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SUBSITE PROFILE OF THE ACTIVE CENTER OF PORCINE PANCREATIC α-AMYLASE. KINETIC STUDIES USING MALTOOLIGOSACCHARIDES AS SUBSTRATES.

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The hydrolysis of several maltooligosaccharides catalysed by porcine pancreatic α-amylase was performed in order to determine their kinetic parameters. Maltose behaves as a substrate. Molecular activity (k_o) increases with chain length up to maltopentaose, remaining practically uncnanged from maltopentaose to maltoheptaose. Maltose shows the highest Km value while the one for maltotriose is the lowest. Only maltose and maltotriose were directly cleaved to glucose. From Km and k_o values, the binding energy of the total complexes and of the productive ones respectively were calculated. The binding energy at each subsite was determined assuming that each substrate forms a single productive complex and that maltose and maltotriose differ in their binding mode from higher oligosaccharides. The model was checked by calculating theorical Km and k_o. Theorical values agree reasonably well with experimental ones.

The amino acid sequence of porcine pancreatic α-amylase (α-1,4 glucan-4-glucanohydrolase EC 3.2.1.1.), a 496 residues long single chain peptide has been recently reported (1,2) as well as its three-dimensional structure at 5 Å resolution (3). The structural knowledge of this enzyme is thus now well documented. Especially a 30 Å long cleft is apparent in the molecule which can accommodate 5 glucose units. Moreover cristallographic studies with substrate analogues methyl and nitrophenylthiomaltosides have shown 2 binding sites, one in the cleft, the second on the surface of the amylase molecule (4). The 2.9 Å resolution structure now in progress will tell us in a near future the nature of the amino acids present in the binding site and the catalytic groups. Still little is known on the position specificity and action pattern of α-amylase. This is due to the use of ill defined substrates as starch and the lack of good techniques for specific quantitative determination of the products. Nitrophenyl-derivatives have given limited information for the understanding of the active site function, because the only glycosidic bond whose hydrolysis can be tested is the one attaching the nitrophenyl moiety (5,6). Using short oligosaccharides (Glc_3 to Glc_8) as substrates, a 5 glucose binding site model in which the catalytic group is located at bond 2 was proposed (7). More precise data on the action pattern and substrate binding have been obtained in the case of exoamylases (8,9).

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In the present work we have measured the initial rate of hydrolysis of maltooligosaccharides including maltose which up to now had not be considered as a substrate and higher oligosaccharides up to maltoheptaose. The values of Km and k_o were determined for each substrate. This allowed to calculate their respective binding energy to amylase and the binding energy at each subsite. A new model is proposed to fit the action pattern.

MATERIAL AND METHODS

Porcine pancreatic amylase was purified according to Granger et al (10). Amylase II was used in our experiments. Its purity was assessed by ion exchange and reverse phase HPLC chromatography and SDS gel electrophoresis. Maltotriose, maltotetraose, maltopentaose, maltohexaose and maltoheptaose, glucose oxidase (aspergillus niger), peroxidase (horse-radish) and ABTS di-ammonium 2,2'-azino-2,2' di(ethyl-3-benzothiazoline-6-sulfonate) were purchased from Boehringer Mannheim. Maltose grade I and neocuproine hydrochloride (2,9-dimethyl-1,10-phenanthroline HCl) were from Sigma. Analysis of all maltosides performed by HPLC chromatography on a RP 18 SHPERT column showed no sizable impurities.

Hydrolysis reactions were carried out at 30°C in 20 mM sodium phosphate buffer containing 1 mM $CaCl_2$ at pH 6.9. Reactions were initiated by adding amylase. 0.5 ml samples were taken at appropriate times and poured each over 2.5 ml of 100 mM Na_2CO_3 to stop the reaction. If necessary new dilutions were made in 100 mM Na_2CO_3 before assay. Total reducing sugars were assayed by reductometry according to Dygert et al (11), glucose was determined according to the technique of Werner et al (12). Appropriate blanks (no amylase added) were taken as the zero time value.

RESULTS AND DISCUSSION

1°) Kinetic parameters.

Maltose is well known as the main product of starch hydrolysis catalysed by α-amylase and also as a weak inhibitor of this enzyme (13) ; we show here that it is also a substrate both for amylase II and I. The k_o value was found constant all along the chromatography peaks at the last stage of purification which separates amylase I and II. It thus appears that this activity is not due to contaminating α-glucosidase. This reaction is catalytic (Fig. 1 A). The reciprocal plot allows the calculation of the kinetic parameters (Fig. 1 B). The Km for maltotriose is 50 times lower than for maltose (Table I). However the molecular activity value is small for both substrates. The hydrolysis of maltotetraose and maltopentaose was mainly followed by increase of the reducing power but it was also tested by glucose assay. The production of glucose is very slow, total reducing sugars are produced over 300 times faster from maltotetraose and 1800 times from maltopentaose. It is thus clear that these substrates are not directly split to glucose. The hydrolysis of maltotetraose therefore differs from the action pattern reported by Robyt

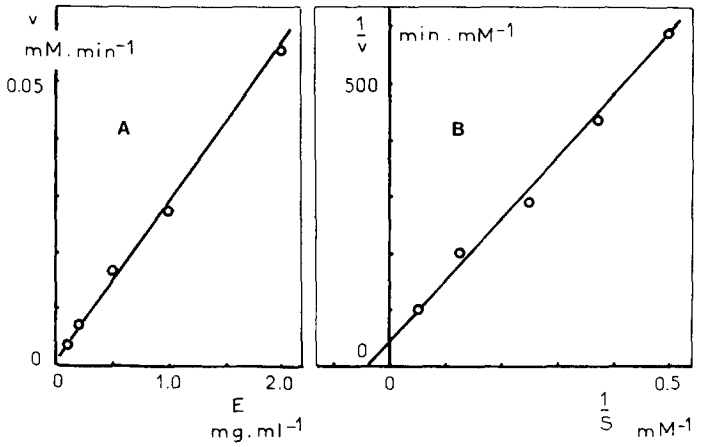

Fig 1 : Amylase catalysed hydrolysis of maltose.
Initial rates were determined by assay of the glucose liberated at various times (up to 30 min) and expressed in mM of glucose produced per minute. The enzyme concentration is in mg/ml.
A. The incubations were carried out at 20 mM maltose concentration.
B. The amylase concentration was 0.2 mg/ml.
The hydrolysis of maltose was more than 90% after a 16 hr incubation period.

and French indicating a 30% cut at the bond joining residues 1 and 2 (7)*. Remarkably the Km of maltotriose is significantly lower than the ones of longer derivatives. Kinetic parameters similar to those of maltotriose have been recently obtained with o-nitrophenylmaltoside (6). Also identical Km value for maltopentaose has been reported (14). In contrast there is a stepwise increase in the molecular activity depending on the chain length from maltose up to maltopentaose. The K_o value is about 700 times higher for the tetraose than for the triose and 8000 times larger for the pentaose

TABLE I : Kinetic parameters for the hydrolysis of maltooligosaccharides

Substrate	Km (mM)	k_o (s^{-1})	assay	number of experiments
Maltose	28.38	0.120	glucose	7
Maltotriose	0.50	0.160	glucose	7
Maltotetraose	0.72	118	reductometry	4
Maltotetraose	1.96	0.36	glucose	2
Maltopentaose	1.08	1360	reductometry	6
Maltopentaose	1.75	0.72	glucose	2
Maltohexaose	0.62	1270	reductometry	2
Maltoheptaose	1.02	1250	reductometry	5

The number of points by experiment was 5. The substrate concentration was varied in a ten fold range. Values of the parameters were calculated by linear regression. Correlation factors thus obtained indicated a significance with $p \leq 1\%$.

* Glucose residues in the substrate are numbered 1, 2, 3, ... from the reducing end.

then plateaus up to heptaose. The plateaus observed in the variation of k_o and Km values as a function of chain length strongly suggest a limited number of binding modes of the substrate-amylase complexes. Especially the slightly lower Km determined for maltotriose as compared with higher homologues indicates a high interaction at 3 subsites as a non productive complex.

2°) Evaluation of the binding energies of total and productive maltooligosaccharides - amylase complexes.

The value of Km allows the calculation of the apparent molecular binding free energy of total (i.e. productive plus non productive) complexes for a given substrate of chain length n (8) :

$$B_{t\ app} = RT \ln \sum_j K_{n,j} + 2.4 \text{ kcal/mole}$$

where $\sum_j K_{n,j}$ is the sum of the affinity constants of total complexes with a substrate of chain length n for all binding modes j. $\sum_j K_{n,j}$ can be considered as the apparent affinity constant for this substrate, and therefore as the reciprocal of its Km. It comes then :

$$B_{t\ app} = RT \ln (1/Km) + 2.4 \text{ kcal/mole}$$

The values so obtained are represented by the void bars on Fig. 2 A. The binding free energy of maltose (4 kcal/mole) is lower by about 1/3 than

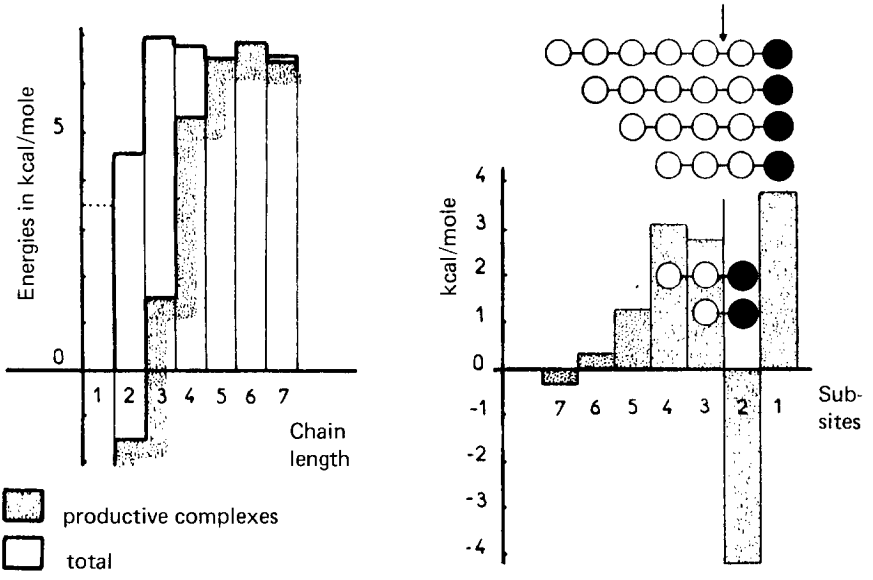

Fig 2 : A. Histograms of the binding energy of total and productive maltooligosaccharides-amylase complexes. n indicates the chain length.
B. Histograms of the binding energy of the subsites of α-amylase and the position of productive complexes. Void round circles indicate glucose residues, filled circles indicate the reducing end. K_{int} was determined by taking $\frac{k_o}{k_{int}} = 1$ for maltopentaose. Arrow indicates the catalytic site.

the one of maltotriose (7 kcal/mole). Remarkably the maltotriose complex has the same binding free energy than those formed by higher oligosaccharides (n up to 7). The binding free energy of glucose (3.5 kcal/mole) was calculated from reported inhibition experiments with α-methylglycoside and is given for comparison (15). The apparent binding free energy of the productive complexes can be calculated from the equation :

$$B_{p\ app} = RT \ln \sum_{p} Kn,p + 2.4 \text{ kcal/mole}$$

Assuming the intrinsic velocity constant, k_{int}, to be independent of substrate length, $\sum_{p} K_{n,p}$ will be related to the kinetic parameters as follows:

$$\sum_{p} K_{n,p} = k_o/(k_{int}.K_m)$$

and then :

$$B_{p\ app} = RT \ln \left(\frac{k_o}{k_{int}.K_m}\right) + 2.4 \text{ kcal/mole}$$

The binding energy of the productive complexes (Fig. 2 A dotted bars) increases with chain length up to maltopentaose and then remains practically constant. The apparent binding free energy of the productive complex of maltose is negative. As shown later, this is a consequence of the negative binding energy at subsite 2. The productive complex binding energy of maltotriose is small, about 1/3 of the one of tetraose. The plateau value reached with maltopentaose is obviously consistent with the 5 subsite hypothesis.

3°) Evaluation of subsite binding energies. Basis of the model.

They were calculated on the ground of three basic assumptions : 1) There is a single productive complex for each substrate ; 2) In productive complexes the binding mode of maltose and maltotriose (b.m. 2) is different from longer substrates (b.m. 1). 3) The total binding free energy of the complex enzyme-maltotriose, B_{t3} , corresponds almost exclusively to a non productive complex.

On the basis of product analysis and from Robyt and French data (7), productive complexes of maltose and maltotriose are bound following mode 2 (Fig. 2 B). The productive complex of maltotetraose, as discussed above, binds in the same way as maltopentaose and higher substrates (our results and 7), those substrates being cleaved between glucose units 2 and 3 (Fig. 2 B).

The binding free energy of most subsites A_j was calculated by substracting the apparent binding energies of the appropriate productive complexes $B_{p,n}$: $A_1 = B_{p4} - B_{p3}$; $A_4 = B_{p3} - B_{p2}$; $A_5 = B_{p5} - B_{p4}$; $A_6 = B_{p6} - B_{p5}$; $A_7 = B_{p7} - B_{p6}$. A_2 was calculated as $A_{1+2} - A_1$; A_3 as $B_{p2} - A_2$, and A_{1+2} as $B_{p5} - B_{t3}$ (Fig. 2 B). The binding energy of subsites 1, 3, 4 and 5 is within 1.2 and 3.7 Kcal/mole. The energy

Table II : Comparison between experimental and calculated kinetic parameters

N *	Subsites occupied	Km (mM) Calculated	Km (mM) Experimental	Subsites occupied	k_o sec^{-1} Calculated	k_o sec^{-1} Experimental
2	3, 4	3.8	28	2, 3	0.008	0.06
	4, 5	44			0.091	
3	3, 4, 5	0.5	0.5	2, 3, 4	0.162	0.162
4	3, 4, 5, 6	0.3	0.7	1, 2, 3, 4	50.6	118
5	1,2,3,4,5	1.1	1.1	1,2,3,4,5	1360	1360
6	1,2,3,4,5,6	0.6	0.6	1,2,3,4,5,6	1360	1270
7	1,2,3,4,5,6,7	1.25	1.0	1,2,3,4,5,6,7	1360	1250

* Substrate chain length as number of glucose residues.

of subsite 2 is negative (-4.2 Kcal), which may be explained by substrate induced strain at the catalytic center (Fig. 2 B, arrow). Such situation is often encountered in the case of other osidases (16). The binding energy of subsites 6 and 7 is much lower, then their signification is uncertain.

4°) Calculation of theoretical parameters.

Theoretical Km and k_o have been calculated for each substrate (Table II). In the case of Km only the complex of the highest binding energy was taken into account. A good correlation was generally obtained with experimental values. Maltose makes an exception since its experimental Km is intermediate between those calculated from two binding modes. The reported K_i values for maltose are within the same range : 4.6 mM (17), 12 mM (unpublished data), 25 mM (13). Calculation of the affinity constant of all possible productive complexes indicates that maltotetraose and higher sugars up to maltoheptaose, almost exclusively occupy the sites as indicated by the model, however maltotriose could also be bound to subsites 1, 2, 3 (results not shown). Still the variation of K_o over a range of 23000 times from maltose to maltopentaose, with a change of only 57 times in Km is explained. The existence of a second distinct substrate binding site is not included in this model.

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