THE KINETICS OF AMYLOLYSIS

PART III. EFFECT OF SELF-INHIBITION ON THE KINETIC CONSTANTS OF BETA-AMYLOLYSIS

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ABSTRACT

For beta-amylolysis, the number of bonds split per effective enzyme–substrate encounter has been determined with amyloses having various degrees of polymerization (d.p.), labelled with ^{14}C. The degradation is shifted in the direction of the "single chain mechanism" with increasing d.p. By taking into account the "inactive, non-reacting" collisions leading to "self-inhibition", the number of bonds split per "reactive" and "non-reactive" enzyme–substrate encounter was established. From these data, and from the inhibition constant of the inner D-glucose residues of the amylose chain, in conjunction with the Michaelis constant extrapolated to d.p. = 4, the actual rate constants for the formation and dissociation of the "reactive" enzyme–substrate complexes and for the formation and dissociation of the "non-reactive" enzyme–substrate complexes were determined. It was found that the rate constants for the formation of the "reactive" complexes decrease slowly with the increase of "self-inhibition", whereas those for the dissociation of these complexes decrease to a greater extent.

INTRODUCTION

beta-Amylase can be coupled not only to the non-reducing end of the amylose chain but also to the "inner" D-glucose residues of the molecule[1,2]. However, the enzyme–substrate complex formed in this way does not induce bond splitting and thus effectively lowers the enzyme concentration. The degree of "non-reactive" enzyme-binding does not depend upon the ratio of enzyme to substrate, and "inactive enzyme–substrate complexes" are in fact formed even in the case of a great excess of substrate. An increase in the d.p. of the substrate raises the number of inactive bond sites, whereas a decrease in the d.p. leads to the smallest substrate molecule which is inclined solely to the formation of a reactive complex. The affinity of beta-amylase for the non-reducing chain-end of the amylose molecule is not the same as its affinity for the inner D-glucose residues[1]. From the dependence of the affinity on the d.p. and from the apparent Michaelis constant referred to the total D-glucose unit, respectively, the conclusion may be drawn that the affinity of crystalline, sweet-potato beta-amylase at 30° and pH 4.8 is 1000 l/mole with respect to the non-reducing chain-end of the substrate, and 146 l/mole with respect to the inner D-glucose residues[2]. Thus, on

average, the affinity of 6.85 inner D-glucose residues is the same as that of the non-reducing chain-end. This value corresponds fairly well with the average number of D-glucose residues of a deformed amylose spiral[3,4]. In other words, this means that the terminal spiral, which contains the non-reducing D-glucose residue of the amylose molecule, has the same probability of forming an enzyme–substrate complex as do the inner spirals of the amylose molecule. Consequently, HO-4 of the non-reducing D-glucose residue of the substrate does not play any role in the formation of the enzyme–substrate complex.

The ratio of "reactive" enzyme–substrate encounters can be calculated from the linkage of the enzyme to the inner spirals of the amylose molecule, *i.e.* from the so-called "self-inhibitor" effect. From the established ratio, it is possible to obtain[5] the actual rate constants for the degradation of an amylose substrate labelled with ^{14}C. This method is suitable for determination of the actual rate constants for formation and dissociation, respectively, of both the "reactive" and "non-reactive" enzyme–substrate complexes[5].

We now report on the manner in which these rate constants are affected by an increase in the d.p. of the substrate.

EXPERIMENTAL

Materials. — beta-Amylase (crystalline, sweet-potato enzyme; Sigma Chemical Co.): 1 mg of the enzyme was capable[6] of forming 875 mg of maltose in 3 min at pH 4.8 and 20°. The enzyme, marketed as a suspension, was centrifuged and dissolved in a 0.02M sodium acetate buffer at pH 4.8.

Wheat amylose was hydrolysed with 0.01M hydrochloric acid for 15 min at 100°, neutralized, and fractionated with ethanol. In this way, three fractions were obtained; the number-average d.p. values were 248, 46, and 22 D-glucose residues, as measured by the Somogyi and Nelson method[7]. The degree of heterogeneity of the individual samples was 1.5, 2.2, and 2.6 (expressed as the ratio of the weight-average d.p. to the number-average d.p.).

D-Glucose residues universally labelled with ^{14}C were coupled to the non-reducing end of the molecules of these samples[8]. D-Glucose 1-phosphate labelled with ^{14}C was prepared from tobacco starch[8], and the addition catalysed by potato phosphorylase[9]. The number of labelled D-glucose residues attached to the molecule was determined by micromethylation, followed by hydrolysis with acid and determination of the ratio of the activities of the tetra- and tri-*O*-methyl-D-glucose. These values were 1.7, 2.1, and 2.1 D-glucose residues in the case of the amyloses having d.p. 250, 48, and 24. The specific activity of the individual products was: d.p. 250 (7.55 nCi/mg), 48 (32.6 nCi/mg), and 24 (70.0 nCi/mg).

Methods. — The amount of maltose formed during beta-amylolysis was measured by the photometric method of Somogyi and Nelson[7]. The values of the degrees of degradation (α) were calculated from these data, taking into account the maximum degree of degradability.

The specific activity of maltose formed during the enzymic reaction was determined after dialysis to an equilibrium state, as previously described[5]. The activity of the dialyzed samples was measured by the liquid scintillation method[5], and the maltose content by the method of Somogyi and Nelson[7].

Determination of the actual rate constants. — The number (n^x) of bonds split per effective encounter of enzyme and substrate was calculated by the formula:

$$n^x = \frac{\alpha N}{4.6 \log [1/(1-\alpha R)]},$$

where α denotes the degree of degradation, N is the number-average d.p. of the individual substrates, and R is the quotient of the specific activities of the product maltose and of the initial amylose.

The rate constant (k^x_{-1}) of the dissociation of the reactive enzyme–substrate complex was obtained from the correlation[5]:

$$k^x_{-1} = k_2/n^x,$$

where k_2 is the rate constant for product formation. The rate constant of the formation of the reactive enzyme–substrate complex (k^x_1) was determined from the affinity value extrapolated[2] to d.p. 4.

The number (n) of bonds split per total encounters (reactive and non-reactive) of enzyme–substrate complexes was established by taking self-inhibition[2] into account. It is then possible to calculate the rate constant of the dissociation of the overall enzyme–substrate complex from the expression $k_{-1} = k_2/n$.

Since the formation and dissociation of the "reactive" and "non-reactive" enzyme–substrate complexes take place in parallel reactions, the rate constants of the dissociation of the inactive "non-reactive" enzyme–substrate complex (k^i_{-1}) are known, and from the value of the inhibition constant the rate constants of the formation of the "non-reactive" complexes (k^i_1) may be established.

Amylose degradations. — (*a*) Labelled amylose (15 mg, d.p. 250) was allowed to swell in 0.3 ml of M sodium hydroxide and dissolved in 4 ml of distilled water, and the pH value of the solution was adjusted to 4.8 with 0.5M sulphuric acid. The resulting solution was diluted to 10 ml with distilled water, and 1 ml of 0.02M sodium acetate buffer (pH 4.8) and 0.01 ml of a diluted solution of beta-amylase were added. The final amylose and beta-amylase concentrations were 3.36×10^{-5} and 1.05×10^{-10} mole/l, respectively. During the hydrolysis carried out at $30 \pm 0.1°$, samples (2.0 ml) were withdrawn after 0, 10, 20, and 40 min. The degree of degradation was obtained from 1-ml portions of these samples, and the specific activity of the maltose was determined (see Table I) on a 0.6-ml portion after dialysis.

(*b*) Labelled amylose (10 mg, d.p. 48) was dissolved and treated with the same amount of enzyme as described in (*a*). The amylose concentration of the reaction mixture was 1.16×10^{-4} mole/l. During hydrolysis at $30 \pm 0.1°$ and pH 4.8 ± 0.02, samples were withdrawn after 0, 30, 60, and 90 min and examined as described in (*a*). The results are summarized in Table I.

TABLE I

CHANGES IN THE DEGREE OF DEGRADATION (α), SPECIFIC ACTIVITY OF MALTOSE, AND VALUE R DURING THE BETA-AMYLOLYSIS OF AMYLOSE[a]

Time (min)	*Degree of degradation* (α)	*Specific activity of maltose* ($nCi/\mu g \times 10^3$)	R
Amylose of d.p. 250 (3.36×10^{-5} mole/l)			
10	0.020	63.2	8.35
20	0.038	60.0	7.95
40	0.073	56.0	7.40
Amylose of d.p. 48 (1.16×10^{-4} mole/l)			
30	0.087	146	4.48
60	0.149	148	4.54
90	0.190	132	4.05
Amylose of d.p. 24 (1.75×10^{-4} mole/l)			
15	0.057	212	3.03
30	0.092	247	3.53
60	0.133	258	3.68

[a]beta-Amylase concentration, 1.05×10^{-10} mole/l; temperature, 30 $\pm$0.1°; pH, 4.8 $\pm$0.02.

(*c*) Labelled amylose (7.5 mg, d.p. 24) was dissolved and degraded as described in (*a*). The concentrations of amylose and enzyme were 1.75×10^{-4} and 1.05×10^{-10} mole/l, respectively. The results are presented in Table I.

Number of bonds split per effective encounter of enzyme and substrate. — The number of bonds split per effective encounter and per total number of collisions for the amyloses of three different degrees of polymerisation is shown in Table II. Since the value of n^x in general decreases with increase in the degree of degradation, the values extrapolated to zero degree of degradation were used.

TABLE II

NUMBER OF BOND SPLITTINGS[a]

D.p.	n^x	n
250	14.60	0.405
48	6.37	0.980
24	5.45	1.88

[a]n^x = Number of bond splittings per effective encounter; n = number of bond splittings per overall encounter.

Rate constants. — The rate constants for the formation of the "reactive", "non-reactive", and overall enzyme–substrate complexes are given in Table III.

TABLE III

RATE CONSTANTS[a] FOR THE FORMATION OF ENZYME–SUBSTRATE COMPLEXES

D.p.	$k_1^{x\,a}$ ($l.min^{-1} \cdot mole^{-1}$)	$k_1^{l\,b}$ ($l.min^{-1} \cdot mole^{-1}$)	k_1^{c} ($l.min^{-1} \cdot mole^{-1}$)
250	3.18×10^8	2.57×10^{10}	2.6×10^{10}
48	6.55×10^8	3.14×10^9	4.84×10^9
24	8.17×10^8	6.98×10^8	1.52×10^9

[a]For the formation of "reactive" enzyme–substrate complexes. [b]For the formation of "non-reactive" enzyme-substrate complexes. [c]Overall rate constant of the "reactive" and "non-reactive" enzyme–substrate complexes.

The rate constants for the dissociation of the "reactive" and "non-reactive" enzyme–substrate complexes, and the overall rate constants of the amylose substrates are listed in Table IV.

TABLE IV

ACTUAL RATE CONSTANTS FOR THE DISSOCIATION OF ENZYME–SUBSTRATE COMPLEXES

D.p.	$k_{-1}^{x\,a}$ (min^{-1})	$k_{-1}^{l\,b}$ (min^{-1})	k_{-1}^{c} (min^{-1})
250	2.04×10^4	7.16×10^5	7.36×10^5
48	8.90×10^4	4.88×10^5	5.70×10^5
24	1.27×10^5	2.40×10^5	3.67×10^5

[a]For the dissociation of the "reactive" enzyme–substrate complexes. [b]For the dissociation of the "non-reactive" enzyme–substrate complexes. [c]Overall rate constant for the dissociation of the "reactive" and "non-reactive" enzyme–substrate complexes. The rate constants for product formation[2] are d.p. 250, 2.98×10^5; d.p. 48, 5.66×10^5; d.p. 24, 6.90×10^5 min^{-1}.

DISCUSSION

The number of bonds split per effective enzyme–substrate encounter increases with increase in d.p. of the amylose substrate (see Table II). Thus, for a substrate with a long chain, degradation is shifted to the single-chain mechanism. However, the correlation is not linear, and the number of bonds split per total number of encounters plotted against d.p. passes through a maximum value (Fig. 1). A result obtained earlier with an amylose of d.p. 128 is included in Fig. 1.

The actual rate constants for the formation of the "reactive" enzyme–substrate complexes decrease only to a small extent with increase in d.p. of the substrate (see Table III), for which, however, the decreasing reactivity of the non-reducing chain-ends of the substrates is not responsible. The longer chain molecules possibly form a more-complicated "coil-structure", and thus part of the non-reducing chain-end may be in a sterically hindered location.

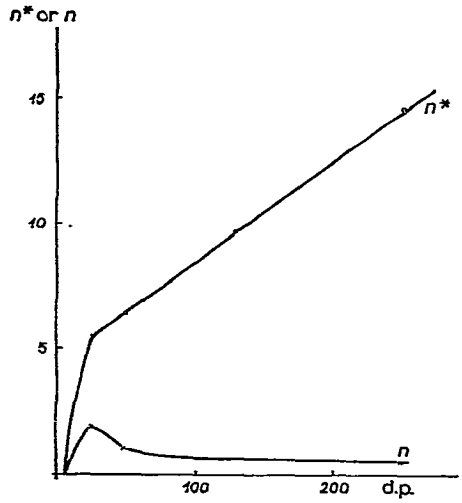

Fig. 1. Changes in the number of bonds split per "reactive" (n^x) and overall (n) encounter, plotted against the d.p. of the substrate.

The rate constants for the formation of the "inactive" enzyme–substrate complexes increase rapidly with increase in the d.p. of the substrate. In this case, the affinity of the enzyme for the individual D-glucose residues did not increase. Still, for the formation of an inactive bond, more D-glucose residues are actually available in a substrate of a higher d.p. than in one of a substrate having lower d.p. Consequently, the k_1^i values given in Table III denote the rates of formation not only of one single "non-reactive" enzyme–substrate complex, but also of all the enzyme–substrate complexes, which are sterically possible, developed with the substrate concerned. Fig. 2 must be interpreted on this basis, where the rate constant (k_2) for product formation, the rate constant (k_1^x) for formation of the "reactive" enzyme–substrate

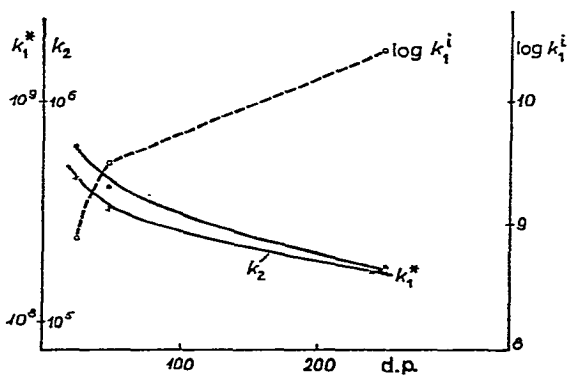

Fig. 2. Actual rate constants for formation of product (k_2), and "reactive" (k_1^x) and "non-reactive" complexes (k_1^i).

complex, and the rate constant for formation of the "non-reactive" enzyme–substrate complexes (k_1^i) were plotted against the d.p. of the substrate applied.

As can be seen from Fig. 2, the rate constant for product formation changes in the same way as does the rate for formation of the "reactive" enzyme–substrate complexes, *i.e.*, the rate of product formation is determined by the latter process. Consequently, in the range of d.p. investigated, the measured Michaelis constant is the quotient k_2/k_1^x, *i.e.*, it can be treated as a kinetic constant. Neglect of k_{-1}^x causes only an error of a few percent.

The dissociation of the "reactive" enzyme–substrate complexes increases also with the decrease of d.p. (see Table IV). This may be ascribed to the lower mobility of the different substrates and to the law of mass action. The compensating effect of these factors manifests itself also in a smaller alteration of the rate constants of dissociation of the "non-reactive" enzyme–substrate complexes (see Table IV).

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