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Frontal Analysis Continuous Capillary Electrophoresis for Protein–Polyelectrolyte Binding Studies

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Summary

A novel technique, frontal analysis continuous capillary electrophoresis (FACCE), has been described as an effective way to study protein–polyelectrolyte binding. FACCE involves continuous sampling, integrating sample injection and separation into one process that provides advantages over conventional frontal chromatography. The method provides rapid and precise determination of binding isotherms, and allows for quantitative binding analysis in terms of binding constant and the binding-site size by considering the protein as the ligand and allowing the polyelectrolyte to bind to a number of proteins with variable levels of cooperativity. FACCE is particularly suitable for binding systems involving rapid binding kinetics because it allows for the determination of the concentrations of free or bound ligands under conditions that avoid perturbation of the binding equilibrium. This chapter focuses on studies of the binding of bovine serum albumin (BSA) to heparin using FACCE. These investigations are demonstrated within the context of this chapter as representative of a model protein–polyelectrolyte system from which extensions to other systems can be made.

Key Words

Binding constant; binding isotherm; binding site size; bovine serum albumin; capillary electrophoresis; heparin; patch binding; protein–polyelectrolyte.

1. Introduction

Capillary electrophoresis (CE) has been used increasingly for protein–ligand binding studies, such as the association of proteins with drugs ***(1–4)***, inorganic ions ***(5,6)***, sugars ***(7)*** and micelles ***(8,9)***. The CE techniques applied involve either the measurement of mobility or the determination of the concentrations of free or bound ligand ***(1–3,10)***. The main problems observed in binding studies based on those CE techniques are: (1) the difficulty in calibration for quan-

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titative studies, and (2) the perturbation of the binding equilibria caused by the dynamic behavior of the systems under study. A novel method that addresses these problems, frontal analysis continuous capillary electrophoresis (FACCE) ***(11,12)***, has been developed for the study of protein–polyelectrolyte (PE) binding.

Because both biological and synthetic polyelectrolytes have contour lengths that are large compared to the polyelectrolyte-binding sites on the protein surface, it is necessary to view the protein in these cases as the ligand, one polyelectrolyte capable of binding a number of proteins. There is no fundamental problem in discriminating between bound and free proteins, and there is a sound theoretical framework for the analysis of such macromolecular binding ***(13)***. Experimentally, the binding of proteins to linear macromolecules has been studied by turbidimetry, light scattering, electrophoretic mobility, viscometry, fluorescence, potentiometric titration, and dialysis equilibrium ***(14)***. However, only the last directly yields binding isotherms, and it is unacceptably slow. Thus, FACCE addresses the need for rapid and precise determination of such binding isotherms.

In contrast to conventional frontal chromatography (CFC), FACCE combines continuous sampling, integrating sample injection and separation into one process as illustrated in **Fig. 1**. The electroosmotic flow that transports all components toward the cathode arises from the negatively charged wall of the fused-silica capillary. CFC employs regular chromatography methods but with a sample volume relatively large compared to the capacity of the separation column. Careful selection of the amount of sample injected, the flow rate of the mobile phase, and the length of the separation column, leads to continuous but distinct plateaus, followed by resumption of detector baseline response as shown in the electropherograms in **Fig. 1A**. In FACCE, the capillary is filled and equilibrated with the run buffer prior to sample introduction. The inlet end of the capillary is then immersed in the sample vial and a voltage is applied across the capillary to initiate the sample introduction and separation process. Species separated by electrophoresis appear as discrete and progressive plateaus in the electropherograms as shown in **Fig. 1B**. The separation profile of FACCE is particularly suitable for the study of binding in systems with rapid binding kinetics, inasmuch as it allows for the determination of the concentrations of free or bound ligands without complete separation avoiding perturbation of the binding equilibrium.

FACCE is particularly effective in multiple complexation equilibria where more than one protein binds to a single ligand; measurement of the concentration of free ligand is determined not from mobility but from the peak height which directly indicates the free protein concentration. The stoichiometric relationship between bound protein and the protein–ligand complex can then be fit to appropriate binding isotherms to yield binding constants and the bind-

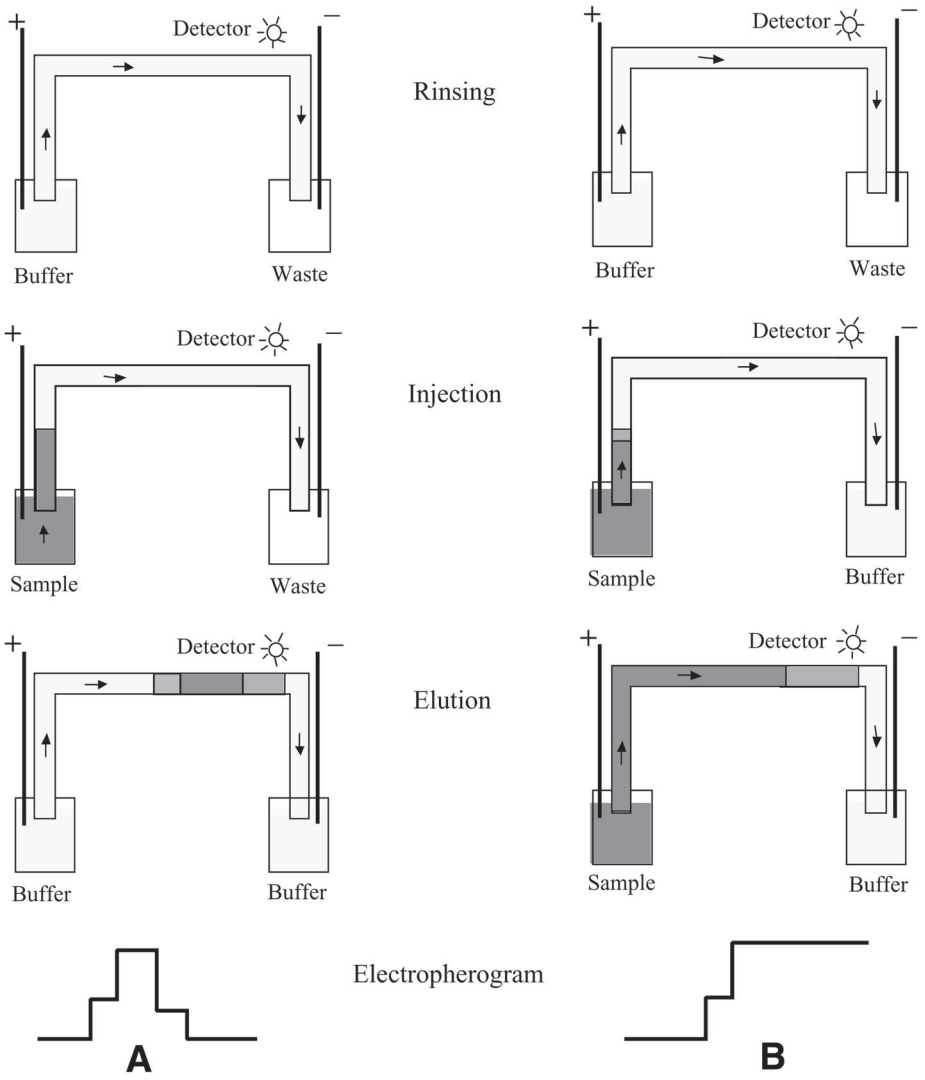

Fig. 1. Schematics of CFC (**A**) and FACCE (**B**). Reprinted with permission from **ref. *12***.

ing-site size, both of which are parameters essential to the elucidation of the binding mechanism. Binding of proteins with both synthetic and biological polyelectrolytes is an example of multiple complexation and has, therefore, been studied with FACCE ***(11–25)***. In this paper, analysis by FACCE of the binding of bovine serum albumin (BSA) and heparin will be discussed as an example. Because heparin is a negatively charged polyelectrolyte, it interacts

with the positive charges on the protein. The positive charges of the protein increase with decreasing pH, and the binding force increases. However, binding often occurs at pH above the isolecetric point (p*I*) of the protein where the protein bears the same net charge as the polyelectrolyte. For example, the p*I* of BSA is 4.9 and binding at $I = 0.01\ M$ occurs at pH 7.0, where both the protein and the polymer bear net-negative charges. This is an indication of "patch binding" in which the electrostatic attraction between polyanion and a local protein positive region overcomes the repulsion between polyanion and the global protein charge ***(15)***.

Many functions have been ascribed to the interaction of heparin with various proteins ***(26)***. Although no specific function has been identified with the heparin–BSA interaction, this system has been used to develop the methodology that would enable application of the technique to, for example, heparin–protein cognate pairs, DNA-binding proteins, and other biological polyelectrolyte–protein systems. The extended use of FACCE to such protein–polyelectrolyte systems will facilitate a better understanding of many biological phenomena.

2. Materials

1. Heparin (sodium salt, porcine intestinal mucosa, Calbiochem, La Jolla, CA, nominal M_r 13,500–15,000).
2. BSA (fatty acid free, M_r 68,000, Boehringer Mannheim, Indianapolis, IN).
3. For turbidimetric titrations: Brinkmann PC800 probe colorimeter detecting at 420 nm, equipped with a 1-cm path-length fiber optics probe, and a pH meter.
4. FACCE rinsing and run buffer: phosphate buffer prepared at desired pH and ionic strength.
5. For FACCE rinsing: 1 *N* NaOH before each use of capillary column, 0.1 *N* NaOH for rinsing between consecutive runs.
6. FACCE instrument: P/ACE 5500 CE (Beckman, Fullerton, CA).
7. Fused-silica capillary of dimensions 50 μm × 27 cm (Polymicro Technologies Inc, Phoenix, AZ).
8. Milli-Q water for all buffer and solution preparations (Millipore, Milford, MA).
9. All protein and PE solutions should be prepared fresh, and complete solubilization should be achieved prior to experiments.

3. Methods

3.1. Identification of Soluble Complex Region for Protein–Polyelectrolyte System

Binding equilibria in any protein–PE system can only be studied under conditions corresponding to the formation of the complex without second-order reactions, such as aggregation or phase separation. Any protein–PE system can be identified in terms of one of these three states. For a polyanion–protein system at fixed ionic strength, the system progresses, upon decrease in pH,

from: (a) noninteracting solution, to (b) soluble complex phase, and finally to (c) complex aggregate or biphasic state ***(27–29)***. Turbidity is sufficiently sensitive to detect the two relevant transitions; therefore, these three regions can be effectively identified by pH-turbidimetric titration. Titrations should be done at various ionic strengths to enable construction of a phase boundary: a plot of transition pH vs ionic strength, which shows the three regions corresponding to (a), (b), and (c) states. This will allow for determination of the working conditions for FACCE experiments, where the pH and ionic strength of the solution should lie in the complex formation region (b).

3.1.1. Preparation of Protein–Polyelectrolyte Solutions for pH-Turbidimetric Titration

1. The concentrations of protein and PE solutions to be prepared should be determined such that protein is in excess when they are mixed. A weight ratio of protein to PE of 10 would be appropriate for a typical titration.
2. Dissolve the protein and PE separately in desired salt solution.
3. Mix appropriate amounts of protein and PE solutions to achieve the mixture with the desired weight ratio.
4. Prepare a blank protein solution for blank titration that has the protein concentration in the mixture.
5. Add 1 *M* NaOH gradually to the mixture to adjust the pH to 10.0.
6. As an example, 4 mg/mL of BSA and 0.4 mg/mL of heparin solutions were prepared in 0.01 *M* NaCl solution, and 10 mL of both were combined to obtain a final mixture with weight ratio of 10, making a total volume of 20 mL. (The required amount of sample volume depends on the size of the probe and the container.) A "blank" protein solution was 4 mg/mL BSA in the same NaCl solution.

3.1.2. pH-Turbidimetric Titration

The soluble complex region lies between pH at the initial point of increasing turbidity (pHc) and pH at the point of the abrupt increment of turbidity (pHϕ) ***(14,15)***.

1. Titrate the protein–PE solution with 1 *M* HCl using, e.g., Gilmont microburet, while monitoring simultaneously pH and % transmittance (%T) at 420 nm.
2. Plot 100-%T, proportional to turbidity vs pH, and identify pHc and pHϕ.
3. A sample plot for heparin and BSA at $I = 0.01$ *M* is shown in **Fig. 2**. The soluble complex exists in region 2 which lies between pHc (7.1) and pHϕ (5.0).

3.2. CE

3.2.1. Equipment

CE is performed using a Beckman P/ACE 5500 CE with programmatic autosampling. Operating temperature is at 25°C. The dimensions of the fused-silica capillary are 50 μm × 27 cm with an effective length (the distance from the inlet end to the UV detector) of 20 cm.

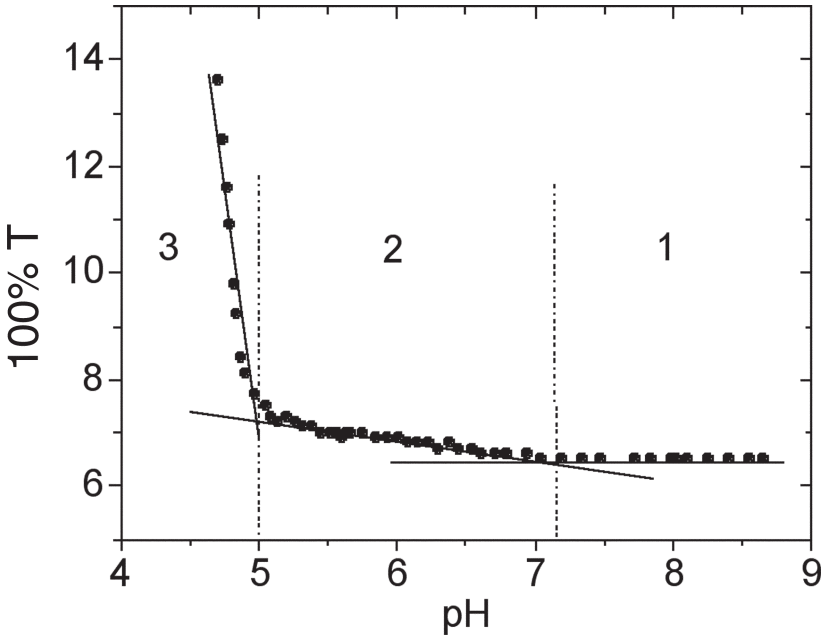

Fig. 2. Turbidimetric titration curve for 1 g/L BSA and 1 g/L heparin at I=0.01 M, Region 1, no complex formation as turbidity does not change. Region 2, complex formation as turbidity starts to increase gradually. Region 3, phase separation as there is an abrupt change in turbidity. The dashed lines represent the defined pHc and pHϕ values dividing regions 1, 2, and 3. Reprinted with permission from **ref. *19***.

3.2.2. Procedure for FACCE

Each measurement follows the procedure given below.

1. Wash the capillary with 0.1 *M* NaOH solution for 5 min. (This rinsing is necessary between each run to remove any adsorbed protein on the capillary surface. *See* **Note 1**.)
2. Rinse the capillary with water for 5 min.
3. Run phosphate buffer solution of desired pH and ionic strength through the capillary for 5 min.
4. Immerse the negative pole tip of the capillary into the sample and the positive pole tip into the buffer solution.
5. Apply a constant voltage and monitor UV absorbed spectra at a certain wavelength. The operating voltage and the wavelength should be determined so that the best resolution is obtained for the desired components to be measured. The typical applied voltage range is 5–15 kV. For most proteins, 200 or 214 nm would be the best detection wavelength.
6. As an example, the electropherograms shown in **Fig. 3** was obtained by applying a voltage of 10 kV at 214-nm wavelength.

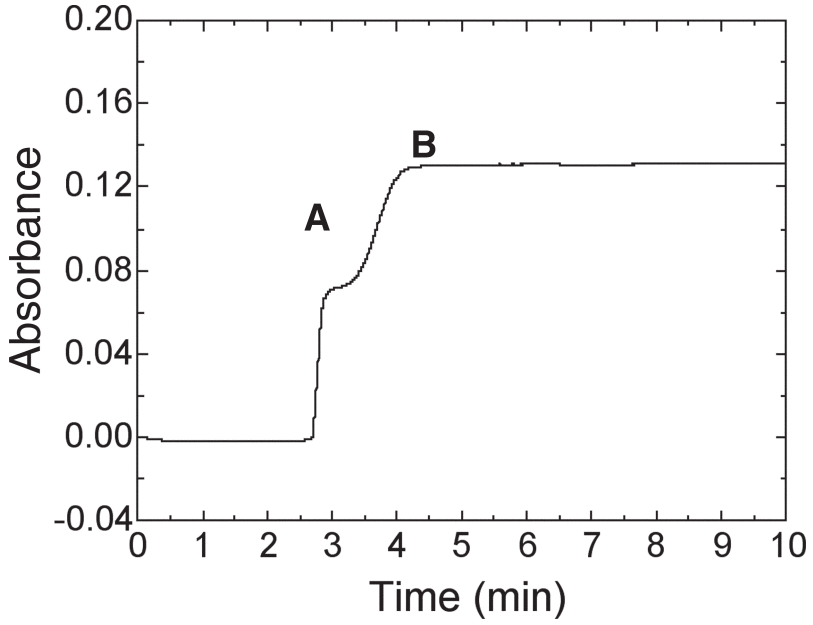

Fig. 3. Electropherogram of a sample FACCE experiment with BSA (4 g/L) and heparin (0.2 g/L) mixture at pH=6.8, *I*=0.01 *M* with plateaus representing (a) free BSA, (b) BSA–heparin complex.

3.3. Determination of Free–Protein Concentration

The electropherogram of FACCE shows multiple plateaus, the number of which depends on the number of components in the mixture. In a protein–PE mixture, two plateaus are typically observed (*see* **Fig. 3**), the first plateau corresponding to free protein and the second to protein–PE complex. The concentration of free protein can be determined by the height of the first plateau using a calibration curve ***(12)***.

3.3.1. Calibration Curve for Free Protein

1. Solutions of protein from 0.1 to 1.5 mg/mL are prepared in NaH_2PO_4–Na_2HPO_4 buffer solution at the desired pH and ionic strength. Measure each sample solution as explained in **Subheading 3.2.2**.
2. Measure the height of the single plateau in the electropherogram at each concentration.
3. Plot absorbance (plateau height) vs concentration of protein.

3.3.2. Determination of Free Protein in a Protein–PE System

1. Make up a series of solutions containing protein and PE dissolved in the appropriate buffer solution. The concentrations needed for the analysis depend on the strength of UV signal. For example, for BSA and heparin, the protein concentration was 0.4–4.0 g/L with heparin at 0.2 g/L.
2. Measure each sample solution as explained in **Subheading 3.2.2**.
3. Determine the concentration of free protein by absorbance of first plateau height using the calibration curve (*see* **Note 2**).

3.4. Data Analysis

There are several procedures for fitting binding isotherm data ***(30,31)***. Particularly appropriate in the present case is the one based on a binding theory of large ligands to a 1D homogeneous lattice given by McGhee and von-Hippel ***(32,33)***. This is appropriate for specific or nonspecific binding involving cooperative or noncooperative interaction between binding sites.

3.4.1. Constructing the Binding Isotherm

The binding isotherms were obtained by plotting the concentration of free protein, L_{free}, which is calculated as explained above vs the average number of bound protein per unit charged group on heparin, ν (*see* **Note 3**) and (**Fig. 4**).

3.4.2. Determination of Binding Parameters Via McGhee and von-Hippel Equation

General equation for McGhee and von Hippel model ***(31)*** is given in **Eq. 1**:

$$\frac{\nu}{L_{free}} = K_b(1 - n\nu)\left[\frac{(2w+1)(1-n\nu)+\nu-R}{2(w-1)(1-n\nu)}\right]^{n-1} \times \left[\frac{1-(n+1)\nu+R}{2(1-n\nu)}\right]^2 \quad (1)$$

with

$$R = \sqrt{\left[1-(n+1)\nu\right]^2 + 4w\nu(1-n\nu)} \quad (2)$$

where ν represents the binding density (in units of moles of bound ligand per mole of total lattice residue), L_{free} is the free-ligand concentration, K_b is the binding constant, n is the number of binding sites, and w is the cooperativity parameter.

For w=1, which is the case for noncooperative binding, **Eq. 1** reduces to **Eq. 3** for noninteracting ligands. In the case of BSA–heparin, **Eq. 3** was used for analysis of binding parameter as no additional cooperativity term was needed to obtain a good fit to the binding isotherms.

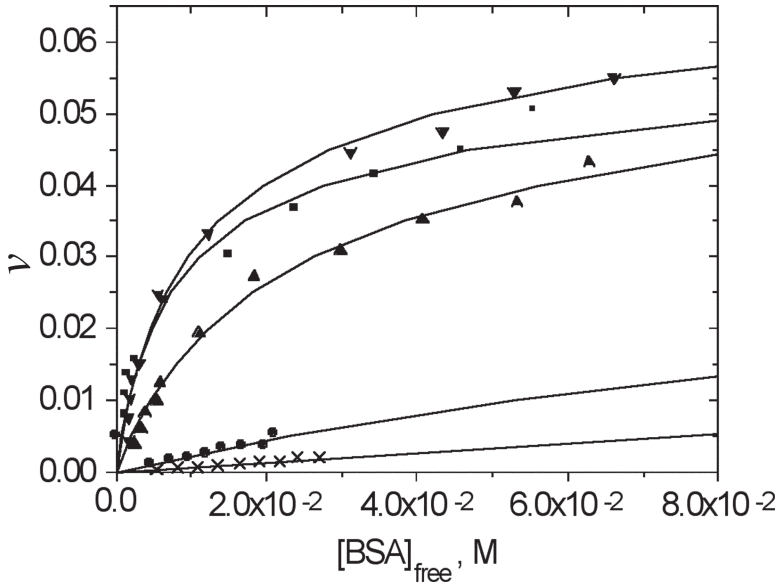

Fig. 4. Binding isotherms for BSA and heparin at ionic strengths of (▼) 2 m*M*, (■) 7 m*M*, (▲) 10 m*M*, (•) 30 m*M*, (×) 50 m*M* in phosphate buffer at pH=6.8; the solid lines are fits to the McGhee von Hippel equation (from **ref. *19***).

$$\frac{\nu}{L_{\text{free}}} = \mathrm{K_b}\left(1 - n\nu\right)\left[\frac{1-n\nu}{1-\left(n-1\right)\nu}\right]^{n-1} \tag{3}$$

Equation 3 yields two parameters, the intrinsic binding constant K_b and the binding-site size *n*. The binding isotherms are fitted to **Eq. 3** where ν is the number of bound BSA per ionic site of heparin, L_{free} is the concentration of free BSA, K_{obs} is the observed binding constant, and *n* is the binding site size in number of heparin charge groups (*see* **Note 3**). The nonlinear curve fitting can be carried out by a software such as Origin (Microcal Software, Inc.) to obtain the parameters K_{obs} and *n*. The fitted curves are also shown in **Fig. 4** for BSA and heparin at various ionic strengths. The calculated parameters are given in **Table 1**.

4. Notes

1. The adsorption of protein on the capillary wall is a significant problem. In case of BSA, strong adsorption of protein was observed at pH < 6.5; therefore, the measurements were made above this pH. The adsorption conditions which may differ for each protein may require the use of a coated capillary.

Table 1
Binding Constants and Binding Site Size for BSA–Heparin Interaction Calculated by Nonlinear Curve Fitting of Fig. 4 Data to Eq. 3

Ionic strength (*M*)	log *K*	n (±0.8)
0.05	1.88	12.7
0.03	2.39	13
0.01	3.44	11.4
0.007	3.89	12.9
0.002	3.85	10.8

2. A typical FACCE electropherogram shows two steps; however, more have also been observed ***(28)***. In addition, in some cases, the electropherogram had a spike peak before the first plateau ***(34)***, which may arise from adsorption of protein onto the capillary wall. Although this behavior did not significantly affect determination of free BSA here, the use of a coated capillary might be necessary to reduce adsorption when the effect becomes more significant.
3. Binding density ν is calculated by dividing the bound protein concentration (in mol/L) by the charge of heparin (in eq.mol/L). Because heparin has an equivalent weight of 200 g/mol, a 0.2 g/L heparin solution contains 1.0×10^{-3} eq. mol/L. Binding density ν is defined in this manner because the binding site on heparin does not correspond to a well-defined portion of molecule, which is the typical case for nonspecific binding arising from long range electrostatic forces. The size of the apparent binding site *n* is defined for the same reason in terms of the number of charges it encompasses. An estimate of the number of disaccharide units involved in binding can easily be obtained by dividing *n* by the average number of charges of one disaccharide, 3.7 for heparin ***(15)***. The length of the binding site can then be calculated from the length of a disaccharide unit, 11 Å.

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