have already been reported. However, most of them were prepared by soaking crystals of the enzyme in the substrate solution. Furthermore, the enzymes were usually crystallized from a solution of high salt or organic solvent concentration. \( \beta \)-Amylase-maltose complex, on the contrary, was crystallized from acetate buffer, pH 4.2. Since amylase is assayed routinely in the same buffer at pH 4.8, the conformational state of the enzyme in the crystals may be quite similar to that under the assay conditions. The dissociation constant of maltose for \( \beta \)-amylase was reported to be 6 mM (19), and that obtained from Fig. 4 was about 24 mM, so the determination of bound maltose was expected to be very difficult. Equilibrium dialysis or Sephadex column chromatography (22) might not be applicable. We therefore developed a method, in which \( \beta \)-amylase-
maltose complex was crystallized and the amount of bound maltose in the crystals was estimated.

Maltotetraose was not found in the crystals of β-amylase-maltose complex, although Hehre et al. (17) reported the rapid formation of maltotetraose from maltose. It is likely that the crystals are those of β-amylase-maltose complex because β-amylase forms crystals in the presence of phenyl-α-maltoside. This compound is a derivative of maltose in which the reducing end is blocked with a phenol group, and it is not hydrolyzed by β-amylase.

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