ALLOSTERIC BEHAVIOR IRRESPECTIVE OF CONFORMATIONAL CHANGE OF ENZYME PROTEIN. SIGMOIDAL CONCENTRATION DEPENDENCE OF RATE OF ACTION OF SACCHARIFYING α-AMYLASE ON MALTOSE

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1. Introduction

The sigmoidal concentration dependence of the rate of enzyme reactions has been termed allosteric behavior or positive cooperativity. There are several kinds of theory which interpret the phenomenon quantitatively: Monod-Wyman-Changeux's [1], Koshland-Némethy-Filmer's [2], Rabin-Frieden-Ainslie's [3–5] and Ricard-Meunier-Buc's [6]. The former two are concerned with oligomeric enzymes only, while the latter two are applicable to monomeric enzymes as well. Of these, the third one predicts that a lag phase in the time course of reaction can be accompanied by positive cooperativity [5]. All of the theories are based on the conformational change of enzyme protein as being responsible for the allosteric behavior.

The reaction of saccharifying α-amylase from *B. subtilis* with maltose as the substrate shows a strong sigmoidal concentration dependence of rate, with the Hill coefficient as high as 3.3–3.8 [7]. Fujimori [8] carefully re-examined the same reaction using purified maltose, following the reaction polarimetrically, and found that a lag phase appears in the initial stage of the reaction and the steady-state rate of the reaction (the linear part after the lag) shows a concentration dependence with a Hill coefficient of 3.8 [8]. The lag and the positive cooperativity observed simultaneously are similar to the feature of monomeric allosteric enzymes with slow conformational transition of the enzyme protein, as proposed by Ainslie et al. [5].

In this paper, we aimed to investigate the origin of the strong cooperativity and the observed lag. Quantitative determination of the substrate and products by paper chromatography was used to follow the reaction. The results showed that no change was detected during the lag phase and thereafter maltotriose and maltotetraose began to appear and finally disappear. Moreover, the addition of a small amount of maltotriose was found to eliminate the lag phase. The computer simulation of the time course of the reaction based on a reaction scheme involving transglycosylation and condensation in addition to hydrolysis has demonstrated that the observed lag phase and the strong positive cooperativity can be reasonably reproduced. The results have provided a novel interpretation of allosteric behavior irrespective of any conformational change of the enzyme protein.

2. Materials and methods

Crystalline saccharifying α-amylase from *B. subtilis* was prepared according to the method of Fukumoto et al. [9]. Maltose was purified by paper chromatography from the product of Hayashibara Biochemical Laboratories, Inc.

The enzyme reaction was performed in 0.02 M acetate buffer, pH 5.4, at 25°C, using maltose anomerically equilibrated as the substrate. Quantitative determination of the substrate and products in the reaction mixture was performed by paper chromatography, with Whatman 3MM filter paper, developed three

times at room temperature using 65% (v/v) *n*-propanol as the solvent.

The reducing sugars were determined by the Somogyi-Nelson method [10] after being eluted separately. For malto-oligosaccharides, glucoamylase was used to increase the sensitivity of the determination [8]. For polarimetric measurements, a high sensitivity polarimeter (± 0.0002°) (Union Giken OR-70) was used at 411 nm to monitor continuously the time course of the reaction or to measure the optical rotation change after stopping the reaction with NaOH and completing the mutarotation.

3. Results and discussion

The time course of the reaction followed polarimetrically showed a clear lag in the initial stage of the reaction (similar to that shown in fig.3), and the steady-state rate obtained from the linear part after the lag showed a concentration dependence very similar to that observed by Shibaoka et al. ([7] see fig.2) from which the Hill coefficient for equilibrated maltose was calculated to be 3.8 [8].

The results of the quantitative determination by paper chromatography are shown in fig.1. No change occurs in the lag phase, indicating that the glucosidic linkage of the substrate is highly resistant to cleavage leading either to hydrolysis or to transglycosylation.

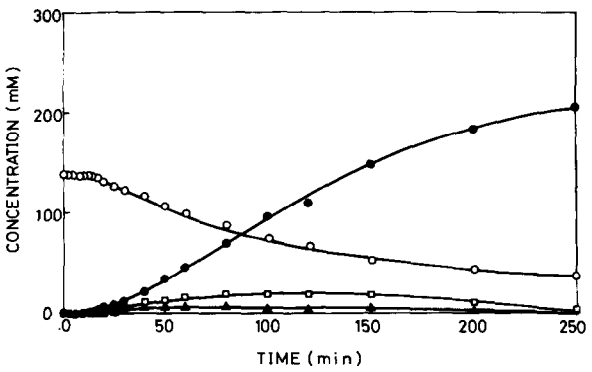

Fig.1. Quantitative determination by paper chromatography of the substrate and products in the time course of the enzymatic reaction of saccharifying α-amylase with maltose. The enzyme concentration was 3.87 μM, and the initial concentration of maltose was 140 mM. (●) Glucose, (○) maltose, (□) maltotriose, (▲) maltotetraose.

After the lag phase, the decrease in substrate concentration starts concomitantly with the appearance of glucose. At the same time, maltotriose and maltotetraose begin to appear, reach their maxima and finally disappear. The addition of small amount of maltotriose to the system (7 mM versus 140 mM maltose) eliminated the lag phase almost completely. These phenomena are quite similar to those observed by Chipman [11] for the lysozyme-catalyzed reaction of oligosaccharides.

The proposed reaction scheme, including transglycosylation and condensation in addition to hydrolysis is presented in fig.2.

Since the subsite structure of this enzyme can be

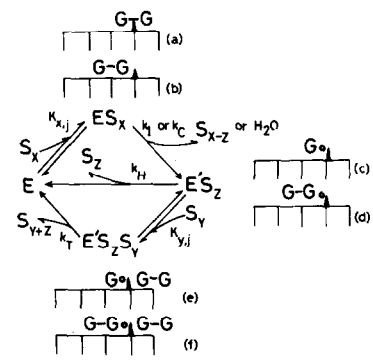

Fig.2. Schematic representation of the mechanism of action of the saccharifying α-amylase from *B. subtilis*. E and S represent enzyme and substrate, respectively. (ES_x). The enzyme–substrate complex ($E'S_z$) the reactive intermediate, ($E'S_zS_y$) the ternary complex formed from ($E'S_z$) and the acceptor S_y. The suffixes x, y and z represent the degree of polymerization of the substrate, integers larger than 1. k_I is the rate constant of reactive intermediate formation from productively bound substrate, k_C the rate constant of reactive intermediate formation from nonproductively bound substrate, k_H the rate constant of hydrolysis and k_T the rate constant of transglycosylation. $K_{x,j}$ and $K_{y,j}$ are the binding constants of which binding modes are specified by j. (a)–(f) show the models of complexes for maltose as the substrate. The four compartments show the four subsites of the enzyme. The wedge represents the catalytic site, and G–G, maltose. G–G° and G° represent the reactive-intermediate forms derived from the non-productively-bound and productively-bound maltose to the subsites of the enzyme, respectively. (a) and (b) are the productive and non-productive complexes of maltose, respectively. (c) and (d) are the reactive intermediates produced from (a) and (b), respectively. (e) and (f) are the ternary complexes with maltose as an acceptor, formed from (c) and (d), respectively.

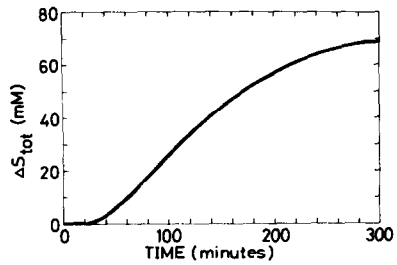

Fig.3. Reaction of saccharifying α-amylase with maltose. The change in total molar concentration of all the species (substrate and products) ($\Delta S_{tot} = \sum_{n=1}^{9} S_n - S_0$) as a function of time, was obtained by computer simulation. The concentration of maltose was 140 mM, and of enzyme, 3.87 μM. The rate constants (fig.2) used were k_I = 3,420 min^{-1}, k_C = 0.0 min^{-1}, k_T/k_H 1,154. The subsite affinities, A_1–A_4, are 2.20, 3.20, 1.38 and 0.52 kcal/mol, respectively. The maximum oligomer length considered in the calculation was 9.

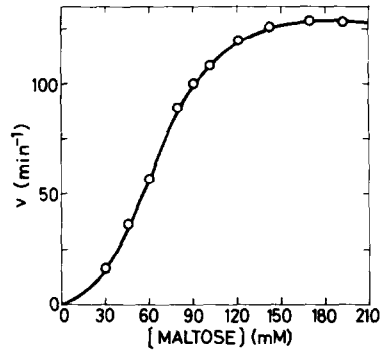

Fig.4. Reaction of saccharifying α-amylase with maltose. The result of computer simulation for the dependence of the steady-state v on the substrate concentration. The steady-state rate v is the rate of increase in the total reducing end for all species per 1 M of enzyme concentration, at which $d^2\Delta S_{tot}/dt^2 = 0$ holds, calculated from the results shown in fig.3. The constants used are the same as those described in the legend to fig.3.

estimated from the dependence of the rate parameters upon the degrees of polymerization n of the linear substrates [12], it is now possible to simulate the time course of the reaction by using two independent adjustable parameters; one subsite affinity A_i out of four in total [12], and the ratio of the rate constants k_T/k_H, assuming that $k_I \ll k_H$ and $k_I \ll k_T$. The details of the calculation will be published elsewhere [13]. An example of the results of the simulation is shown in fig.3, in which the change in total molar concentrations of all species, ΔS_{tot}, which is proportional to the change in optical rotation, is plotted against time. The lag phase followed by the approximately linear part is clearly seen.

It should be emphasized that the plot of the simulated steady-state rate v, (the rate at which $d^2\Delta S_{tot}/dt^2 = 0$ holds) versus the initial substrate concentration shows a strong sigmoidal character as seen from fig.4. The Hill coefficient calculated from the plot was 3.5, which is quite close to the experimental value of 3.8. Thus the scheme shown in fig.2 and the parameters used (see legend to fig.3) have satisfactorily reproduced the main feature of the experimental results, the lag phase and the strongly positive cooperativity.

The positive cooperativity has so far been accounted for by assuming, explicitly or implicitly, the conformational change of the enzyme protein. However, the present study has clearly demonstrated that a Hill coefficient as high as 3 or more can arise from the reaction mechanism, irrespective of any conformational change of the enzyme protein.

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