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KINETIC STUDIES ON THE INTERACTION OF *Rhizopus* GLUCOAMYLASE WITH MALTODEXTRIN AND MALTOTRIOSE, UTILIZING THE ABSORBANCE CHANGE NEAR 300 nm*†

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ABSTRACT

Maltodextrin (high-d.p. malto-oligosaccharides) was found to produce a trough at 303 nm in the difference spectrum of glucoamylase (E.C. 3.2.1.3) from *Rhizopus niveus* upon binding with the enzyme; this trough disappears upon hydrolysis. The trough, which was ascribed to a change in the electrostatic environment of a tryptophan residue at the terminal subsite of the enzyme, was found closely related to the formation of the enzyme–substrate complex. The kinetics of binding of maltodextrin and maltotriose to the enzyme were studied at pH 4.5 and 5°, by monitoring the trough by the stopped-flow method. The result was consistent with a two-step mechanism, in which a fast, bimolecular association is followed by a slower, unimolecular isomerization-process. The latter process involves an environmental change of the tryptophan residue, and is considered to be closely connected to the formation of the productive complex essential for the catalysis.

INTRODUCTION

Rhizopus glucoamylase, which hydrolyzes starch completely to D-glucose from its nonreducing ends by an exo type of degradation, was first crystallized by Tsujisaka *et al.*[1]. Because of its simple action-pattern, the enzyme has been an excellent object for kinetic studies[2–19]. The structure of its active site has been characterized in terms of subsites, and the subsite affinities have been estimated[12,13]. Chemical modification of the enzyme with *N*-bromosuccinimide (NBS) has revealed that probably two tryptophan residues are in the active site[16]. The tryptophan fluorescence of the enzyme protein has been found to be partially quenched by the addition of maltodextrin‡[17], D-glucono-1,5-lactone[18], and low molecular-weight malto-oligosaccharides[19]. This property has been utilized to study the kinetics of interaction

*Dedicated to Professor Dexter French on the occasion of his 60th birthday.

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‡This term is used to denote a malto-oligosaccharide mixture of relatively high degree of polymerization (d.p. >10).

of the enzyme with these substrates or analogs[17–19]. It has also been found that a characteristic trough near 300 nm in the difference spectrum of the enzyme appears upon binding with maltose and D-glucono-1,5-lactone, but not with D-glucose[14]. It was strongly suggested that the trough is due to some electrostatic, environmental change of a tryptophan residue, which is located in the terminal subsite where the nonreducing-end D-glucose residue is situated in the productive binding-mode of substrates[14].

In this paper, we have aimed to study kinetically the mechanism of interaction between the enzyme and substrates, maltodextrin and maltotriose, by utilizing the trough near 300 nm as a probe for monitoring the reaction.

EXPERIMENTAL

Materials. — Glucoamylase from *Rhizopus niveus* (the purest grade, lyophilized) was purchased from Seikagaku Kogyo Co., and was used without further purification. The enzyme sample was confirmed to be free from alpha amylase by examining the relationship between the blue value and the reducing value, as has been described elsewhere[20]. The enzyme concentration was determined spectrophotometrically at 280 nm, by using the value of $E^{1\%}_{280} = 13.5\ cm^{-1}$ and the molecular weight[15] of 56,000.

Maltodextrin (malto-oligosaccharides) of number-average degree of polymeri-

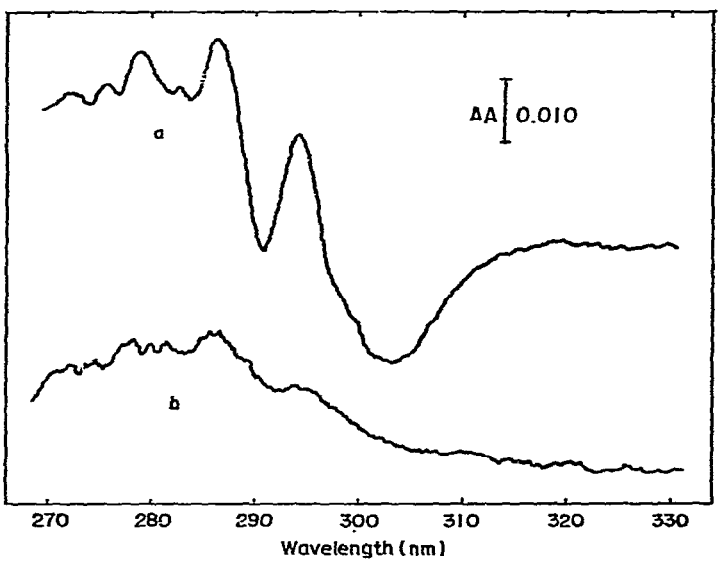

Fig. 1. Difference spectra of glucoamylase as produced by maltodextrin; pH 4.5, 25°; enzyme concentration $e_0 = 22.0\ \mu M$. The initial substrate concentration $s = 0.38\%$ (1.4 mM). The scanning speed was 20 nm per sec. Curve *a*: The difference spectrum taken within 12 sec after mixing, which corresponds to the enzyme–substrate complex in the steady-state of the reaction. Curve *b*: The difference spectrum at 7 min after mixing, which corresponds to the enzyme–product (D-glucose) system.

zation $\overline{d.p.}_n$ = 17, and maltotriose are the products of Hayashibara Biochemical Laboratories, Inc.

Methods. — The difference spectrum was taken with a spectrophotometer (Union Giken SM-401) at 25° using tandem cells, as described elsewhere[14]. A mixing apparatus (Union Giken MX-7) was used for the measurement of difference spectra in the steady state of the enzyme reaction (see Fig. 1).

Two types of stopped-flow apparatus (Union Giken SF-70 and RA-401) equipped with 200 W D_2 lamps as the light source were used. The former (SF-70) is of the conventional type equipped with driving syringes for mixing two solutions[21], whereas the latter (RA-401) is of a new type that utilizes pressure of nitrogen gas and an electromagnetic valve for driving and stopping the solutions[22]. A data-averaging system (Union Giken RA-450) was used in connection with the apparatus, if necessary. The optical path employed was 1 cm, and the dead time of the apparatus was about 1 msec.

The reactions were performed at pH 4.5 with 0.02M acetate buffer and at 25 or 5°.

RESULTS

The difference spectrum between the enzyme–substrate system in the steady state and the enzyme alone was observed with maltodextrin as the substrate, with a spectrophotometer and a mixing apparatus. Fig. 1 shows the result, where (*a*) is the difference spectrum taken within 12 sec after mixing (the scanning speed was 20 nm

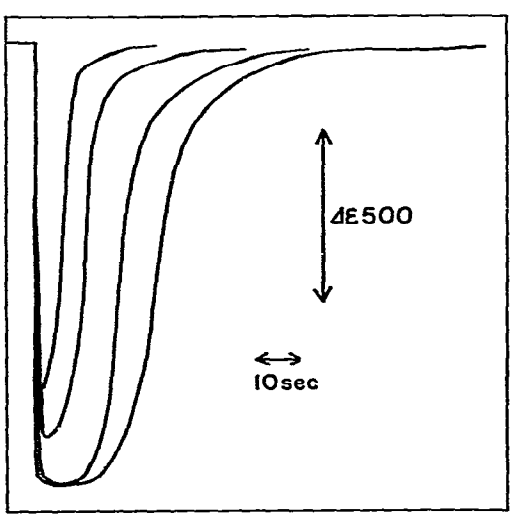

Fig. 2. The time-course of the absorbance change at 303 nm observed for maltodextrin by the stopped-flow method at 25°. The curve represents the overall reaction; e_0 = 24.3 μM; $\Delta\varepsilon$ represents the apparent change in molar absorptivity, which is equal to the absorbance change divided by the molar enzyme concentration e_0. The initial substrate concentration (from left to right): s = 0.05, 0.1, 0.2 and 0.3%.

per sec), which can be regarded as the difference spectrum between the enzyme–substrate complex and the enzyme, and (*b*) is the difference spectrum 7 min after mixing, which refers to the enzyme-D-glucose system. The transient appearance of a trough near 300 nm is clearly seen. The difference spectrum (*a*) is very similar to that observed for maltose[14]. The disappearance of the trough when the enzyme reaction is terminated is due to the fact that D-glucose does not show this trough[14] and to its low affinity for the enzyme[7].

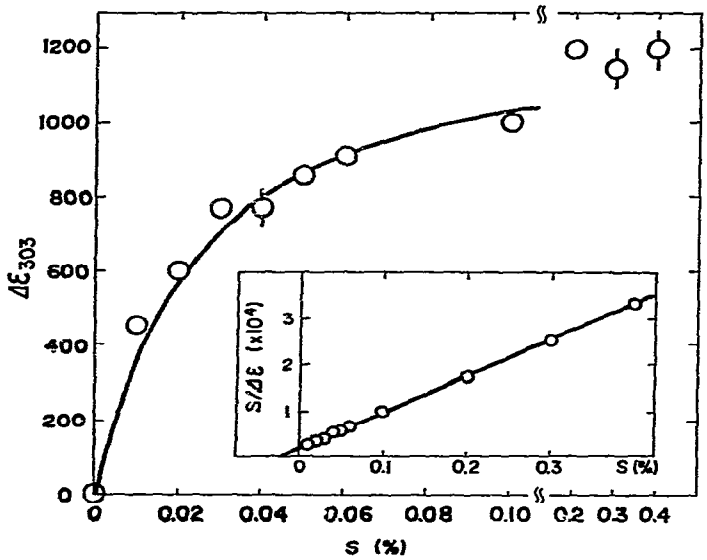

Fig. 3. The dependence of the apparent change in molar absorptivity at 303 nm ($\Delta\varepsilon_{303}$) on the initial concentration of maltodextrin. The value of $\Delta\varepsilon_{303}$ was obtained from the depth of the curve in Fig. 2. The insert is the linear plot of $s/\Delta\varepsilon_{303}$ against s; pH 4.5, 25°.

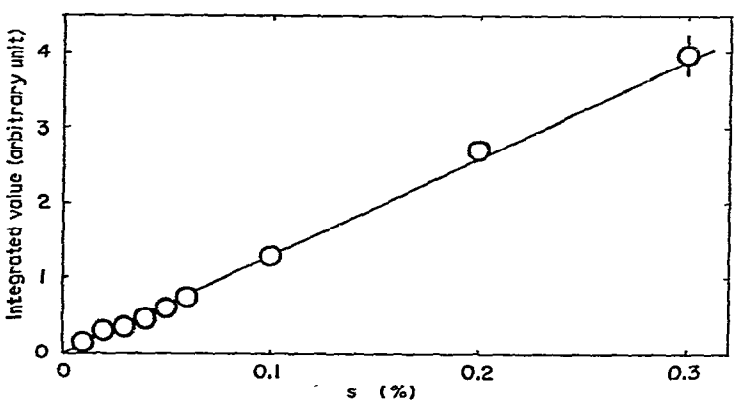

Fig. 4. The proportionality between the integrated absorbance-change and the initial concentration of substrate. The ordinate shows the area surrounded by the reaction curve in Fig. 2 (in arbitrary units); pH 4.5, 25°.

Time courses of the change in absorbance at 303 nm accompanying the overall reaction observed with the stopped-flow method at 25° are shown in Fig. 2 for various substrate concentrations. Each curve shows a rapid initial decrease in absorbance, which recovers to the initial state after achievement of steady states following various time-lapses. That these curves reflect the formation and disappearance of the enzyme–substrate complex may be confirmed in the following manner[17].

The apparent change in molar absorptivity at 303 nm, $\Delta\varepsilon_{303}$, which can be obtained from the depth of the individual curve in Fig. 2, is plotted as a function of the substrate concentration s in Fig. 3. The insert of the figure shows the linearity of the plot of $s/\Delta\varepsilon_{303}$ versus s, indicating a concentration dependence of the Michaelis–Menten type. The maximum change in molar absorptivity ($\Delta\varepsilon_{303}^{max}$) is estimated to be 1220 cm^{-1}, and the substrate concentration for half-saturation $s_{1/2}$ is determined as 0.022% (0.080 mM). The latter value of $s_{1/2}$ is in good agreement with that obtained[17]

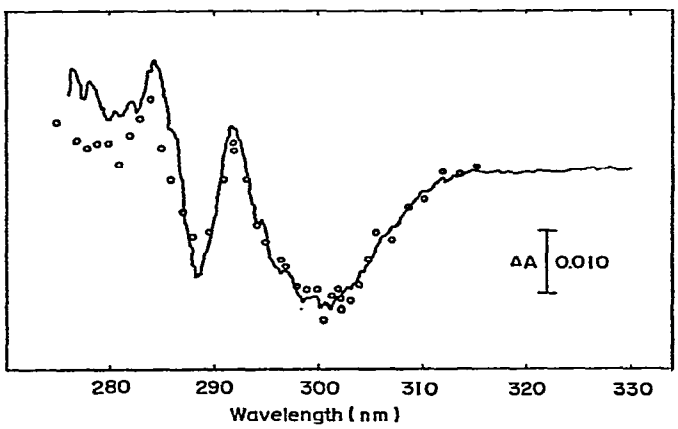

Fig. 5. Comparison between the statically obtained, difference spectrum and that obtained by the stopped-flow method for maltodextrin at 5°. The solid line shows the difference spectrum obtained with a spectrophotometer as in Fig. 1. The open circle shows the absorbance change at various wavelengths obtained by the stopped-flow method (such as shown in Fig. 2); e_0 = 22.0 μM; s = 0.38% (1.4 mM).

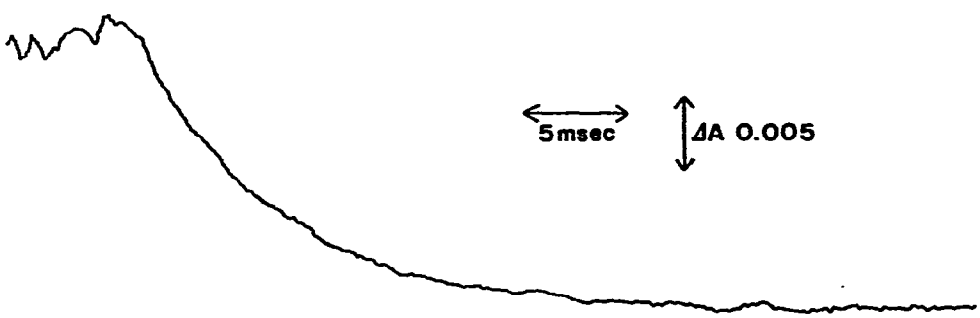

Fig. 6. The time course of the binding of maltodextrin with the enzyme, as observed from the absorbance change at 303 nm at 5°. The curve was obtained with the stopped-flow apparatus (RA-401) and the data-averaging system (RA-450); 5 accumulations; e_0 = 24.3 μM; s = 0.10% (0.36 mM).

from the fluorescence stopped-flow method ($s_{1/2} = 0.027\%$) with maltodextrin at 25°.

Moreover, the area surrounded by the reaction curve in Fig. 2 is proportional to the initial substrate-concentration s, as shown in Fig. 4.

These results have provided us with sufficient evidence for considering that the trough at 303 nm is due to the formation of an essential intermediate of the reaction, the enzyme–substrate complex.

The total change in absorbance (ΔA_{303}) observed with the stopped-flow method (such as shown in Fig. 2) was measured at various wavelengths at 5°, and was compared with the difference spectrum statically obtained with a spectrophotometer at 5°, as shown in Fig. 5. The result demonstrates a reasonable agreement between the two spectra. The discrepancy below 285 nm might be due, in part at least, to the effect of stray light.

In this paper, we focus attention on the binding of the substrate to the enzyme, which can be observed through the rapid decrease in absorbance at 303 nm. The reaction temperature (5°) was chosen so as to lower the rate and facilitate observation. A typical example of the time-course of the reaction obtained with a stopped-flow apparatus (RA-401) and a data-averaging system (RA-450) is shown in Fig. 6.

The reaction fits first-order kinetics, and the apparent first-order rate-constant (k_{app}) can readily be evaluated. The dependence of k_{app} on the substrate concentration s is shown in Fig. 7. Unfortunately, it is rather difficult to judge whether the plot is linear or whether it tends to saturate at higher substrate-concentrations, a factor that is important in the interpretation of the results.

To make this point clear, the same kinetic study was made with maltotriose as the substrate, for which it is much easier to work at higher molar concentrations. An example of a stopped-flow trace and the concentration dependence of k_{app} on s are shown in Figs. 8 and 9, respectively. The hyperbolic concentration-dependence of k_{app} is consistent with a two-step mechanism of the interaction, involving fast,

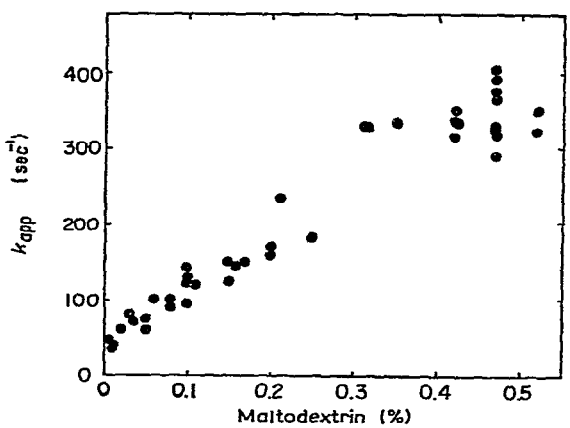

Fig. 7. The dependence on substrate concentration of the apparent first-order rate-constant (k_{app}) for the binding of maltodextrin with the enzyme at pH 4.5 and 5°.

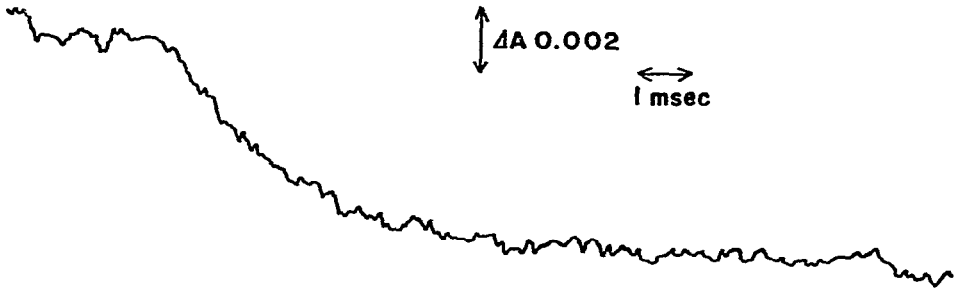

Fig. 8. The time-course of the binding of maltotriose with the enzyme, as observed from the absorbance change at 303 nm at 5°. The curve was obtained with the stopped-flow apparatus and the data-averaging system as in Fig. 6; 17 accumulations; e_0 = 10.9 μM; [Maltotriose] = 5.0 mM.

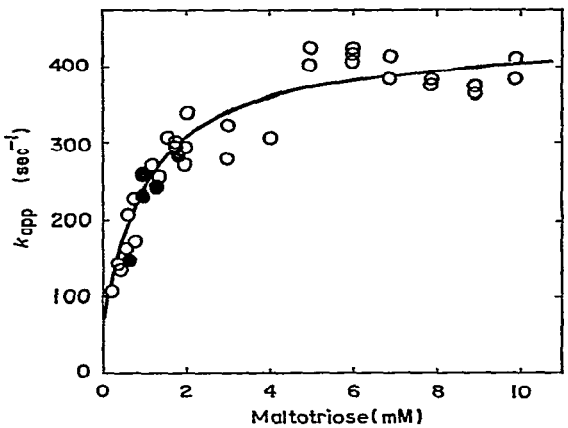

Fig. 9. The dependence on substrate concentration of the apparent first-order rate-constant (k_{app}) for the binding of maltotriose with the enzyme at pH 4.5 and 5°; e_0 = 10.9 μM(○) and 21.3 μM (●). The solid line is the theoretical curve drawn from Eq. 5, by using the following values: K_{-1} = 1.1 mM, k_{+2} = 370 sec^{-1}, k_{-2} = 70 sec^{-1}.

bimolecular association followed by a slower, unimolecular, isomerization-process[23]:

$$E + S \underset{k_{-1}}{\overset{k_{+1}}{\rightleftarrows}} ES \underset{k_{-2}}{\overset{k_{+2}}{\rightleftarrows}} ES^* \qquad (1),$$

where ES and ES* are structural isomers of the enzyme–substrate complex.

Relaxation kinetics[23] predicts that, at most, two relaxation-times τ_1 and τ_2 ($\tau_1 < \tau_2$) may be obtained from the two-step mechanism (Eq. *1*). When the initial substrate-concentration s is sufficiently larger than the enzyme concentration e_0, the reciprocal relaxation-times (which are equal to apparent first-order rate-constants) are given as follows[24]:

$$1/\tau_1 = k_{-1} + k_{+1}s \qquad (2)$$

and

$$1/\tau_2 = k_{-2} + \frac{k_{+2}s}{K_{-1} + s} \quad (3),$$

where K_{-1} ($= k_{-1}/k_{+1}$) is the dissociation constant of the rapidly formed enzyme–substrate complex, ES. The shorter relaxation-time τ_1 may not be obtained if the bimolecular process is too fast to be observed by the method employed, or if no observable change for detection inherently accompanies the process, or both.

The result obtained for maltotriose (Fig. 9) is undoubtedly consistent with the interpretation that k_{app} is equal to $1/\tau_2$, which bears mainly the characteristics of the slower, unimolecular, isomerization-process.

DISCUSSION

The hyperbolic concentration-dependence of k_{app} observed for maltotriose is of the same type as those commonly observed for many enzyme–ligand interactions[25]. The most acceptable interpretation of the result is that the enzyme–substrate interaction is consisted of two steps, a fast, bimolecular association ($E + S \rightleftarrows ES$) followed by a slower, unimolecular, isomerization-process ($ES \rightleftarrows ES^*$), as shown in Eq. *1*. The rate constant (k_{app}) obtained is now given by:

$$k_{app} = k_{-2} + \frac{k_{+2}s}{K_{-1} + s} \quad (4).$$

The overall dissociation-constant (K_s), which can be obtained from the static measurement (see, for example, Fig. 3), is related to K_{-1} and k_{+2}/k_{-2} as follows[24]:

$$K_s = \frac{K_{-1}}{1 + (k_{+2}/k_{-2})} \quad (5).$$

The value of k_{-2} may readily be obtained from the vertical intercept of the plot k_{app} versus s. K_{-1} and k_{+2} may then be obtained in the same manner as for the Michaelis–Menten plot. These values for maltotriose are as follows: $K_{-1} = k_{-1}/k_{+1} = 1.1$mM, $k_{+2} = 370\ sec^{-1}$, and $k_{-2} = 70\ sec^{-1}$. K_s is thus calculated from Eq. *5* as $K_s = 0.17$mM, which is comparable to the value of Michaelis constant K_m (=0.36mM) obtained[13] from the steady-state kinetics at 25°. It may be reasonable to consider that ES* is the essential productive-complex from which the product D-glucose is formed.

The data with maltodextrin as the substrate (Fig. 7) are not sufficient to conclude the two-step mechanism, because of the limited range of substrate concentration studied. However, the values of k_{-2} (the vertical intercept of the plot in Fig. 7) and k_{+2}/K_{-1} (the initial slope of the plot, about $3 \times 10^5\ M^{-1}sec^{-1}$) are similar in magnitude to those of maltotriose (Fig. 9). Moreover, Yamashita has found hyperbolic substrate-concentration dependence of k_{app} similar to that of maltotriose for a series of malto-oligosaccharides (d.p. = 2–7), by monitoring the fluorescence quenching accompanying the enzyme–substrate interaction[19]. Therefore, it is quite

reasonable to consider that the same two-step mechanism (*Eq. 1*) is also valid for maltodextrin.

It is now interesting to see in which of the two processes the characteristic trough at 303 nm does appear. This can be done by comparing the total change in absorbance ΔA_{tot} (see, for example, Fig. 5) with the absorbance change observed in the stopped-flow trace (Fig. 6)[18]. If the trough appears solely in the unimolecular step, the absorbance change observed by the stopped-flow method (Fig. 6), which is corrected to the dead-time of the apparatus*, ΔA_{SF}, must be equal to ΔA_{tot}.

If the fast, bimolecular process were accompanied with appreciable change in absorbance, ΔA_{SF} and ΔA_{tot} would be different.

Now the value of ΔA_{tot} from Fig. 5 at 303 nm may be compared with the value of ΔA_{SF} which can be obtained from Fig. 6. With $s = 0.38\,\%$ and $e_0 = 22\ \mu M$, ΔA_{tot} is 0.024. By using the concentration dependence of $\Delta\varepsilon_{303}$ (Fig. 3), and correcting for the enzyme concentration, this value of ΔA_{tot} is converted into that for $s = 0.1\,\%$ and $e_0 = 24.3\ \mu M$, the conditions for the stopped-flow experiment of Fig. 6. Thus we have, ΔA_{tot} ($s = 0.1\,\%$, $e_0 = 24.3\ \mu M$) = 0.023. On the other hand, Fig. 6 gives the absorbance change actually observed with the stopped-flow method, $\Delta A_{obs} = 0.020$. When this value is corrected for the dead time ($t_d = 1$ msec) by using Eq. 7 with $k_{app} = 130\ sec^{-1}$ (Fig. 7), we have: $\Delta A_{SF} = 0.020 \times e^{0.13} = 0.023$ for $s = 0.1\,\%$ and $e_0 = 2.43\ \mu M$. The excellent agreement between ΔA_{tot} and ΔA_{SF} thus confirms that the trough at 303 nm appears solely in the isomerization process (ES $\rightleftarrows$ ES*), but not in the fast, bimolecular step (E + S $\rightleftarrows$ ES).

As the tryptophan residue responsible for the trough is considered to be located in the first subsite adjacent to the catalytic site[14], which is occupied by substrates only in productive binding-modes[13], it is most likely that the trough due to the environmental change of the tryptophan residue is closely related to the formation of productive complex essential for the catalytic process. Thus the minimal mechanism for the glucoamylasecatalyzed reaction may be represented as follows:

$$\mathrm{E} + \mathrm{S} \underset{k_{-1}}{\overset{k_{+1}}{\rightleftarrows}} \mathrm{ES} \underset{k_{-2}}{\overset{k_{+2}}{\rightleftarrows}} \mathrm{ES}^* \xrightarrow{k_{+3}} \mathrm{E} + \mathrm{P} \qquad (8)$$

where ES* is the essential productive complex that shows the trough at 303 nm, and P is the product. The rate constant k_{+3} may correspond to the formerly defined "intrinsic rate-constant k_{int} for the bond cleavage" in the productive complex[13]. As k_{+2} (= 370 sec^{-1} for maltotriose at 5°) is appreciably larger than k_{int} (= 77 sec^{-1}

*Let the process be of a (pseudo)first order having a rate constant k_{app}, and ΔA_{obs} be the fast change in absorbance actually observed in the stopped-flow apparatus having a dead time t_d. Then, the correction for the dead time may be made with the following equation:

$$k_{app} t_d = \ln(\Delta A_{SF}/\Delta A_{obs}) \qquad (6)$$

or

$$\Delta A_{SF} = \Delta A_{obs} \cdot \exp(k_{app} t_d). \qquad (7)$$

at 25°)[13], the rate-limiting step is considered to be the bond-cleavage process (the k_{+3} step).

The k_{+2} value for glucoamylase, which is closely related to the productive complex-formation, is much larger than the corresponding rate-constant for lysozyme–oligosaccharide interaction obtained by Holler *et al.*[26] (k_{23} = 10–20 sec^{-1} at 25° for β-process), and rather similar in magnitude to those for the unproductive complex-formation for lysozyme[24,26].

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REFERENCES

1 Y. TSUJISAKA, J. FUKUMOTO, AND T. YAMAMOTO, *Nature*, 181 (1958) 770–771.
2 S. ONO, K. HIROMI, AND M. ZINBO, *J. Biochem. (Tokyo)*, 55 (1964) 315–320.
3 K. HIROMI, Z. HAMAUZU, K. TAKAHASHI, AND S. ONO, *J. Biochem. (Tokyo)*, 59 (1966) 411–418.
4 K. HIROMI, K. TAKAHASHI, Z. HAMAUZU, AND S. ONO, *J. Biochem. (Tokyo)*, 59 (1966) 469–475.
5 K. HIROMI, M. KAWAI, AND S. ONO, *J. Biochem. (Tokyo)*, 59 (1966) 476–480.
6 N. SUETSUGU, E. HIROOKA, H. YASUI, K. HIROMI, AND S. ONO, *J. Biochem. (Tokyo)*, 73 (1973) 1223–1232.
7 K. HIROMI, M. KAWAI, N. SUETSUGU, Y. NITTA, T. HOSOTANI, A. NAGAO, T. NAKAJIMA, AND S. ONO, *J. Biochem. (Tokyo)*, 74 (1973) 935–943.
8 S. ONO, K. HIROMI, AND Z. HAMAUZU, *J. Biochem. (Tokyo)*, 57 (1965) 34–38.
9 Z. HAMAUZU, K. HIROMI, AND S. ONO, *J. Biochem. (Tokyo)*, 57 (1965) 39–41.
10 K. HIROMI AND S. ONO, *J. Biochem. (Tokyo)*, 53 (1963) 164–166.
11 K. HIROMI, K. OGAWA, N. NAKANISHI, AND S. ONO, *J. Biochem. (Tokyo)*, 60 (1966) 439–449.
12 K. HIROMI, *Biochem. Biophys. Res. Commun.*, 40 (1970) 1–6.
13 K. HIROMI, Y. NITTA, C. NUMATA, AND S. ONO, *Biochim. Biophys. Acta*, 302 (1973) 362–375.
14 M. OHNISHI, H. KEGAI, AND K. HIROMI, *J. Biochem. (Tokyo)*, 77 (1975) 695–703.
15 M. OHNISHI, T. YAMASHITA, AND K. HIROMI, *J. Biochem. (Tokyo)*, 79 (1976) 1007–1012.
16 M. OHNISHI AND K. HIROMI, *J. Biochem. (Tokyo)*, 79 (1976) 11–16.
17 K. HIROMI, M. OHNISHI, AND T. YAMASHITA, *J. Biochem. (Tokyo)*, 76 (1974) 1365–1367.
18 M. OHNISHI, T. YAMASHITA, AND K. HIROMI, *J. Biochem. (Tokyo)*, 81 (1977) 99–105.
19 T. YAMASHITA, Master's Thesis, Kyoto University, Faculty of Agriculture, 1976.
20 M. KATO, K. HIROMI, AND Y. MORITA, *J. Biochem. (Tokyo)*, 75 (1974) 563–576.
21 K. HIROMI, S. ONO, S. ITOH, AND T. NAGAMURA, *J. Biochem. (Tokyo)*, 64 (1968) 897–900.
22 B. TONOMURA, H. NAKATANI, M. OHNISHI, J. YAMAGUCHI-ITO, AND K. HIROMI, *Anal. Biochem.*, 84 (1978), in press.
23 M. EIGEN AND L. DE MAEYER, in A. WEISSBERGER (Ed.), *Technique of Organic Chemistry*, Interscience Publishers, New York, 1963, Vol. 8, Part 2, pp. 895–1054.
24 S. E. HALFORD, *Biochem. J.*, 149 (1975) 411–422.
25 G. G. HAMMES AND P. R. SCHIMMEL, in P. D. BOYER (Ed.), *The Enzymes*, Academic Press, New York, 1970, Vol. 2, pp. 67–114.
26 E. HOLLER, J. A. RUPLEY, AND G. P. HESS, *Biochemistry*, 14 (1975) 2377–2385.