Biochimica et Biophysica Acta, 302 (1973) 362–375

BBA 66852

SUBSITE AFFINITIES OF GLUCOAMYLASE: EXAMINATION OF THE VALIDITY OF THE SUBSITE THEORY

KEITARO HIROMI*, YASUNORI NITTA, CHIKASHI NUMATA AND SÔZABURO ONO
Laboratory of Biophysical Chemistry, College of Agriculture, University of Osaka Prefecture, Sakai (Japan)

(Received October 13th, 1972)

SUMMARY

1. The dependence of Michaelis constant (K_m) and the molecular activity (k_0) on the degree of polymerization (n) of maltooligosaccharides for the hydrolysis catalyzed by glucoamylase of *Rhizopus delemar* (α-1,4:1,6-glucan 4:6-glucohydrolase, EC 3.2.1.3) was studied at pH 4.50 and 25 °C, for a range of n from 2 to 15.5.

2. The results were analyzed to evaluate the subsite affinities (A_i) and the intrinsic rate constant of hydrolysis of substrate linkage in a productive complex (k_{int}) according to the theory[1] formerly developed by one of the authors (Hiromi, K. (1970) *Biochem. Biophys. Res. Commun.* 40, 1–6), which assumed the independency of k_{int} on n and the additivity of A_i.

3. The values of K_m and k_0 calculated with the above determined values of A_i and k_{int} were in excellent agreement with the experimentally obtained ones for $n = 2$–7, which confirmed the validity of the theory.

INTRODUCTION

The success of X-ray analysis of lysozyme and its complex with tri-*N*-acetylglucosamine provided us with a detailed picture of the active site of the enzyme and the mode of interaction between the enzyme and the substrate[2–4]. The active site, which forms a cleft capable of accommodating six *N*-acetylglucosamine residues, may be regarded as consisting of six "subsites" (termed A–F), each of which interacts specifically with a substrate residue through hydrogen bonds and hydrophobic bonds. The catalytic site, the two carboxyl groups of Glu-35 and Asp-52, is situated in the cleft between the fourth and the fifth subsites (D and F) counting from the terminal for the nonreducing end residue of the substrate. In the stable enzyme–substrate complex studied by X-ray analysis, the tri-*N*-acetylglucosamine molecule is bound in a nonproductive form away from the catalytic site, occupying the first three subsites

* Present address: Laboratory of Enzyme Chemistry, Department of Food Science and Technology, Faculty of Agriculture, Kyoto University, Kyoto (Japan).

(A–C). However, the fact that tri-*N*-acetylglucosamine is hydrolyzed by lysozyme, even although very slowly, indicates that the substrate can be bound also in a productive form upon the catalytic site, although the binding probability may be low. This provides evidence for the existence of multiple binding modes of substrates with the enzyme.

It is reasonable to suppose that a similar situation will hold also for other homopolymer-degrading glycoside hydrolases such as amylases and cellulases. For these enzymes which act on linear homopolymer substrates, the specific interaction between a certain subsite and a substrate residue (glucose residue) may be considered to be the same, regardless of the chain length and the binding mode of the substrate. Therefore, we may assign a definite subsite affinity for a given subsite to represent the strength of interaction with which the subsite in question would bind a substrate residue[1,5]. The subsite affinity of the ith subsite* will be denoted by A_i (kcal/mole), which is equal to the decrease in the unitary part[6,7] of free energy caused by the binding of a substrate residue with the subsite[1].

One of the most important manifestations of the substrate specificity of amylases resides in their action patterns, by which we mean: (1) the dependence of rate of hydrolysis on the degree of polymerization, n, of linear substrates, and (2) the mode of cleavage of oligosaccharides[8]. We shall focus our attention particularly on the n-dependence of the hydrolysis rate, or more precisely, the Michaelis constant K_m and the molecular activity k_0 (the maximum velocity V divided by the molar concentration of enzyme e_0). In many glycoside hydrolase-catalyzed reactions, it has been noticed that k_0, as well as $1/K_m$, increases with n for linear substrates within a given range of n[9–12]. Since the rate of acid-catalyzed hydrolyses of these substrates is scarcely dependent on n[13], such an n-dependence of k_0 seems to be characteristic for enzyme catalysis. However, how such an n-dependency of k_0 arises cannot readily be explained, in so far as k_0 is simply regarded as a true rate constant for the hydrolysis of a substrate linkage in an ES complex according to the Michaelis–Menten mechanism:

$$E + S \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} ES \xrightarrow{k_{+2}} E + P \qquad (1)$$

where k_{+2} is equal to k_0.

There are two alternative ways of interpreting the observed n-dependency of k_0, according to the following concepts: (1) In enzyme catalysis, the true rate constant of hydrolysis of substrate linkage, k_{int}, is dependent on n. (2) The true rate constant k_{int} is independent of n, but the observed n-dependency of k_0 arises merely from the multiplicity of the binding modes of substrate to the enzyme.

In the first concept, which does not necessarily require the multiple binding modes, some special mechanism should be assumed, such as cooperativity of subsites which leads to an enhanced rate of hydrolysis of the substrate linkage in an ES complex depending on n. However, there is no *a priori* basis for predicting such cooperativity. The magnitude of this effect could only be taken as an adjustable parameter in a theoretical treatment. Therefore, we would not take this concept as a starting point for our theory.

* The subsites are numbered counting from the terminal one for the binding of nonreducing end residue of substrate (see Fig. 3 below).

On the other hand, the second concept, which assumes that k_{int} is independent of n as in the case of acid hydrolysis, is much more mechanical and does not require any special mechanism such as postulated in the first concept. In this case, the multiplicity of binding modes could lead to the n-dependency of k_0 through the statistical weights of productive and non-productive complexes, which are determined by subsite affinities[1].

Several attemps have been made to correlate subsite affinities with the rate parameters of linear n-mer substrates[1,11] and with the mode of cleavage of malto-oligosaccharides[14]. We proposed, by assuming the independency of k_{int} with respect to n, a method for evaluating subsite affinities from the n-dependency of rate parameters, and the method has been applied to several amylases[1,11,15–18]. On the other hand, Thoma *et al.*[14] evaluated the subsite affinities of *Bacillus liquefacience* α-amylase from the product distribution ratios with end-labeled oligosaccharides. In the latter case, however, the rate parameters calculated with the subsite affinities thus evaluated were not consistent with the experimentally obtained values, in so far as k_{int} was assumed to be independent on n[10]. To account for this discrepancy, they claimed that k_{int} should not be constant but must vary with n, as was considered in the first concept above.

In this paper, we aimed to examine thoroughly the validity of the assumption for the constancy of k_{int}, as based on the second concept. Glucoamylase, a typical exo-amylase which splits off glucose from nonreducing ends of substrates[19], was chosen as the best object for this purpose, because of the simplicity of its action pattern.

The procedure involves: (i) The determination of A_i and k_{int} from the observed rate parameters of n-mer substrates assuming that k_{int} is independent of n. (ii) To calculate the rate parameters with these A_i and k_{int} values and to compare them with the experimentally obtained rate parameters. If the theory is valid, we should have an agreement between the calculated and observed rate parameters for various values of n.

The results clearly demonstrated that the assumption of independency of k_{int} is actually valid for glucoamylase.

THEORETICAL

The detail of the theory which relates rate parameters for subsite affinities A_i and the intrinsic rate constant k_{int} for the hydrolysis of substrate linkage in a productive complex(es) have been described elsewhere[1,11,20–22]. The outline of the theory will be mentioned briefly below.

When an n-mer substrate S_n is bound with an enzyme E to give several productive and nonproductive complexes, represented by $ES_{n,p}$ and $ES_{n,q}$, respectively, the reaction scheme may be written as follows:

$$E + S_n \underset{K_{n,q}}{\overset{K_{n,p}}{\rightleftharpoons}} \begin{matrix} ES_{n,p} \\ ES_{n,q} \end{matrix} \overset{k_{int}}{\longrightarrow} E + P \qquad (2)$$

where it is assumed that k_{int} is constant irrespective of both the substrate chain

length n and the binding mode of productive complex(es) as was stated above*. $K_{n,p}$ and $K_{n,q}$, which will be generally represented by $K_{n,j}$, are the association constants of the n-mer substrate in a binding mode specified by the subscript p (productive), q (nonproductive), or j (either productive and nonproductive).

$$K_{n,j} = [ES_{n,j}]/[E]\,[S_n] \tag{3}$$

The numbers of the subscripts p, q or j, specifying the binding mode, will be conveniently taken as being equal to the number of the subsite at which the nonreducing end-residue of the substrate is situated (see Fig. 3 below).

By assuming rapid equilibrium between E and S_n, a rate equation of the same form as the familiar Michaelis–Menten type is obtained, where K_m and k_0 are expressed by the following formula:

$$1/K_m = \sum_j K_{n,j} = \sum_p K_{n,p} + \sum_q K_{n,q} \tag{4}$$

$$k_0 = k_{int} \cdot \sum_p K_{n,p} \Big/ \sum_j K_{n,j}$$

$$= k_{int}\,(1 + \sum_q K_{n,q} \Big/ \sum_r K_{n,p})^{-1} \tag{5}$$

$$k_0/K_m = k_{int} \cdot \sum_p K_{n,p} \tag{6}$$

It is an important property that k_0/K_m involves only the productive terms, while K_m and k_0 contain nonproductive terms in addition.

The association constant $K_{n,j}$ is related to the molecular binding affinity, $B_{n,j}$, which is the unitary part[6,7] of standard affinity $-\Delta G_{n,j}$.

$$RT \ln K_{n,j} = -\Delta G_{n,j} = B_{n,j} - 2.4 \text{ kcal/mole} \tag{7}$$

The term 2.4 kcal/mole arises from the contribution of the mixing entropy in water at 25 °C[6,7].

Now we assume that $B_{n,j}$ is simply expressed by the sum of A_i values of the subsites occupied by the substrate molecule in that mode of binding. Thus

$$B_{n,j} = (\sum_i^{cov.} A_i)_{n,j} \tag{8}$$

where A_i is the subsite affinity of the i-th subsite, expressed in free energy units, and $\sum_i^{cov.}$ implies that the sum is taken for the occupied subsites. Then $K_{n,j}$ becomes

$$K_{n,j} = (0.018) \exp (B_{n,j}/RT) = (0.018) \exp (\sum_i^{cov.} A_i/RT)_{n,j} \tag{9}$$

If we substitute Eqn 9 into Eqns 4–6, the rate parameters K_m, k_0 and k_0/K_m are expressed in terms of k_{int} and A_i values. Therefore, we can calculate all the rate parameters for any linear substrate, when the A_i values and k_{int} are known. It has been shown that the n-dependency of k_0 can actually arise merely from the term $\sum_p K_{n,p}/\sum_j K_{n,j}$, which represents the statistical weight of productive complex(es)[1,11].

The above equations are applicable both for exo- and endo-amylases. In the

* Enzyme–substrate complexes in which more than one substrate molecules are bound (at the active site) will not be considered here. This problem has been discussed elsewhere[27,28].

case of exo-amylases, however, there exists only one productive complex for an n-mer substrate, as is apparent from their mode of action. In this case, the sum $\sum_{p} K_{n,p}$ in Eqns 4–6 reduces to a single term:

$$\sum_{p} K_{n,p} = K_{n,1} \tag{10}$$

where $K_{n,1}$ is the association constant of n-mer substrate in the productive binding mode $j = 1$.

EXPERIMENTAL

Enzyme

Crystalline glucoamylase from *Rhizopus delemar*, which was kindly provided by Dr Y. Tsujisaka of Osaka Municipal Technical Research Institute was dissolved in distilled water and kept in a refrigerator as a stock solution. The enzyme concentration was determined from the absorbance at 280 nm, assuming $A_{1\,cm}^{1\,\%}$ to be 14.5 and a molecular weight of 70 000*.

Substrates

Maltooligosaccharides of degree of polymerization ranging from 2 to 7 were prepared from the acid hydrolyzate of cycloheptaamylose as described elsewhere[11]. Crystalline maltodextrin of average degree of polymerization of 15.5 was prepared by the method of Hizukuri *et al.*[23].

Method

The reaction was carried out at 25.0 °C in 0.02 M acetate buffer at pH 4.50 throughout. The initial rate was determined in the usual way from the measurement of reducing-end concentrations by a modified Somogyi–Nelson method[24]. The enzyme activity of the stock solution was calibrated on a standard run using amylose as the substrate.

RESULTS

Determination of rate parameters

The Michaelis constant K_m and the maximum velocity V were determined from s/v *versus* s plots using the least square method, where v and s are the initial velocity and the substrate concentration, respectively. Some typical examples of the plots are shown in Fig. 1.

The molecular activity k_0 was obtained as V/e_0, where e_0 is the molar concentration of enzyme. Table I summarizes the values of K_m and k_0 for maltooligosaccharides studied, together with their standard deviations calculated according to Youden's formula[25]. The plots of $\log(1/K_m)$, $\log k_0$ and $\log(k_0/K_m)$ against n (degree of polymerization of linear substrates) are shown in open circles in Fig. 2. The dependency of these rate parameters on n, where the increase in k_0 together with

* This value was obtained from sedimentation and diffusion measurements assuming a partial specific volume of 0.73.

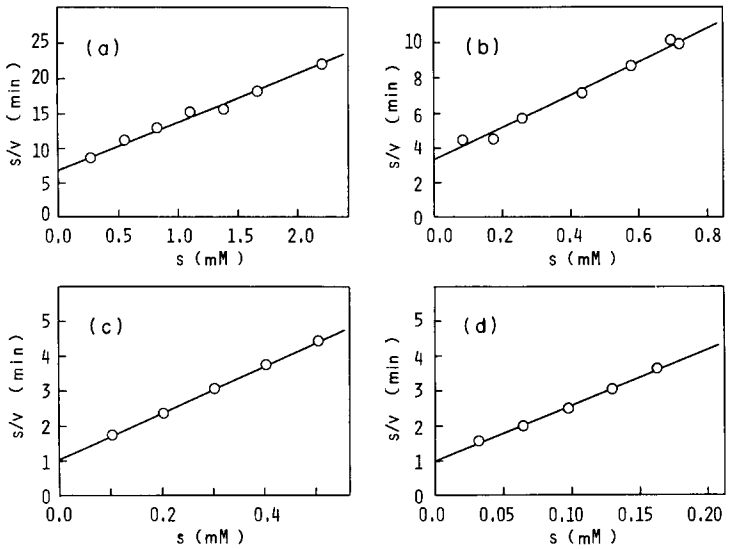

Fig. 1. Typical examples of *s/v versus s* plots for glucoamylase-catalyzed hydrolyses of various maltooligosaccharides as substrates (25 °C, pH 4.50). a, maltose, $e_0 = 5.18 \cdot 10^{-7}$ M; b, maltotriose, $e_0 = 7.82 \cdot 10^{-8}$ M; c, maltopentaose, $e_0 = 7.69 \cdot 10^{-8}$ M; d, maltodextrin with an average degree of polymerization of 15.5, $e_0 = 4.27 \cdot 10^{-8}$ M, where e_0 is the enzyme concentration.

$1/K_m$ between $n = 2$ and 4 is noticed, is essentially the same as those observed earlier with the same enzyme at 15 °C for more limited number of substrates[12].

Analysis of the results (evaluation of A_i and k_{int})

The values of rate parameters experimentally obtained for a series of maltooligosaccharides allow us to evaluate the subsite affinities, A_i, and the intrinsic rate constant, k_{int}. The procedure for the evaluation will be described below.

Fig. 3 shows a schematic model of the active site of glucoamylase which involves m subsites, and several binding modes of n-mer and $(n+1)$-mer substrates. The binding modes with $j = 1$ and $j \geqq 2$ refer to productive and nonproductive modes, respectively. For glucoamylase, which is an exo-enzyme, the simple formula of Eqn 10

TABLE I

EXPERIMENTALLY OBTAINED RATE PARAMETERS WITH STANDARD DEVIATIONS FOR GLUCOAMYLASE-CATALYZED HYDROLYSES OF MALTOOLIGOSACCHARIDES AT pH 4.50 AND 25 °C

Substrate	n*	K_m ($10^4 \times M$)	k_0 (s^{-1})	k_0/K_m ($10^{-4} \times M^{-1} \cdot s^{-1}$)
Maltose	2	11 ± 1.1	4.6 ± 0.25	0.42 ± 0.025
Maltotriose	3	3.6 ± 0.51	23 ± 2.1	6.4 ± 0.34
Maltotetraose	4	2.5 ± 0.33	33 ± 1.9	13 ± 1.5
Maltopentaose	5	1.6 ± 0.02	32 ± 0.2	20 ± 0.1
Maltohexaose	6	1.2 ± 0.14	28 ± 1.1	23 ± 3.1
Maltoheptaose	7	1.1 ± 0.11	31 ± 1.5	28 ± 2.4
Maltodextrin	15.5	0.65 ± 0.06	24 ± 1.0	37 ± 2.5

* The degree of polymerization of the substrate expressed as glucose units. For maltodextrin only, the average degree of polymerization is shown.

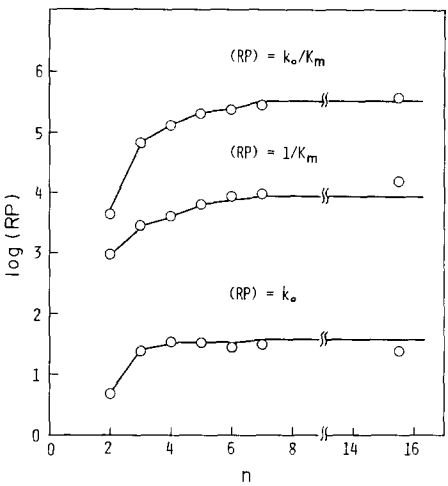

Fig. 2. Dependence of rate parameters (RP) on the degree of polymerization (n) of maltooligosaccharides. K_m, Michaelis constant in M units; k_0, molecular activity (V/e_0) in s^{-1}. ○, and —, values obtained experimentally and the value theoretically (calculated by Method I), respectively (see text).

can be used. By substituting Eqns 9 and 10 into Eqn 6, we have an expression for k_0/K_m for an n-mer substrate:

$$(k_0/K_m)_n = k_{int} \cdot K_{n,1} = (0.018)\, k_{int} \exp\left(\sum_{i}^{cov.} A_i/RT\right)_{n,1} \tag{11}$$

When n does not exceed the number of subsites, m, we have:

$$\sum_{i}^{cov.} A_i = \sum_{i=1}^{n} A_i = A_1 + A_2 + \cdots\cdots + A_n \tag{12}$$

Therefore, with $(n+1)$-mer and n-mer substrates, we have:

$$\ln(k_0/K_m)_{n+1} - \ln(k_0/K_m)_n = \left(\sum_{i=1}^{n+1} A_i - \sum_{i=1}^{n} A_i\right)/RT = A_{n+1}/RT \tag{13}$$

as is apparent from Fig. 3. Thus the subsite affinity of the $(n+1)$-th subsite, A_{n+1},

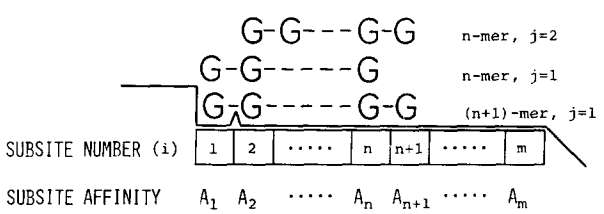

Fig. 3. Schematic representation of the active site of glucoamylase and the binding modes of n-mer and $(n+1)$-mer substrates. G represents a glucose residue. A reducing end is on the right. The wedge signifies the catalytic site of the enzyme. Subsites are numbered counting from the terminal on which a glucose residue of a productive ES complex is situated. A_i ($i = 1, 2, \cdots, m$) indicates the subsite affinity (in free energy units, kcal/mole) of the i-th subsite. The binding modes with $j = 1$ and $j = 2$ refer to productive and nonproductive complexes, respectively.

can be obtained from k_0/K_m values of $(n+1)$-mer and n-mer substrates. In this way, $A_3, A_4, \ldots, A_7$ can be evaluated using the k_0/K_m values for $n = 2$–7 listed in Table I. The A_i values are: A_3, 1.59 kcal/mole; A_4, 0.43 kcal/mole; A_5, 0.22 kcal/mole; A_6, 0.11 kcal/mole; A_7, 0.10 kcal/mole. Of these, A_3 is the largest. On the other hand, the apparent molecular binding affinity for maltose, $B_{2,app}$, is calculated to be:

$$B_{2,app} = RT \ln(1/K_m) + 2.4 \text{ kcal/mole} = (4.01 + 2.4) \text{ kcal/mole} = 6.4 \text{ kcal/mole}$$

which is much higher than A_3 and even than (A_3+A_4). Since $B_{2,app}$ includes contributions from all the possible binding modes ($j = 1,2,\ldots$), this implies that either A_1 or A_2, or at least the sum of them, should be appreciably larger than A_3. Hence the binding mode which do not involve A_1 and A_2 ($j \geqq 3$) may be ignored in comparison with the two main binding modes, $j = 1$ (productive) and $j = 2$ (nonproductive). This situation is also valid for any n-mer substrate. Thus the sum $\sum_j K_{n,j}$ is approximated as:

$$\sum_j K_{n,j} \doteqdot K_{n,1} + K_{n,2} \tag{14}$$

Then Eqns 4 and 5 reduce to:

$$(1/K_m)_n = K_{n,1} + K_{n,2} \tag{15}$$

$$(k_0)_n = k_{int} \cdot K_{n,1}/(K_{n,1} + K_{n,2}) = k_{int} \cdot (1 + K_{n,2}/K_{n,1})^{-1} \tag{16}$$

Using Eqn 8, the two association constants, $K_{n,1}$ and $K_{n,2}$ for $n < m$ are expressed in terms of A_i as follows:

$$\begin{aligned} K_{n,1} &= (0.018) \exp[(A_1 + A_2 + \cdots + A_n)/RT] \\ K_{n,2} &= (0.018) \exp[(A_2 + A_3 + \cdots + A_n + A_{n+1})RT] \end{aligned} \tag{17}$$

Substituting Eqn 17 into Eqn 16, we have:

$$\exp(A_{n+1}/RT) = [k_{int}/(k_0)_n - 1] \cdot \exp(A_1/RT) \tag{18}$$

Since the values of $(k_0)_n$ and A_{n+1} for $n = 2$–6 have now been obtained, the plot of $\exp(A_{n+1}/RT)$ against $(1/k_0)_n$ can be drawn. This plot should be linear in so far as k_{int} is independent of n. Fig. 4 shows the plot drawn using the values of $A_3, \ldots A_6$ and those of $(k_0)_n$ for $n = 2$–6. The linearity of the plot supports the validity of the assumption of the constancy of k_{int}. The vertical and the horizontal intercepts give $-\exp(A_1/RT)$ and $1/k_{int}$, respectively. Thus we have: $k_{int} = 77\ s^{-1}$ and $A_1 = 0$ kcal/mole.

The apparent zero value of the subsite affinity A_1 does not necessarily imply the absence of interaction between Subsite 1 and the glucose residues. More reasonably, it may be interpreted that the positive interaction affinity (A_1') is compensated by the distortion free energy (D) produced by the binding of a glucose residue at this subsite*. The distortion of the pyranose ring of a substrate molecule, which would greatly facilitate the hydrolysis of the substrate linkage, was actually substantiated

* In one of our earlier papers[1], the net interaction affinity A_1' and the distortion free energy were considered separately. However, it is more convenient to define subsite affinity as the algebraic sum of A_1' and distortion free energy D, as was employed in the later paper already published[11]. Thus $A_1 = A_1' - D$.

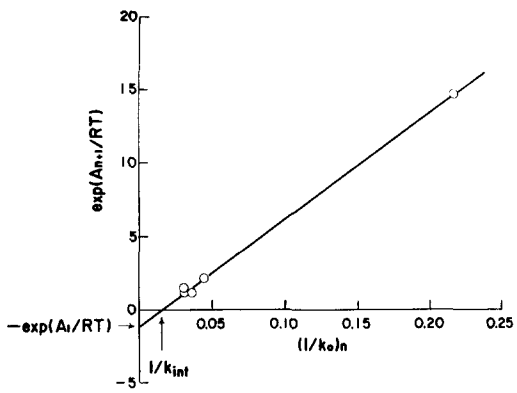

Fig. 4. A plot of exp (A_{n+1}/RT) *versus* $(1/k_0)_n$. The values of A_{n+1} and $(k_0)_n$ for $n = 2$–6 were taken from Tables III and I, respectively. A_1 and k_{int} are evaluated from the vertical and the horizontal intercepts, respectively. $A_1 = 0$ kcal/mole, $k_{int} = 77\ s^{-1}$.

from the model building based on the results of the X-ray crystallography of lysozyme[2–4].

The intrinsic rate constant of hydrolysis of a substrate linkage in a productive complex, k_{int}, (77 s^{-1}) is about twice as large as the largest of k_0 value observed (33 s^{-1} for maltotetraose). This fact implies that the contribution of the nonproductive complex ($j = 2$) is always involved, leading to the selfinhibition of substrate as was indicated by Thoma and Koshland[26] for β-amylase, and that $K_{n,2}$ (nonproductive term) contributes to a similar degree as $K_{n,1}$ (productive term) (see Eqn 16).

Next, the second subsite affinity A_2 can be evaluated by the following two methods. First, K_m for n-mer substrates can be expressed, with Eqns 15 and 17, as follows:

$$(1/K_m)_n = K_{n,1} + K_{n,2} = (0.018)\exp(A_2/RT)\cdot\exp[(A_1 + A_3 + \cdots + A_n)/RT] + \\ + (0.018)\exp(A_2/RT)\cdot\exp[(A_3 + A_4 + \cdots + A_{n+1})/RT] \quad (19)$$

where only A_2 is unknown. This equation can then be solved for A_2. The values of A_2 were calculated with Eqn 19 using A_i values obtained above and K_m values for $n = 2$–6 (Table I). The results are summarized in Table II (the column specified by Method I). The average value for $n = 2$–4 becomes $A_2 = 4.85 \pm 0.05$ kcal/mole.

The second method involves the use of k_{int} and k_0/K_m (instead of K_m in the first method). k_0/K_m for an n-mer is given by:

$$(k_0/K_m)_n = k_{int}\cdot K_{n,1} = (0.018)\,k_{int}\exp[(A_1 + A_2 + \cdots + A_n)/RT] \quad (20)$$

Therefore, we can calculate A_2 from k_{int} (77 s^{-1}), other A_i values and k_0/K_m according to Eqn 20. The results are also included in Table II (the column specified by Method II). The value of A_2 is reasonably constant for $n = 2$–7 ($A_2 = 4.79 \pm 0.03$ kcal/mole).

The discrepancy between the two values of A_2 obtained by the two different methods is insignificant.

TABLE II

THE VALUE OF SUBSITE AFFINITY A_2 CALCULATED BY TWO DIFFERENT METHODS

Values following Method I were obtained with Eqn 19 using K_m values. Values following Method II were obtained with Eqn 20 using k_{int} and k_0/K_m values. A_2 is expressed as kcal/mole.

Method	*Degree of polymerization (n)*					
	2	*3*	*4*	*5*	*6*	*7*
I	4.80	4.84	4.90	4.91	4.98	—
II	4.77	4.79	4.78	4.82	4.79	4.81

As concerns the slight tendency for A_2 values obtained by Method I to increase with n, a comment will be made later.

All the values of A_i and k_{int} have now been determined from the rate parameters of substrates with $n = 2$–7. These values are listed in Table III.

TABLE III

VALUES OF SUBSITE AFFINITIES A_i AND k_{int} FOR GLUCOAMYLASE AT 25 °C AND pH 4.50

Subsite (i)*	1	2	3	4	5	6	7
Subsite affinity (A_i)	A_1	A_2	A_3	A_4	A_5	A_6	A_7
Value of A_i (kcal/mole)	0	4.85**	1.59	0.43	0.22	0.11	0.10
		4.79***					
k_{int} (s^{-1})		77					

* The subsites are numbered from the nonreducing-end side. The catalytic site is situated between Subsites 1 and 2.

** Obtained by Method I.

*** Obtained by Method II (see text).

DISCUSSION

Since the values of A_i and k_{int} have been determined, it is now possible to calculate the rate parameters, K_m, k_0 and k_0/K_m, using Eqns 14–17. These calculated rate parameters should of course be in agreement with the experimentally obtained ones for all the substrates, insofar as the theoretical treatment is valid. In other words, the validity of the theory, including that of the basic assumptions on which the theory was based, can be checked by comparing the calculated and observed rate parameters. Relevant equations to be used for the calculation of K_m, k_0 and k_0/K_m from A_i and k_{int}, which are readily obtained from Eqns 14–17, are as follows:

$$(1/K_m)_n = K_{n,1} + K_{n,2} = (0.018)\,[\exp\,(\sum_{i=1}^{n} A_i/RT) + \exp\,(\sum_{i=2}^{n+1} A_i/RT)] \tag{21}$$

$$(k_0)_n = k_{int} \cdot K_{n,1}/(K_{n,1} + K_{n,2}) = k_{int}/[1 + \exp\,\{(A_{n+1} - A_1)/RT\}] \tag{22}$$

$$(k_0/K_m)_n = k_{int} \cdot K_{n,1} = (0.018)\,k_{int} \exp\,(\sum_{i=1}^{n} A_i/RT) \tag{23}$$

where the values of A_i for i exceeding seven may reasonably be assumed to be zero.

TABLE IV

COMPARISON BETWEEN OBSERVED AND CALCULATED RATE PARAMETERS FOR GLUCOAMYLASE

n*	K_m ($10^4 \times M$)			k_0 (s^{-1})			k_0/K_m ($10^{-4} \times M^{-1} \cdot s^{-1}$)		
	Obsd**	Calcd***	Ratio*** (obsd/calcd)	Obsd**	Calcd***	Ratio*** (obsd/calcd)	Obsd**	Calcd***	Ratio*** (obsd/calcd)
2	11	10.0	1.10	4.6	4.87	0.94	0.42	0.487	0.86
		11.3	0.97		4.92	0.93		0.435	0.97
3	3.6	3.55	1.02	23	25.1	0.92	6.4	7.07	0.91
		3.94	0.91		25.1	0.92		6.37	1.00
4	2.5	2.57	0.97	33	31.5	1.05	13	12.3	1.06
		2.39	1.05		31.4	1.05		13.2	0.98
5	1.6	1.60	1.00	32	34.1	0.94	20	21.4	0.94
		1.83	0.87		34.9	0.92		19.1	1.05
6	1.2	1.35	0.89	28	34.3	0.82	23	25.4	0.91
		1.53	0.78		35.3	0.79		23.0	1.00
7	1.1	1.16	0.95	31	38.6	0.80	28	33.2	0.84
		1.42	0.77		38.5	0.80		27.2	1.03
15.5	0.65	1.16	0.56	24	38.6	0.62	37	33.2	1.11
		1.42	0.46		38.5	0.62		27.2	1.36

* n, degree of polymerization of substrate in glucose units. For maltodextrin only, the average degree of polymerization is shown.

** Experimentally obtained values (see Table I).

*** The values in upper and lower rows indicate those calculated by using the values of A_i and k_{int} listed in Table III with two different A_2 values, obtained by Method I and II, respectively, (*cf.* Table II also).

The values of rate parameters calculated in this way with the values of A_i and k_{int} listed in Table III, are summarized in Table IV, together with the ratios between the observed and the calculated ones. For all the maltooligosaccharides except for $n = 15.5$, the ratio is satisfactorily close to unity with a maximum deviation of about 20%, which is comparable to the percentage of standard deviation for experimentally obtained rate parameters (see Table I). This confirms the essential validity of the theory, in which it was assumed that k_{int} is independent of n and that A_i values are additive.

It is noted, however, that for maltodextrin with an average degree of polymerization of 15.5, the discrepancy between the calculated and the observed rate parameters are more than the experimental error. Both the observed K_m and k_0 are appreciably lower than those expected from the theory, although $(k_0/K_m)_{obsd}/(k_0/K_m)_{calcd}$ is not very much different from unity. This tendency has also been noticed previously for the hydrolyses of maltooligosaccharides and amylose catalyzed by the same enzyme at 15 °C[12]. This phenomenon may be attributed to the participation of nonproductive complexes of the overhedge type such as shown in Fig. 5, which have not been taken into account in the above treatment. The interaction between the substrate chain and enzyme surface other than the active site, though

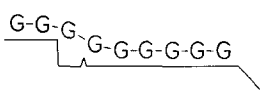

Fig. 5. A schematic model for a nonproductive complex of the overhedge type.

weak and nonspecific, may contribute to the formation of a nonproductive complex of this type, especially with longer substrates. The slight tendency of A_2 to increase with n in the column Method I in Table II is considered to be a reflection of such an interaction.

The agreement between the calculated and observed rate parameters for glucoamylase obtained in this study seems quite excellent in spite of the simplicity of the theory. Recently Thoma *et al.*[10] evaluated subsite affinities of liquefying α-amylase from *Bacillus subtilis* (an endo-amylase) by product analysis with terminally-labeled maltooligosaccharides. Their method is different in principle from that used in this study. They calculated the rate parameters with the subsite affinities thus obtained, and compared them with those obtained experimentally. However, as far as k_{int} is assumed to be independent of n, the discrepancy between the calculated and observed rate parameters are quite serious, amounting to a factor of some tens for several substrates. They attributed this discrepancy to one of the basic assumptions that k_{int} is constant, and claimed that k_{int} should vary with n, postulating some cooperative interactions between the subsites. The results obtained in this study, however, clearly showed that the assumption of the constancy of k_{int} is valid at least for glucoamylase. For endo-amylases, the situation is much more complicated due to the fact that multiple productive complexes can exist, and moreover, the possibility of formation of complexes having more than one substrate molecule bound with the enzyme (termed Bi-ES complexes) cannot be ignored[20–22,27,28]. These factors make the examination of the validity of the theory more difficult with endo-amylases than with exo-amylase. Even with endo-amylase, however, a reasonable agreement between the theory and experiment has been obtained by considering the participation of Bi-ES complexes for Taka-amylase A[27,28]. Thus the assumption that k_{int} is constant irrespective of n is essentially valid for certain amylases.

It is of interest to compare the arrangement of subsite affinities of glucoamylase (exo-amylase) with that of Taka-amylase A (endo-amylase), which was obtained by Nitta *et al.*[11]. Fig. 6 shows the arrangements of subsite affinities of these amylases in histograms.

Reflecting the difference in action patterns of these amylases, the arrangement of subsite affinities of glucoamylase is much simpler than that of Taka-amylase A.

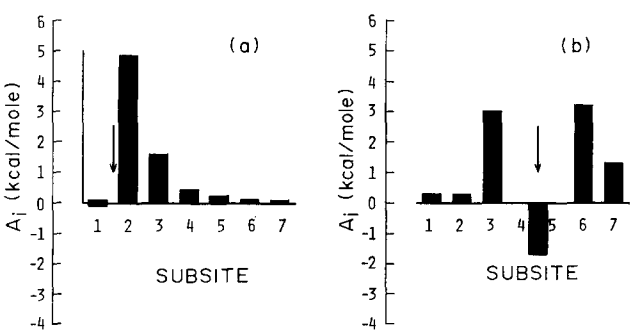

Fig. 6. Histograms showing the subsite affinities of glucoamylase from *Rh. delemar* (a) and Taka-amylase A from *Asp. oryzae* (b). The number on the abscissa denotes the subsite number counting from the non-reducing-end side of bound substrates (left side in the figure). The arrow shows the position of the catalytic site at which the substrate linkage is cleaved.

The second subsite has the largest affinity ($A_2 = 4.79$–4.85 kcal/mole), and A_i decreases, towards the reducing end side, with increasing distance from the catalytic site. There is no subsite which has a negative A_i value as was observed with Taka-amylase A[11].

The value of A_2 is comparable with the molecular binding affinity of α-glucose, B_G, obtained from the inhibitor constant K_i of α-glucose[29–31]:

$$B_G = RT \ln(1/K_i) + 2.4 \text{ kcal/mole} = 4.2 \text{ kcal/mole}$$

The close similarity between these values (A_2 and B_G) suggests that α-glucose may well be bound at the second subsite.

The magnitude of subsite affinity is indicative of the nature of the interaction between subsite and glucose residue. From the study of inhibitor constants for glucose-like monosaccharides, the free energy decrease caused by the formation of one hydrogen bond between glucoamylase and a glucose molecule is estimated at about 0.6–0.9 kcal/mole[29–31]. If we take 0.75 kcal/mole as the average value per hydrogen bond, the binding of glucose residues at Subsite 2 and 3 may be considered to involve about six and two hydrogen bonds, respectively. Lower A_i values for Subsites 4 to 7 suggests that there are nonspecific rather than specific interactions at these subsites.

The arrangement of subsite affinities, by which the population of productive and nonproductive complexes of various linear substrates are determined, are also quite useful in understanding the substrate specificity of amylase. For example, the extremely lower rates of hydrolysis of phenyl α-glucosides by glucoamylase compared with those of phenyl α-maltosides have been quantitatively accounted for in terms of the subsite affinities of glucoamylase obtained in this study[29,30].

Finally, it is apparent from the arrangement of subsite affinities of glucoamylase that there is a negligible probability of simultaneous binding of more than one substrate molecule (even for the smallest substrate maltose) at the active site of this enzyme. Therefore, the scheme in Eqn 2, from which the fundamental Eqns 4–6 are derived, is justified, where only one molecule of substrate was assumed to be bound at the enzyme active site.

For endo-amylases, however, the probability of binding of two substrate molecules at the same time is not always negligible, since there could be two subsites with large affinities on the both sides of catalytic site, as seen for Taka-amylase A in Fig. 6.

ACKNOWLEDGEMENTS

The authors are extremely grateful to Emeritus Professor Juichiro Fukumoto of Osaka City University and Dr Yoshio Tsujisaka of Osaka Municipal Technical Research Institute for their generous gift of the crystalline enzyme, and also to Mr Masatoshi Kato for his assistance in the calculation of standard deviations.

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