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A STUDY BY MEANS OF LACTONE INHIBITION OF THE ROLE OF A "HALF-CHAIR" GLYCOSYL CONFORMATION AT THE ACTIVE CENTRE OF AMYLOLYTIC ENZYMES*†

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ABSTRACT

The kinetics of inhibition of porcine-pancreatic alpha amylase, sweet-potato beta amylase, and *Aspergillus niger* glucamylase enzymes have been studied by use of D-glucono-1,5-lactone and maltobiono-1,5-lactone as transition-state analogs. With D-glucono-1,5-lactone, alpha amylase can be inhibited, to a degree, non-competitively ($K_i = 0.81$mM, $\beta \sim 0.2$), whereas with maltobionolactone, the inhibition is competitive ($K_i = 0.31$mM). The effect of beta amylase can be inhibited with maltobionolactone in a completely competitive way ($K_i = 0.11$mM), whereas with D-gluconolactone the inhibition is very poor ($K_i = 21$mM). Glucoamylase cannot be inhibited with maltobionolactone, whereas with D-gluconic acid, a completely mixed inhibition may be observed ($K_i = 1.3$mM). The ratio of the binding affinity of the lactones, products, and substrates, permits the conclusion that ring distortion takes place in the transition state with all three enzymes.

INTRODUCTION

Hydrolysis of the (1 → 4)-α-D-glucosidic bonds of the starch molecule by amylolytic enzymes takes place 10^{10}–10^{12} times faster than by proton catalysis with mineral acids[1]. According to Thoma[2], this difference in hydrolytic rate cannot be explained even by the combined effect of the carboxylate anion and imidazolium cation functional groups detected in amylolytic enzymes. Thoma presumed that enzyme-induced ring distortion takes place during catalysis with distortion of one of the D-glucosyl groups from the $^4C_1$ to a "half-chair" conformation. This ring distortion decreases the enthalpy of activation and increases the susceptibility of the glucosyl moiety to nucleophilic attack by a water molecule or a carboxylate group.

Distortion of the D-glucosyl group has been verified by X-ray diffraction[3] on lysozyme–sugar conjugates, by kinetic isotope-effects[4] and by dynamic n.m.r., ring-

*Dedicated to Professor Dexter French, on the occasion of his 60th birthday.
†Part I of the series: The Active Centre of Amylolytic Enzymes.

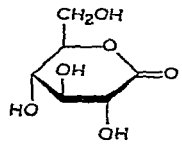
flattening studies[5]. It has also been confirmed, for lysozyme, that the inhibiting effect of chitotetraonolactone is about 3600 times as strong as that of chitotetraose[6]. X-Ray diffraction studies indicate that D-glucono-1,5-lactone (**1**) closely approximates a "half-chair" conformation[7]. This also explains the strong inhibitory effect of various aldonolactones on glycosidases[8,9]. This inhibition is always stereospecific and competitive with regard to the substrate of the respective enzyme. This specific inhibition also indicates the presence and role of a glycosylic moiety having a distorted conformation in reactions catalyzed by the foregoing enzymes.

Thus far, for lactone inhibition of amylolytic enzymes, only the effect of **1** has been investigated. Reese *et al.* found[10] that **1** in 17mM concentration caused 27% inhibition of *Aspergillus oryzae* alpha amylase and 7% inhibition of beta amylase, whereas 4.2–6mM of lactone was required for 50% inhibition of *Aspergillus niger* glucoamylase. The data published are not sufficient, however, to show the role of the distorted glucosyl conformation. Therefore, in addition to investigations with **1**, we also performed detailed kinetic studies on the effect of maltobionolactone (**2**) on the action of pancreatic alpha amylase, sweet-potato beta amylase, and *A. niger* glucoamylase.

MATERIALS AND METHODS

The present experiments employed crystalline porcine pancreatic alpha amylase (Reanal, Hungary), crystalline sweet-potato beta amylase (Sigma) and purified homogeneous *A. niger* glucamylase[11]. The specific activities of the enzymes[12] were as follows: alpha amylase, 480 E/mg protein; beta amylase, 650 E/mg protein; and glucoamylase, 5.6 E/mg protein.

Lactones used as inhibitors were: D-glucono-1,5-lactone (**1** "glucono-δ-lactone", Fluka), lactone content (determined as described later), 99.5%; content of reducing substances, 4,5 mg of glucose/g. Maltobiono lactone (**2**) was prepared by electrolytic oxidation of maltose, according to the procedures given for production of D-gluconic acid. Oxidation was performed with hypobromite obtained by the electrolysis of calcium bromide[13]. Purification and lactonization were performed by the method applied for lactobiono-1,5-lactone[13]. Although the product did not crystallize, it was chromatographically homogeneous. The syrup contained 40% of lactone, 88 mg/g of reducing substances (expressed in terms of maltose), and 41% of maltobionic acid.



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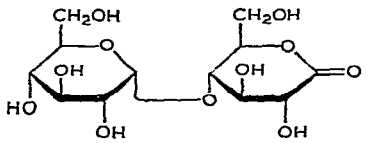

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Soluble starch (Merck) reduced by sodium borohydride served as substrate. After reduction, the content of reducing materials in the product was 2.5 mg/g, expressed in terms of glucose.

The content of free acid and lactone was determined by means of an automatic titrimeter (Radiometer). Acid content up to pH 5.5 and lactone content up to pH 9.0 were measured with a 0.01M solution of sodium hydroxide by the pH-stat method. The content of reducing substances in, and the rates of decomposition of, inhibited and non-inhibited amylolytic starch were determined by the Somogyi–Nelson method[14].

EXPERIMENTAL

Prior to our investigations of inhibition, optimal pH values and the decomposition rates of **1** and **2** were determined at 25°. The pH values of lactone solutions were adjusted by an automatic titrimeter to pH 4.2 (optimum pH for glucoamylase), 4.8 (for beta amylase), and 6.0 (for alpha amylase). After thermostating at 25°, the consumption of alkali was measured by the pH-stat method. From the consumption of 0.01M sodium hydroxide against time, the decomposition of lactone was calculated and is presented as a percentage of initial lactone content in Fig. 1.

As may be seen in Fig. 1, hydrolysis of **1** is more rapid than that of **2**. As, however, in our investigations of inhibition, the time-lag between dissolution of the lactones and the end of the enzymic reaction was, in each case, 5 min (actual reaction-time, 2.5 min), the amount of lactone decomposed during this period was small, even at pH 6 and in the case of **1** only ~5%. No corrections were made, therefore, in the calculation of the experimental results.

Mixtures (3 ml) containing various amounts of soluble starch and lactone were used to investigate inhibition. Before the addition of enzymes, the mixtures were preincubated for 2.5 min and were then incubated at 25° with each enzyme for a further 2.5 min. The reactions were stopped by the addition of 3 ml of Somogyi A solution. From the amount of products determined by the Somogyi–Nelson method, the initial rates could be calculated, and with the use of these results, the inhibitions of the individual lactones were determined graphically as described later.

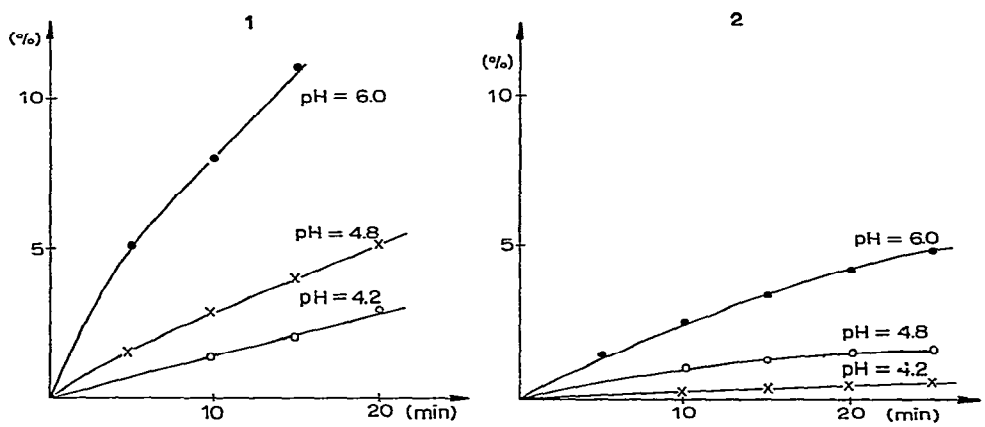

Fig. 1. Decomposition of lactones **1** (left) and **2** (right), at 25°.

The effect of **2** on the reaction catalyzed by alpha amylase was studied at 0.5, 0.2, 0.1, 0.067 and 0.05% (w/v) concentrations of soluble starch, and at 0.93, 1.86, and 3.03mM concentrations of lactone. The enzyme concentration was 1.16×10^{-8}M. The diagram obtained by the Dixon method is given in Fig. 2a, and shows competitive type of inhibition ($K_i = 0.31$mM).

The effect of **1** on alpha-amylolysis was also examined at the foregoing 5 concentrations of soluble starch and 3 concentrations of lactone (1.4, 2.8, and 5.6mM). The concentration of enzyme was the same as in the first series of investigations. The Lineweaver–Burk plot of the results indicates an almost noncompetitive type of inhibition. However, plotting the experimental results according to Dixon gave the diagram depicted in Fig. 2b, which unambiguously points to a partially non-competitive type of inhibition.

The inhibition of beta-amylolysis with **2** was studied similarly at the foregoing 5

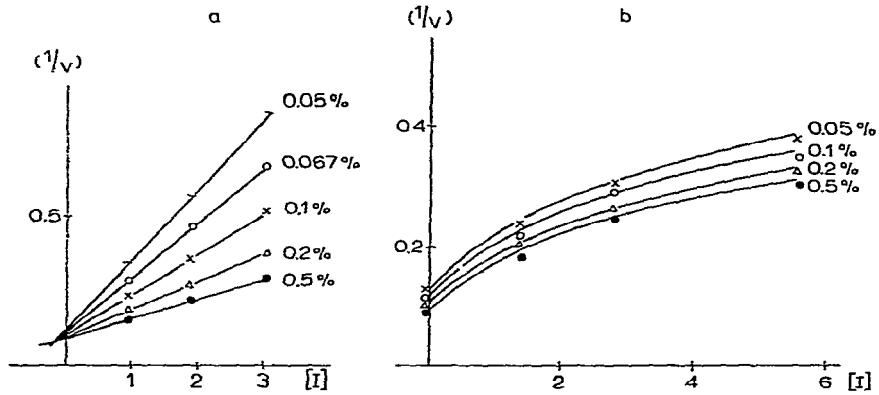

Fig. 2. Inhibition of alpha amylase with maltobionolactone (**2**, a) and D-glucono-1,5-lactone (**1**, b).

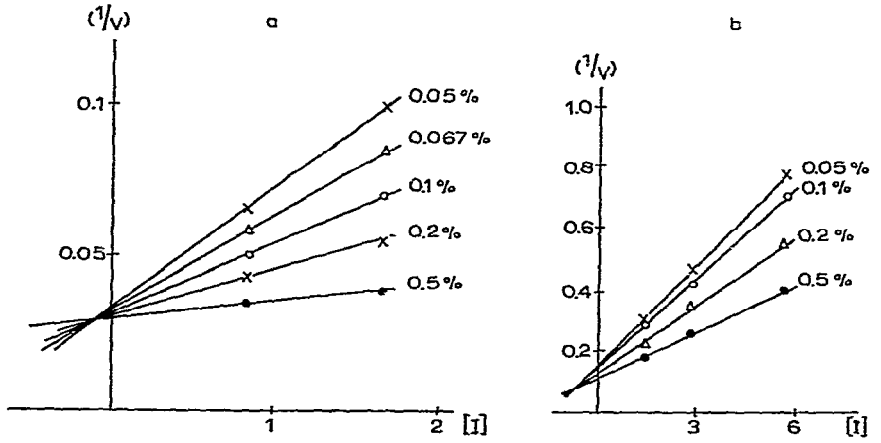

Fig. 3. Inhibition of beta amylase with maltobionolactone (**2**, a) and of glucoamylase with D-glucono-1,5-lactone (**1**, b).

concentrations of soluble starch and 2 concentrations of lactone (0.83 and 1.66mM). The concentration of enzyme was 3.2nM. Plotting the experimental results according to Dixon (Fig. 3a) showed purely competitive inhibition (K_i = 0.1mM). The effect of **1** on beta amylolysis was investigated at the foregoing concentrations of soluble starch and enzyme, and at lactone concentrations of 28 and 56mM. Inhibition proved to be very weak and was nearly competitive ($K_i \sim$ 21mM).

The inhibition of the effect of glucoamylase was investigated at the foregoing 5 concentrations of soluble starch and at 3 concentrations of **1** (1.4, 2.8, and 5.6mM). The diagram obtained on plotting the results according to Dixon is shown in Fig. 3b, which indicates a competitive or mixed inhibition, whereas plotting according to Lineweaver and Burk verifies that a mixed type of inhibition is involved. No inhibiting effect of **2** on glucoamylase could be observed at concentrations of either 0.77 or 1.54mM.

DISCUSSION

Substantial inhibition by lactones was observed with all three enzymes studied, although the inhibition was weaker than experienced, in general, with glycosidases[8]. This is probably because the amylolytic enzymes have more D-glucosyl groups participating in the formation of the enzyme–substrate complex, and a greater number of subsites (at least 4–5 for all three enzymes) are present in the active centre of the enzymes than occur in the glucosidase enzymes (where this number does not exceed 2). Inhibition is not always completely competitive (as in the case of D-glucosidase), and this may also be explained by the foregoing facts.

The inhibition of the effect of alpha amylase with **2** appears to be competitive, according to Fig. 2a and value of K_i (0.31mM).

For comparative purposes, Table I also records the values of K_i and affinities for other competitive inhibitors and substrate. It may be stated on the basis of Table I that the binding to enzyme was strongest in the case of **2**, which was bound four times more strongly than an optimal substrate (maltopentaose) and 40 times as strongly as of the similarly sized molecule, maltose. In the case of a real affinity-ratio, however, the latter value is much lower. As the active centre of pancreatic alpha

TABLE I

AFFINITY VALUES OF ALPHA AMYLASE

Inhibitors or substrate	$K_i \times 10^4$ (M)	*Affinity* (M^{-1})
Methyl α-D-glucoside	1700 (17)	6.0
Maltose	125 (15)	80.0
Maltopentaose	11.7 (16)	852.0
2	3.1	3226.0

amylase is composed of five different subsites[18,19] it is capable of binding several maltose molecules in different ways. From the relative affinity of the individual subsites, the real affinity of the maltose molecule binding competitively with **2** at an identical site may be calculated. The $K_i = 0.22$M value thus obtained and the affinity ratio related to **2** is $0.22/3.1 \times 10^{-4} \approx 7.1 \times 10^2$.

The ratio of rate constants of the hydrolysis[1] catalyzed by alpha amylase and by protons at 25° is $k_E/k_N = 8 \times 10^9$. Because of thermodynamic changes occurring in the course of enzymic hydrolysis (change of enzyme conformation, desolvation, and other interactions), in practice a ratio of binding affinity of the order of 10^2–10^4 could be observed[9,20].

According to Jencks[20], these values may be considered as illustrative of transition-state analogues. On the basis of the foregoing, in the case of pancreatic alpha amylase, compound **2** may be considered as a fair transition-state analogue.

According to Fig. 2b, the inhibition of alpha amylase by **1** is partially non-competitive. This means that, although the ternary complex (enzyme–inhibitor–substrate) is formed with the same probability as the enzyme–substrate complex, the decomposition depends on the value of the beta-coefficient[21]. The beta value may be determined by plotting the experimental results in a v_o/v_i-$1/I$ diagram. This gave a straight line whose perpendicular intercept is equal to $1/(1 - \beta)$ and, from this, β is $\sim$0.2. This result suggests the presence of a water-binding site from where the water molecule may attack the activated, transition-state complex. This assumption is not new[2], although it has been neglected so far because of lack of evidence. If **1** is bound near the water site and the formation of the enzyme–substrate complex is not affected by this binding, an enzyme–inhibitor–substrate (ternary) complex may form. Thus, either attack by the water molecule or distortion corresponding to the transition state is hindered. Therefore the ternary complex is decomposed at a rate of only $\sim$0.2. Obviously, the postulate of the water site must be supported by other methods, and continuing investigations concern this aspect. The calculated value[21] for K_i is 80mM, which is almost identical to the partial inhibition-constant of the lactone part of the molecule of **2**.

The inhibition of beta amylolysis, by both **2** and by **1** is completely competitive. This means that either an enzyme–substrate or an enzyme–inhibitor complex, but no ternary enzyme–inhibitor–substrate complex, is formed. Products are formed only from the enzyme–substrate complex. The K_i value characteristic of the inhibition is 0.11mM for **2** and 21mM for **1**. These results, together with similar values for other competitive inhibitors and substrates, are summarized in Table II.

It may be seen from the data that **2** is bound to beta amylase more strongly than is malto-pentaose (which has an optimum molecular size). This also shows the role of the "half-chair" D-glucosyl group. The ratio of **2**/**1** affinities is in good agreement with the affinity observed for the binding of maltose. This result means that the D-glucosyl group of **2** is bound to subsite 1 with the same strength as maltose, that is, maltose is also bound only at subsite 1, by its non-reducing glucosyl group. Numbering of the subsites constituting the active centre starts at the non-reducing

TABLE II

AFFINITY VALUES OF BETA AMYLASE

Inhibitors or substrate	$K_1 \times 10^4$ (M)	*Affinity* (M^{-1})
2	1.0	10,000
1	210.0	48
Maltose	50.0 (22)	200
Me α-D-glucoside	400 (23)	25
Maltopentaose	12.5 (24)	800

end of the substrate chain. If the reducing D-glucose residue of maltose is bound to the enzyme only weakly or not at all, we may suppose that the substrate molecule can be bound without ring distortion. The actual binding affinity of the maltose molecule at the same site as **2** has also been determined in this case. The value calculated for K_i was 0.17M and the affinity ratio related to **2** was: $0.17/1 \times 10^{-4} \approx 1.7 \times 10^3$. Based on the foregoing, **2** is also a transition-state analogue for sweet-potato beta amylase.

The effect of glucoamylase is not at all inhibited by **2**. This result supports the assumption of a "steric-boundary subsite" in the enzyme. This steric hindrance of the active centre is responsible for the production of D-glucose alone in the enzymic reaction. A similar explanation may be given for the formation of only maltose in beta amylolysis, although this proposal has not yet been verified experimentally (another approach to the problem may be the use of maltotrionolactone).

Compound **1** causes a completely mixed inhibition of glucoamylase, where $\beta = 0$ and α changes from 3 to 7.2 with the increase of inhibitor concentration in the range of our examinations, This result implies that a ternary enzyme–inhibitor–substrate complex may be formed[21], with the amount decreasing with the increase of inhibitor concentration, but no product is formed from the enzyme–inhibitor–substrate complex. The value of K_i was found to be 1.3mM. This value, together with the data for other competitive inhibitors and substrates, are summarized in Table III. As the

TABLE III

AFFINITY VALUES OF GLUCOAMYLASE

Inhibitors or substrate	$K_1 \times 10^4$ (M)	*Affinity* (M^{-1})
1	13.0	769.0
β-D-Glucose	1600.0 (25)	6.3
Maltose	14.0 (26)	714.0
Maltotriose	2.6 (26)	3846.0

binding of the lactone and of maltose are nearly identical, it may be presumed that here also, maltose is bound only to subsite 2, and the transition form of the D-glucosyl moiety having a half-chair conformation is bound to subsite 1. The ratio of apparent inhibition-constant given for β-D-glucose and the inhibition constant measured for **1** was found to be: $0.16/1.3 \times 10^{-3} \approx 128$. In the case of glucoamylase–**1**, the real dissociation constant cannot be calculated on the basis of this ratio and currently available data, therefore, **1** may only be considered as a poor transition-state analogue.

According to the kinetic analysis of lactone inhibition, we may suppose a role for a "half-chair" glycosyl conformation for all three amylolytic enzymes. On the basis of the foregoing, it may be stated that, in the case of amylolytic enzymes, the distorted glycosyl conformation in the transition state is a significant rate-determining step. However, lactone inhibition cannot establish whether distortion takes place during substrate bonding, as with lysozyme, or after bond splitting, as in the case of proton catalysis.

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