Carbohydrate Research, 195 (1989) 138–144
Elsevier Science Publishers B.V., Amsterdam – Printed in The Netherlands

Note

Binding of maltose to *Rhizopus niveus* glucoamylase in the pH range where the catalytic carboxyl groups are ionized*

MASATAKE OHNISHI† AND KEITARO HIROMI

Laboratory of Enzyme Chemistry, Department of Food Science and Technology, College of Agriculture, The University of Kyoto, Sakyo-ward, Kyoto-city, Kyoto 606 (Japan)

(Received November 25th, 1988; accepted for publication in revised form, May 1st, 1989)

From steady-state-kinetic and thermodynamic studies with maltose as substrate, Hiromi *et al.*[1] concluded that the catalytic groups of *Rhizopus* glucoamylase are $-CO_2^-$ and $-CO_2H$, having pK values 2.9 and 5.9, respectively; the latter is not concerned in substrate binding, but it has an essential role in the catalysis as its deprotonation inactivates the enzyme[2]. Chemical modification with water-soluble carbodiimide supports the conclusion that carboxyl groups are essential for enzymic activity[3].

At the optimal pH (4.5) for the glucoamylase-catalyzed reaction, binding of substrate maltooligosaccharides to the enzyme subsites has been observed by the stopped-flow method, monitoring u.v. absorption and fluorescence of tryptophan residue(s) of the enzyme. The binding has been predicted[4–6] to be two-step mechanism, in which a fast bimolecular association-process is followed by a slow unimolecular isomerization-process, for all substrate linear maltoooligosaccharides (G_2–G_7) examined.

This paper provides evidence on the relation of substrate binding with catalysis for the glucoamylase from *Rhizopus niveus*, using the stopped-flow method and difference-absorption and fluorescence spectrophotometry. The results suggest that glucoamylase recognizes and binds its substrates even at pH 7.9, where a catalytic carboxyl group is ionized in the inactive form, $-CO_2^-$.

EXPERIMENTAL

Materials. — Glucoamylase from *Rhizopus niveus* (the purest grade, lyophilized, Toyobo Biochemicals Co., Tokyo) was used without further purification. The enzyme concentration was determined spectrophotometrically at 280 nm,

*Supported in part by a Grant-in-aid for Scientific Research from the Ministry of Education, Science and Culture of Nippon (Japan).

†To whom correspondence should be addressed.

0008-6215/89/$ 03.50 © 1989 Elsevier Science Publishers B.V.

using the absorption unit $A^{1\%}_{280\,nm}$ = 15.6 cm^{-1} and the molecular weight[7] of 58,000. Maltose, grade HHH (purest) was purchased from Hayashibara Biochemical Laboratories Inc., Okayama and other chemicals employed were guaranteed-grade products of Kanto Chemical Co., Tokyo.

Methods. — The time-course of the fluorescence intensity-change (decrease), produced by the binding of maltose to the enzyme was observed with a Union Giken (recently revised to PHOTAL, Otsuka Electronics) RA-401 stopped-flow spectrophotometer with a 200W D_2 lamp as the light source, using the same procedure described previously[4,5]. Reaction curves were accumulated usually 9 times by using a Union Giken (PHOTAL) RA-450 data-averaging processor to improve the signal-to-noise ratio.

The u.v.-difference absorption-spectrum and fluorescence spectrum of the enzyme, produced by the binding of maltose, were measured by using a Union Giken (PHOTAL) SM-401 high-sensitivity spectrophotometer with tandem quartz cells and a Union Giken (PHOTAL) FS-401 fluorescence spectrophotometer, respectively, as described elsewhere[8–10].

For the steady-state kinetic studies, hydrolysis of maltose catalyzed by *Rhizopus* glucoamylase was observed at pH 7.5 and 4.5 by the "Gluco-stat" enzymic method (using glucose oxidase and peroxidase), with reagents purchased from Toyobo Biochemicals Co., Tokyo.

Reactions and observations (kinetic and static) on the binding of maltose to the enzyme were generally performed at pH 7.9 with 0.02M phosphate buffer at 0.5°. Kinetic and binding parameters were determined by using an NEC PC9801-VM2 personal computer with Ookubo Mycom "BIO-GRAPH" software.

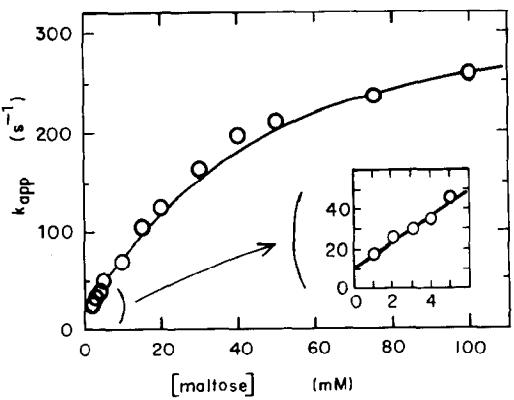

Fig. 1. Dependence of the apparent first-order rate-constant k_{app} on the initial concentration of maltose. The fluorescence stopped-flow method. Experimental conditions: pH 7.9 and 0.5°. The solid line was obtained with the values, K_{-1} = 47mM, k_{+2} = 370 s^{-1}, and k_{-2} = 11 s^{-1} according to Eq. 2. "Two-step mechanism" for maltose binding to glucoamylase: the dependence of k_{app} on $[maltose]_0$ predicts a two-step mechanism, involving a fast bimolecular association-process followed by a slow isomerization-process.

RESULTS AND DISCUSSION

Stopped-flow observation on the binding of maltose to glucoamylase at pH 7.9. — Over the concentration range of maltose employed, only one apparent relaxation was found; obeyed first-order kinetics, and hence the apparent first-order rate-constant (k_{app}) was evaluated from a Guggenheim plot. Fig. 1 (open circles) shows the plots of k_{app} thus obtained against [maltose], the initial concentration of maltose. This hyperbolic dependency of k_{app} on [maltose] cannot be interpreted by a one-step mechanism, but is certainly consistent with the binding manner expected from a "two-step mechanism" in which a fast bimolecular association-processs is followed by a slow unimolecular isomerization process:

$$E + M \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} (EM)^* \underset{k_{-2}}{\overset{k_{+2}}{\rightleftharpoons}} (EM) \qquad (1),$$

where E, M, (EM)*, and (EM) denote respectively the enzyme, maltose, the maltose–enzyme complex, and an isomerized form of the complex, and k_{+1}, k_{-1}, k_{+2}, and k_{-2} are the rate constants for each elementary process represented in Eq. *1*. For this mechanism, two relaxation times can be expected as maxima, and the reciprocal of the slow one, the apparent rate-constant k_{app}, is given under conditions where $[M]_0 \gg [E]_0$ as follows:

$$k_{app} = k_{+2}[M]_0/(K_{-1} + [M]_0) + k_{-2} \qquad (2),$$

where K_{-1} is the dissociation constant of the (EM)* complex, and hence $K_{-1} = [E][M]/[(EM)^*] = k_{-1}/k_{+1}$, and $[M]_0$ is the initial concentration of maltose ([maltose] in Fig. 1). Based on the plot of k_{app} against $[M]_0$, the binding parameters, K_{-1}, k_{+2}, and k_{-2} can be evaluated as described elsewhere[4,5] and are represented in Table I, together with those obtained previously[6] at pH 4.5.

A theoretical curve of the k_{app} *vs.* $[M]_0$ plot may be drawn with the binding parameters obtained according to Eq. *2*. As shown in Fig. 1 (solid line), the theoretical curve is consistent with the experimental points (open circles), indicating that the maltose binding at pH 7.9 fits with the two-step mechanism. The difference in binding parameters at pH 4.5 and pH 7.9 (Table I) are as yet unexplained. In the enzyme subsites, some groups ionizable at alkaline pH (at least 7.9) may be concerned with substrate binding. Based on the two-step mechanism, the overall dissociation constant K_d is represented as follows:

$$K_d = ([E][M])/\{[([EM]^*)] + [(EM)]\}$$
$$= K_{-1}/(1 + k_{+2}/k_{-2}) \qquad (3)$$

TABLE I

BINDING PARAMETERS[a] FOR THE MALTOSE-GLUCOAMYLSE INTERACTION

Conditions	K_{-1} (mM)	k_{+2} (s^{-1})	k_{-2} (s_{-1})	K_d (mM)
pH 7.9				
0.5°	47 ±4.8	370 ±15	11 ±1	1.4
8.0°	38 ±7.5	550 ±80	20 ±2.9	1.5
25.0°				1.6 ±0.3[b]
pH 4.5[c]				
0.5°	7.3 ±0.74	1,500 ±55	55 ±5	0.28

[a]The K_d value is calculated according to Eq. *3*. [b]The K_d value evaluated experimentally by u.v.-difference spectrophotometry from Eqs. *4* and *5*. [c]The binding parameters at pH 4.5 are those reported previously[6], and ± is the standard deviation.

With the values k_{+2}, k_{-2}, and K_{-1} already obtained, the dissociation constant K_d may be calculated according to Eq. *3*, as the results are shown in Table I.

The binding of maltose at pH 7.9 is also significantly inhibited by D-glucose (data not shown). For example, in the presence of D-glucose (92.5mM), apparent values of K_{-1}, k_{+2}, and k_{-2} (pH 7.9, 8°) were determined to be 73 ±33mM, 600 ±170 s^{-1}, and 20 ±9.5 s^{-1}, respectively, by the procedure described previously[5] at pH 4.5. The binding parameters, k_{+2} and k_{-2}, in the presence of D-glucose are almost equal to those obtained in the absence of D-glucose (pH 7.9, 8°), except for K_{-1} (see Table I), suggesting that D-glucose inhibits exclusively the fast, bimolecular association-process of maltose binding. This type of inhibition of the fast bimolecular-process has been found[5] in the binding of maltose at pH 4.5. This is additional evidence for binding of maltose to the enzyme subsites at pH 7.9 by the same mechanism as that at the optimal pH.

From steady-state kinetic observations, the molecular activity k_0 at pH 7.5, 25° for the glucoamylase-catalyzed hydrolysis of maltose was determined to be 0.074 ±0.011 s^{-1}, which is less than 1/50 of that (4.6 s^{-1}) observed at pH 4.5, 25°. These kinetic experiments were carried out at pH 7.5 instead of a pH 7.9, because the rate at pH 7.9 is too low for observation. The Michaelis constant K_m is 1.9 ±0.2mM, and thus k_0/K_m is about 1/60 of that at pH 4.5. The binding of maltose at pH 7.5, 25° was also observed by the fluorescence stopped-flow method. The binding parameters, K_{-1}, k_{+2}, and k_{-2}, thus evaluated are 40 ±6.5mM, 2000 ±190 s^{-1}, and 30 ±2.9 s^{-1}, respectively, and hence K_d is 0.60mM while the binding parameters, K_{-1}, k_{+2}, and k_{-2} at pH 4.5, 5.0° are 5.61 ±0.6mM, 2000 ±80 s^{-1}, and 45.1 ±3 s^{-1}, respectively[5], and hence K_d is calculated to be 0.12mM. It is noteworthy that the difference (5–7 fold) in K_{-1} and K_d between pH 7.5 and pH 4.5 is much less than that (40–60 fold) in k_0 and k_0/K_m.

These findings suggest that maltose is bound to glucoamylase subsites even at pH 7.9, where a catalytic group -CO_2H (whose pK is 5.9 for both the free enzyme and the ES complex[2]) is almost fully ionized into the inactive -CO_2 form, and the

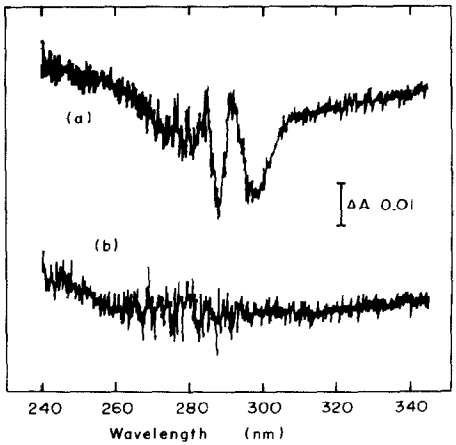

Fig. 2. U.v.-difference absorption-spectrum of glucoamylase produced by the binding of maltose. pH 7.9, 25.0°, Glucoamylase, 16.4 μM; maltose, (*a*) 400mM; (*b*) 0mM.

group carries a decisive role not in substrate binding but in the catalytic process, as reported by Hiromi *et al.*[2].

U.v.-difference absorption- and fluorescence-spectrophotometry studies on the maltose binding to glucoamylase at pH 7.9. — U.v.-difference absorption-spectra of glucoamylase were observed at pH 7.9, 25° with addition of maltose to the enzyme solution. A typical example is shown in Fig. 2; the spectra show a trough around 302 nm. We described earlier[9,11] that, at pH 4.5, substrate maltose is bound at specialized subsites to interact with a tryptophan residue and consequently produces a u.v.-difference absorption-spectrum having a trough at 302 nm attributable to a change in state around the tryptophan residue located at Subsite 1. The difference absorption-spectrophotometry thereby supports the idea that maltose is bound at the enzyme subsites, even at pH 7.9, in the same way that it is as bound at pH 4.5. The same u.v.-difference absorption-spectra were also observed at pH 3.0, 25° and gradually disappeared with progress of the enzyme-catalyzed reaction (data not shown), indicating that the maltose binding itself does produce a characteristic difference spectrum over the wide pH range (3–8) examined. A change in microenvironment of the tryptophan residue located at Subsite 1 may be induced by the binding of maltose.

The dependence of the difference absorption at 302 nm per molar concentration of the enzyme ε_{302} upon the initial concentration of maltose [maltose] was examined at pH 7.9 for the elucidation of K_d, as shown in Fig. 3. Based on the following mechanism:

$$E + M \underset{K_d}{\rightleftharpoons} (EM) \qquad (4),$$

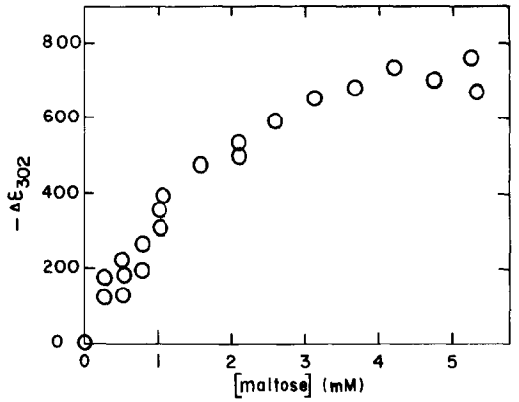

Fig. 3. Dependence of $-\Delta\varepsilon_{302}$ on the initial concentration of maltose [maltose]. Glucoamylase; 16.4 μM, pH 7.9, 25.0°.

the K_d value of the maltose–enzyme complex ([M][E]/[(EM)]) may be determined as follows. The $\Delta\varepsilon_{302}$ value is proportional to [(EM)] and should vary with $[M]_0$ in conformity with the Michaelis equation:

$$\Delta\varepsilon = \Delta\varepsilon_{max}[M]/(K_d + [M]) \quad (5).$$

As $[M]_0 \gg [E]_0$, based on a linear equation derived from Eq. 5, the values of K_d and $\Delta\varepsilon_{max}$ (of which the latter is the maximum change in the difference absorption and would be observed when the enzyme is saturated with maltose) were evaluated by the same procedure as described previously[9,10] and are shown in Table I. The K_d value is 1.6mM almost the same as that observed[9,12] at pH 4.5. On the other hand,

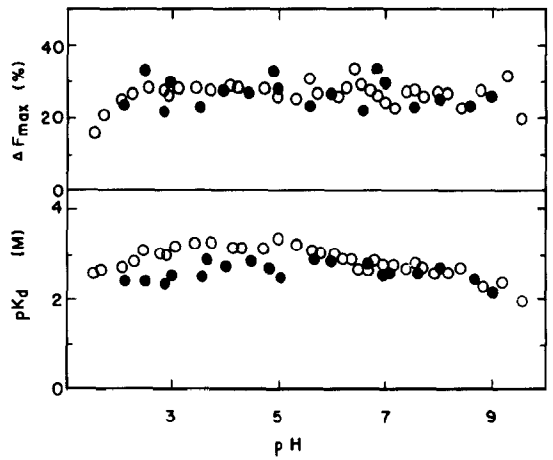

Fig. 4. Dependence of the K_d and ΔF_{max} values on pH. Glucoamylase; 0.97 and 1.1 μM at 5.0° (open circles) and 25.0° (closed circles), respectively. K_d and ΔF_{max} were obtained according to Eq. (6). pK_d denotes $-\log K_d$.

the K_d values (10–500mM) for analogues examined at pH 4.5 (except for D-glucono-1,5-lactone[9,10,12–14]) are much larger than that obtained for maltose at pH 7.9 (K_d = 1.6mM), indicating that binding of maltose, even at pH 7.9, is stronger than that of substrate analogues at the optimal pH.

A change (decrease) in fluorescence, which has a peak at 340 nm with excitation at 280 nm and relates to the tryptophan residue, was also found at pH 7.9 upon binding of maltose to the enzyme (spectra not shown), as also found at pH 4.5. The dependence of the fluorescence intensity-change (ΔF) on the initial concentration of maltose, $[M]_0$, gives the dissociation constant K_d, according to the scheme shown in Eq. *4*. The ΔF value is proportional to [(EM)] and the experiments were carried out under conditions where $[M]_0 \gg [E]_0$, and thus K_d may be obtained as already described:

$$\Delta F = \Delta F_{max}[M]_0/(K_d + [M]_0) \qquad (6),$$

where ΔF_{max} is the ΔF value that would be observed when the enzyme is saturated with maltose. The K_d and ΔF_{max} values were evaluated over the pH range 2.2–9.3, at 5° and 25°, as illustrated in Fig. 4. These values are almost independent of pH between 3 and 8. The K_d and ΔF_{max} values at pH 7.9, 25° are nearly equal to those at pH 4.5, supporting the suggestion already made on maltose binding.

These results indicate that glucoamylase recognizes its own substrate and binds it at the active site, even in an inactive state of the enzyme at pH 7.9, far from the optimal, and a catalytic carboxyl group ($-CO_2H$) has little role in substrate binding. The binding-process for the substrate thus does not necessarily induce a change in state of the catalytic group to initiate catalytic process. Binding of substrate in the pH range where the catalytic carboxyl groups are ionized may be a form of regulation of the glucoamylase-catalyzed reaction.

REFERENCES

1 A. FERSHT, *Enzyme Structure and Mechanism*, W. H. FREEMAN, San Francisco, 1977, pp. 244–287.
2 K. HIROMI, K. TAKAHASHI, Z. HAMAUZU, AND S. ONO, *J. Biochem. (Tokyo)*, 59 (1966) 469–475.
3 D. FRENCH AND M. OHNISHI, in preparation; C. J. GRAY AND M. E. JOLLEY, *FEBS Lett.*, 29 (1973) 197–200; N. INOKUCHI, M. IWAMA, T. TAKAHASHI, AND M. IRIE, *J. Biochem. (Tokyo)*, 91 (1982) 125–133.
4 M. OHNISHI AND K. HIROMI, *Carbohydr. Res.*, 61 (1978) 335–344.
5 A. TANAKA, M. OHNISHI, AND K. HIROMI, *Biochemistry*, 21 (1982) 107–113.
6 A. TANAKA, T. YAMASHITA, M. OHNISHI, AND K. HIROMI, *J. Biochem. (Tokyo)*, 93 (1983) 1037–1043.
7 A. TANAKA, M. OHNISHI, K. HIROMI, S. MIYATA, AND S. MURAO, *J. Biochem. (Tokyo)*, 91 (1982) 1–9.
8 M. OHNISHI, *J. Biochem. (Tokyo)*, 69 (1971) 181–189.
9 M. OHNISHI, H. KEGAI, AND K. HIROMI, *J. Biochem. (Tokyo)*, 77 (1975) 695–703.
10 M. OHNISHI AND D. FRENCH, *Carbohydr. Res.*, 165 (1987) 155–160.
11 K. HIROMI, M. OHNISHI, AND A. TANAKA, *Mol. Cell. Biochem.*, 51 (1983) 79–95.
12 M. OHNISHI, S. WADA, T. YAMADA, A. TANAKA, AND K. HIROMI, *J. Jpn. Soc. Starch Sci. (Tokyo)*, 30 (1983) 57–61.
13 K. HIROMI, A. TANAKA, AND M. OHNISHI, *Biochemistry*, 21 (1982) 102–107.
14 M. OHNISHI, *Introduction to Enzyme Science*, Wiley Japan, Tokyo, 1984, p. 88.