Effect of potential binding site overlap to binding of cellulase to cellulose: a two-dimensional simulation

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Abstract A computer simulation model for the binding of ligands to a totally anisotropic surface (infinite two-dimensional square lattice) with overlapping binding sites has been developed. The validity of the simulation has been proven by comparison with cases where the correct results are known. The simulation of kinetics shows that when the lattice is close to saturation, the true equilibrium state is reached extremely slowly due to a lot of rearranging of the ligands on the lattice. Based on these findings, the terms 'apparent saturation' and 'apparent maximum coverage' have been introduced and defined. The largest discrepancies between 'apparent maximum coverage' and the theoretically predicted value were observed for ligands of large size and/or irregular shape. As an example, the model has been applied to describe the binding of cellobiohydrolase-I core to Avicel. A formula for calculation of the intrinsic binding constant, maximal binding capacity and specific surface of cellulose from real binding data has been derived.

Key words: Cellulase; Cellulose; Adsorption; Overlapping binding sites; Mathematical model

1. Introduction

The adsorption of cellulases onto the insoluble, partially heterogeneous substrate is central in cellulose hydrolysis and is reflected in the structural organization of many cellulases [1]. Adsorption-based kinetic models for enzymatic cellulose hydrolysis have been developed [2,3] and the adsorption processes have also been studied in detail [4–7]. Several equations, many of which are based on Langmuir adsorption theory, have been employed to describe these phenomena [8–10].

The presence of two different types of substrates in cellulose which differ in their susceptibility to the binding of enzyme or enzymatic attack was proposed by Sattler et al. [11] and Wald et al. [12]. Ståhlberg et al. [13] showed that the experimental data fitted that type of model, but discussed the possibility of a broader 'continuous' affinity spectrum. We must point out that there is no structural information about the cellulose itself that could serve as strong support for any model with discrete classes of binding sites.

The assumption about two or more discrete classes of binding sites is mainly based on the deviation from linearity of a Scatchard plot showing bound enzyme/free enzyme versus free enzyme. However, this interpretation is generally valid only for ligands that interact with independent binding sites. The dimensions of cellulases (5–20 nm) greatly exceed those of the repeating cellobiose lattice units (ca. 1 nm), so adsorption will undoubtedly involve interaction with or at least masking of more than one lattice unit. This suggests that the surface of the cellulose crystal can be treated as a two-dimensional lattice comprised of an array of overlapping potential binding sites [14,15]. As previously shown for a one-dimensional lattice, the binding of any large ligand to an array of overlapping potential binding sites results in a nonlinear Scatchard plot for which classical analysis is inapplicable [16]. It is evident that such effects should be still more important in the two-dimensional case.

Contrary to the one-dimensional case, there is to the best of our knowledge no universal analytical expression derived which describes the binding of large ligands to a two-dimensional lattice with overlapping potential binding sites. Stankowsky [17,18] has given a formula to treat the limiting cases of very thin and of very bulky ligands. An attempt has also been made to extend this formula empirically to include ligands of any shape and also to cooperative interactions [19]. However, no clear evidence is given to show the applicability of this formula in the case of ligands with complicated shapes like cellulases. In this work a different approach has been developed to estimate the amount of bound ligands as a function of the amount of free ligands: a simulation process in which the completely anisotropic lattice surface is randomly sampled with ligands for available sites at any degree of lattice saturation.

2. Materials and methods

2.1. Simulation methods

2.1.1. Infinite surface. The infinite surface where the ligands will bind consists of repeating rectangular surface units (RSU) with common borders (like an infinite number of chessboards laid side by side in every direction). Every RSU contains at least 1024 elementary binding sites (EBS) but can be arbitrarily larger depending only on the memory resources of the computer. The term common borders of RSU means in our model that in order to avoid edge effects the ligands can be bound also over the border of one RSU. If one ligand happens to bind, such as to overlap the right border of one RSU then the remaining part of the ligand will appear on the left edge of the adjacent RSU (Fig. 1). The lattice was defined as completely anisotropic which means the nonequivalence of two dimensions, polarity in the vertical dimension and polarity in the horizontal dimension. The ligands are defined as asymmetric and binding in only one of the possible orientations. We expect this mode of interaction to be relevant to the cellulose/cellulase system.

2.1.2. Association and dissociation of ligands. The simulations were carried out to mimic an experimental situation where a surface is introduced at time zero into a solution containing the adsorbing ligands. The binding proceeds step by step. At every step a certain number of ligands

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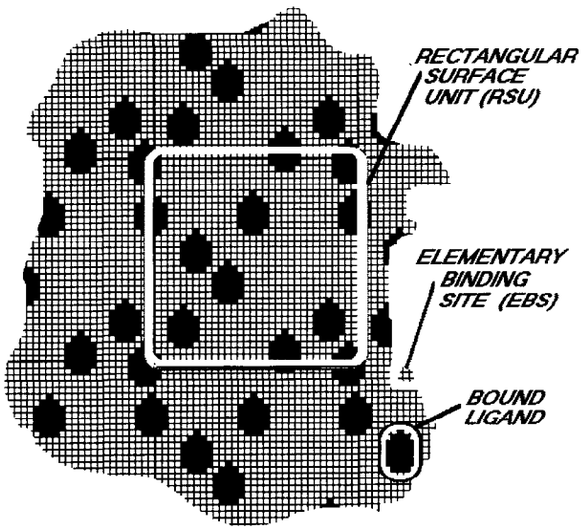

Fig. 1. The illustration of computer simulation of binding of ligands to a totally anisotropic surface with overlapping potential binding sites. The ligand has a complicated shape with a total area equal to 27 EBS (elementary binding sites). The size of the repeating RSU (rectangular surface unit) on the figure is very small (32 × 32 EBS).

try to bind to the surface and at the same time a certain number of ligands will dissociate from the surface. The number of ligands trying to associate to the RSU is calculated according to the mass action law:

$$L_{pot.ass} = k_1 \times L_{free} \times Nr_{EBS}$$

The number of dissociating ligands in the same step is:

$$L_{diss} = k_2 \times L_{bound}$$

where L_{diss} and $L_{pot.ass}$ are the number of ligands dissociating and trying to associate; L_{free} is the concentration of free ligand and L_{bound} is the number of bound ligands; Nr_{EBS} is the total number of EBS in the RSU; k_1 and k_2 are the corresponding rate constants.

When removing or attempting to add bound ligands, a bound ligand or potential surface binding site, respectively, is chosen at random. Binding will occur only when the randomly chosen site in the lattice is completely uncovered. The number of associating ligands L_{ass} is therefore always less than or equal to $L_{pot.ass}$.

In the case of binding equilibrium simulation, the key parameter is only the *ratio* of the rate constants k_1/k_2. This *intrinsic binding constant* $K_{int.bind}$ characterizes the binding ability of ligand at infinitively low ligand concentration where the spacial problems of binding are practically negligible.

2.2. Establishing of best fitting equations for formal description of the results of simulation of ligand binding

This part of work has been done using the software SYSTAT (SYSTAT Inc.).

3. Results and discussion

To demonstrate the influence of the size of the RSU to the accuracy and reproducibility of the results we simulated the binding of the 1 × 1, 2 × 2, 4 × 4 and 8 × 8 square ligands to the RSU with the size of 32 × 32, 64 × 64 and 128 × 128 EBS (Table 1).Every simulation was repeated 5 times. The ligands were chosen to be able to compare the simulation results with calculations by Stankowski's formula [18] (2 × 2, 4 × 4 and 8 × 8 ligands) or Langmuir isotherm (1 × 1 ligands). The equality of results obtained by different methods indicates the applicability of the simulation also at more complex cases where no analytical formula is available as the used method does not depend on the size and shape of ligands.

The results of simulation show that the reproducibility and accuracy of the results is somewhat better at larger RSU (Table 1). However, the time of simulation is linearly dependent on the area of the RSU and therefore the strategy to obtain the results with needed accuracy can be either to repeat the simulation more times using smaller RSU or vice versa. The reproducibility of results should be checked at all simulations, as it depends also on size and shape of ligands. It is important to notice, however, that the average values show no significant differences between the RSU tested except for the extreme case of 8 × 8 squares on 32 × 32 RSU.

A problem could arise when the true equilibrium state is reached very slowly. This situation is most likely to occur when the lattice is close to saturation and a lot of rearranging is required to allow the binding of each additional ligand. For demonstration of this the kinetics of binding of 3 × 3 ligands at high concentrations to RSU with the size 64 × 64 EBS is shown on Fig. 2. The kinetics of binding consists of the fast first stage and the slow second stage where the rate of rearrangement limits the rate of the whole process. At high concentrations of the ligands the rate of rearrangement depends mainly on the rate of dissociation as every newly formed vacant binding site is practically *in situ nascendi* filled with ligand due to the high association rate. The results of simulation of binding at high ligand concentration after different times are shown in Table 2. Our results show that a practically stable coverage, which is almost independent of ligand concentration, is

Table 1
Coverage (%) of the surface by the square ligands with different size to the RSU with the size of 32 × 32, 64 × 64 and 128 × 128 EBS

Size of ligand (EBS)	[Ligand]	Coverage (%)			Theoretical coverage
		Size of RSU (EBS)			
		32 × 32	64 × 64	128 × 128	
1 × 1	1	50.1 ± 0.2	50.0 ± 0.1	50.0 ± 0.1	50
1 × 1	10	90.7 ± 0.2	91.0 ± 0.1	insuff. progr. mem.	90.9
2 × 2	1	54.6 ± 0.8	54.7 ± 0.4	55.1 ± 0.2	54.9
2 × 2	10	77.8 ± 0.6	77.6 ± 0.5	77.4 ± 0.2	77.2
4 × 4	1	57.6 ± 0.8	57.5 ± 0.5	57.8 ± 0.2	58.0
8 × 8	1	55.6 ± 3.2	59.7 ± 2.2	60.8 ± 1.3	61.6

The mean value and standard error are calculated using results of 5 simulations. Theoretical binding is calculated by Stankowsky's formula [18] or by Langmuir's isotherm (1 × 1 ligands). Dissociation equilibrium constant = 1.

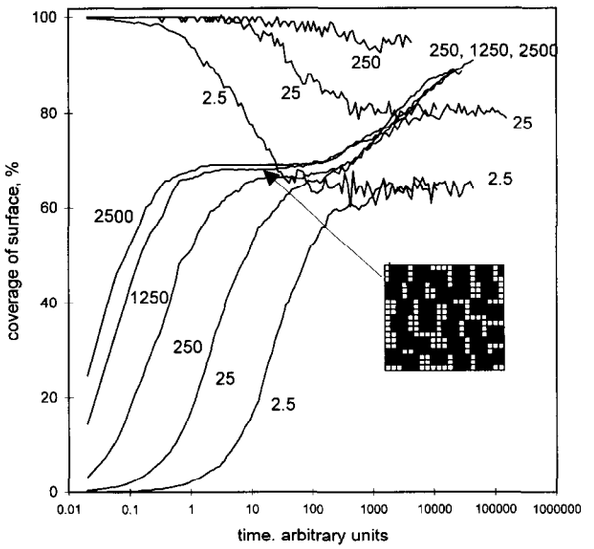

Fig. 2. Simulation of the kinetics for binding of square ligands with size 3 × 3 EBS to the infinite surface with RSU = 48 × 48 EBS. Dissociation equilibrium constant = 0.11, rate constant for association = 2.3, rate constant for dissociation = 0.256. Numbers at curves indicate the free ligand concentration in arbitrary units. The arrow indicates the 'apparent equilibrium' region for high ligand concentration. The insert shows a typical surface coverage at 'apparent equilibrium'. The true equilibrium coverage calculated by Stankowsky's formula [18] was 64.4%, 78.0%, 86.0%, 89.5% and 90.7% at the free ligand concentrations 2.5, 25, 250, 1250 and 2500, respectively.

achieved at high concentration of ligands. This differs significantly not only from that of a Langmuir isotherm but also from the theoretical value calculated by Stankowsky's formula [18]. Hereby we would like to introduce the term 'apparent equilibrium' for indicating the state achieved at a time when the amount of ligand bound will increase less than 2% if time is increased an additional 10 times. The 'apparent maximum coverage' can be introduced respectively to indicate the coverage of the surface at ligand concentrations where 'apparent equilibrium' differs not more than 1% in comparison with 10 times higher ligand concentration. Both criteria would be regarded as adequate to indicate that 'true' equilibrium or 'true' saturation has been reached in a real experiment. These new terms would be significant in description of biochemical systems where the true saturation never will be reached in experiments due to both the large and irregular shape of ligands and the comparatively low dissociation (rearranging) rates. The considerable difference between true and apparent maximum coverage, respectively, for ligands of the same size but different shapes is shown in Fig. 3. and for ligands of the same shape but different size in Fig. 4. Fig. 5 compares apparent equilibrium, true equilibrium and Langmuir isotherm at different concentrations of 6 × 6 ligand.

To obtain binding parameters from experiments, the possibility for fast calculation of binding isotherms is needed. In this sense, the simulation of binding is too slow. Therefore, after initial simulation of binding it is advantageous to find an analytical expression that gives a good *numerical* fit to the simulated isotherm. This whole procedure is here applied to published data on the adsorption of the core domain of *Trichoderma reesei* cellobiohydrolase I (CBH I) to microcrystalline cellulose (Avicel) [13]. CBH I is the quantitatively dominant cellulase of *Trichoderma reesei* and several other fungi [20]. Limited proteolysis with papain cleaves the enzyme into its two functional domains [21], which can then be assayed separately.

We choose the cellobiose residues as the elementary binding sites on the cellulose surface with the size 1 × 0.5 nm [22]. The size of CBH I core is approximately 6 × 4 nm and the shape is given in data measured by SAXS [23] and X-ray crystallography [24]. The shape of the ligand as built from cellobiose-sized units is shown in Fig. 6. We also assumed that the shape was unaffected by binding and simulated the binding of these ligands to the periodic boundary surface with repeating surface units of 320 × 320 elementary binding sites. The concentration of free ligand was expressed as moles of free CBH I core per 320 × 320 mole of cellobiose residues on the cellulose surface. Total binding was expressed as the degree of covered surface.

After simulation of binding we tried different equations for the best-fit to the resulting data. The simulation results indicated a divergence between the apparent and the true equilibrium as the surface coverage exceeds ~58%. As the further analysis shows ,the experimental binding isotherm [13] was measured up to 50% of the surface coverage. To obtain the adequate analytical expression for this binding curve,we used the results of simulating from the same region of surface coverage (0–50%) where experiment was performed. The results of fitting are shown in Table 3.

As expected, none of the functions showed a perfect fit. The sum of hyperbola and Hill's model has the smallest sum of residues squared, but since the sum of two hyperbola has one parameter less, the latter is preferable. Rather unexpectedly, it was impossible to fit the formulas of Stankowsky [18] and

Table 2
The time curves of simulated binding of square ligands with size 6 × 6 EBS at high concentration

[Ligand]	Coverage (% of the whole surface)				
	Time (arbitrary units)				Theoretical binding
	1	10	100	1000	
25	52.5 ± 0.9	66.6 ± 0.9	67.9 ± 0.9	74.7 ± 0.9	85.9
125	66.6 ± 1.5	68.8 ± 1.5	69.4 ± 1.3	75.4 ± 0.7	89.5
250	66.8 ± 0.9	67.2 ± 0.9	68.1 ± 1.1	75.1 ± 1.5	90.7

Size of RSU was 48 × 48 EBS. Dissociation equilibrium constant = 0.11, rate constant for association = 2.3, rate constant for dissociation = 0.256. Mean value and standard error of binding were calculated using the results of 5 simulations. Theoretical binding equilibrium is calculated by Stankowsky's formula [18].

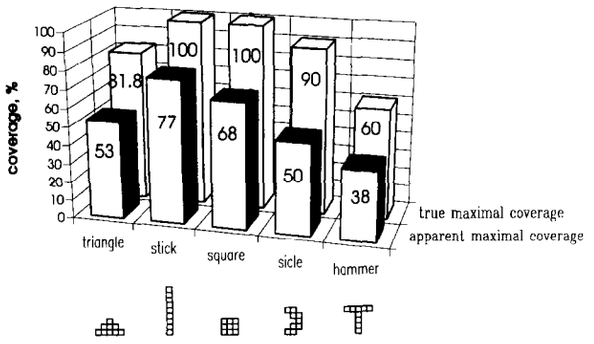

Fig. 3. The apparent maximal coverage of a totally anisotropic surface by ligands with different shapes (pale bars) in comparison with the true maximal 'crystal-like' covering of the surface (dark bars) .

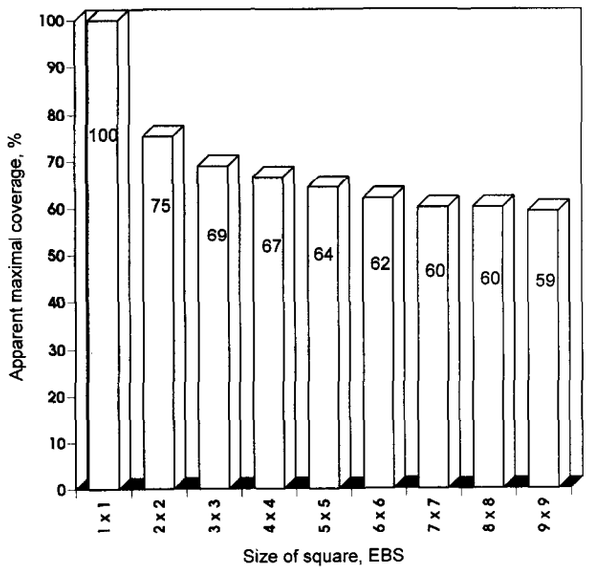

Fig. 4. The apparent maximal coverage by square ligands with different size. The true maximal coverage is here 100%.

Andrews [25] to the simulated data freely varying all parameters because the parameters showed a strong linear correlation in our case.

The sum of two hyperbolas has already been used quite successfully by many authors to describe experimental data. This good fit has served as the main evidence for the hypothesis about two discrete types of binding sites with different properties [11–13]. The equally good fit between our simulation data and the two-hyperbola function stresses that a good numerical correlation between the experimental binding data and this function does not necessary reflect a real two-site binding. Still, this function finds practical use, e.g. as a standard curve for binding.

We also choose the sum of two hyperbolas as a formal model in order to get an equation for the binding isotherm in the case of ligands with the shape of CBH I core [23]. After simulation of binding with a set of different intrinsic binding constants we found out that, in all cases, the constants C_1, C_2 and C_4/C_3 were equal to 0.37, 0.23 and 32.3, respectively. This results in an empirical analytical formula which fits well in this case:

$$S_{\text{covered}} = \frac{0.37 \cdot [\text{Core}_{\text{free}}]}{C_3 + [\text{Core}_{\text{free}}]} + \frac{0.23 \cdot [\text{Core}_{\text{free}}]}{C_3 \cdot 32.3 + [\text{Core}_{\text{free}}]} \quad (1)$$

where S_{covered} = degree of covered surface, $[\text{Core}_{\text{free}}]$ = concentration of non-adsorbed CBH I core domain, C_3 = the formal scaling constant depending on the intrinsic binding constant.

In Avicel it is impossible to define the total available surface of cellulose and therefore we have fitted the experimental data of [13] to the formal model:

$$\frac{[\text{Core}_{\text{bound}}]}{[\text{Cellulose}]_0} = \frac{S_{\text{spec}} \cdot 0.37 \cdot [\text{Core}_{\text{free}}]}{C_3 + [\text{Core}_{\text{free}}]} + \frac{S_{\text{spec}} \cdot 0.23 \cdot [\text{Core}_{\text{free}}]}{C_3 \cdot 32.3 + [\text{Core}_{\text{free}}]} \quad (2)$$

where S_{spec}= the specific binding capacity of cellulose (μmol of core-protein per mg of cellulose); S_{spec} and C_3 varied as free parameters.

The quality of the fit is essentially the same as that obtained by using the two-site binding model (as it ought to be) (Fig. 6). However, in an explicit two-site binding model two additional assumptions are made illegally: (1) that the binding sites for

Table 3
Comparison of fitness of various analytical expressions with data obtained from simulation of binding of CBH I core domain (intrinsic binding constant equal to 1; 125 points of simulation; repeating rectangular surface unit 320 × 320)

Formula	Sum of squared residuals
Sum of Hill's model [17] and hyperbola	2.40E + 03
$L_{\text{bound}} = \frac{C_1 \times L_{\text{free}}}{C_3 + L_{\text{free}}} + \frac{C_2 \times L_{\text{free}}^{C_5}}{C_4 + L_{\text{free}}^{C_5}}$	
Sum of two hyperbolas	4.30E + 03
$L_{\text{bound}} = \frac{C_1 \times L_{\text{free}}}{C_3 + L_{\text{free}}} + \frac{C_2 \times L_{\text{free}}}{C_4 + L_{\text{free}}}$	
Hill's model [17]	2.21E + 04
$L_{\text{bound}} = \frac{C_2 \times L_{\text{free}}^{C_5}}{C_4 + L_{\text{free}}^{C_5}}$	
Sum of hyperbola and straight line	3.39E + 04
$L_{\text{bound}} = \frac{C_4 \times L_{\text{free}}}{C_3 + L_{\text{free}}} + C_2 \times L_{\text{free}}$	
Hyperbola	1.29E + 05
$L_{\text{bound}} = \frac{C_1 \times L_{\text{free}}}{C_{\text{free}} + L_{\text{free}}}$	
Monod-Wyman-Changeux' model for cooperative binding [26]	did not converge
Stankowsky's model for binding to the overlapping binding sites [18]	did not converge
Andrew's model for binding to the overlapping binding sites [25]	did not converge

these ligands on the cellulose surface are separate (do not overlap); (2) that there exist two distinctly different types of binding sites. According to Occam's principle we should give preference to the model that takes into account overlapping of potential binding sites because this model requires fewer additional assumptions.

The intrinsic binding constant can be calculated as follows:

$$K_{\text{int.bind.}} = \frac{[\text{Core}_{\text{bound}}]}{[\text{Cellulose}_{\text{free}}] \cdot S_{\text{spec}} \cdot [\text{Core}_{\text{free}}]} = \lim_{\text{Core}_{\text{free}} \to 0} \left(\frac{[\text{Core}_{\text{bound}}]}{[\text{Cellulose}]_0 \cdot S_{\text{spec}} \cdot [\text{Core}_{\text{free}}]} \right) = \frac{0.37}{C_3} + \frac{0.23}{C_3 \cdot 32.3} \quad (3)$$

In our example we obtained values for: (1) intrinsic binding constant = 0.175 mM^{-1}; and (2) specific binding capacity of cellulose = 0.36 mmol core/g cellulose (in surface units 5.20 m^2/g).

The simulation procedure described above can be adapted for any case of ligand binding to overlapping binding sites. The model construction consists of four principal stages.

(1) Simulation of the binding of ligands with their particular qualities (size, shape, cooperativity in binding, etc.) to the surface.

(2) Finding the mathematical function that gives the best fit to the simulated data. Identifying the physical meanings (intrinsic binding constant, maximal capacity, etc.) of the function parameters (or their combinations).

(3) Finding numerical values for parameters of the selected function by nonlinear parametrization using experimental data.

(4) Interpretation of the function parameters according to (2).

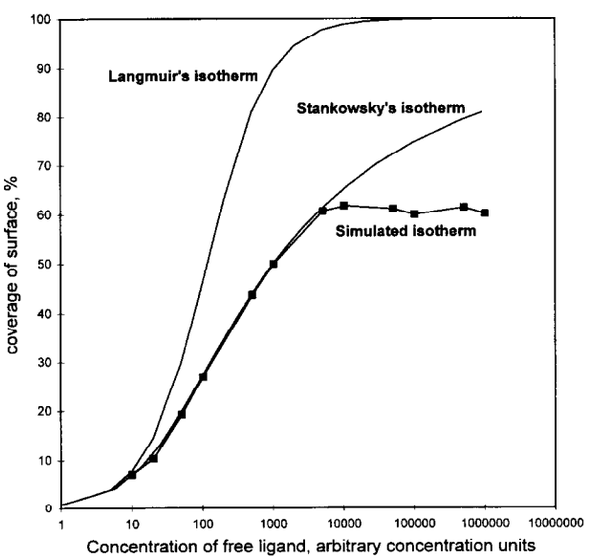

Fig. 5. Simulated binding equilibrium (apparent equilibrium for [Free ligand] > 5000) in comparison with true binding equilibrium calculated by Stankowsky [18] and with a normal Langmuir isotherm. Dissociation constant = 114 arbitrary concentration units.

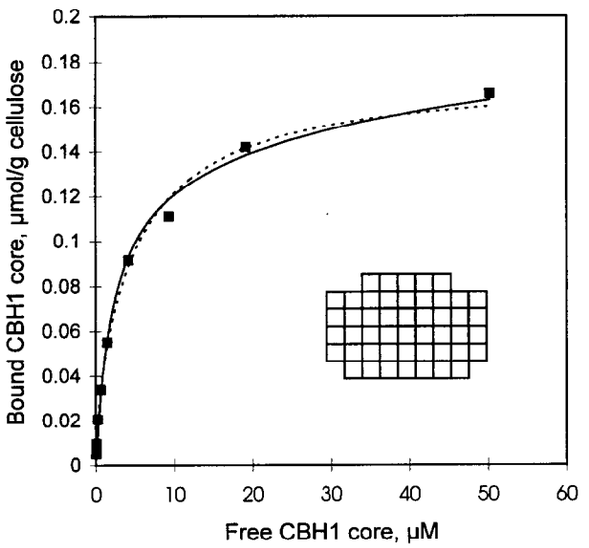

Fig. 6. The fit between experimental data (points), simulated model (line) and a formal two-site binding model (dotted line) for the CBH1 core domain. The insert shows the assumed shape of the CBH I core when built from cellobiose-sized elementary binding sites.

Besides providing numerical values for the intrinsic binding constant, maximum available surface for binding, etc., this procedure can be employed to discriminate among various hypotheses about the size, shape and orientation of bound ligands.

Besides the cellulase/cellulose system, other relevant objects for this type of modeling include systems as diverse as amylases/starch, cells/adsorption surface and antibodies/antigen saturated surface.

The computer program is available free of charge on written request to the authors.

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