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Isothermal microcalorimetric study of the pH dependence of the interactions between a cellulase and a β-blocker

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Abstract

The influence of the pH on the complexation equilibria between (*S*)- or (*R*)-alprenolol and the cellulase Cel7A was investigated by isothermal titration calorimetry. The results obtained agree with those of previous, similar studies of the same equilibria in which the protein was immobilized on silica particles, packed in a chromatographic column. The association constant and the complexation enthalpy and entropy of the (*S*)-enantiomer increase with increasing pH. For (*R*)-alprenolol, the binding is endothermic at all pH values. Thus, for both enantiomers in the pH range 5.5–6.8, the binding is an entropically driven process.

Keywords: pH effects; Isothermal titration calorimetry; Complexation equilibria; Cellulase; β-Blockers; Alprenolol

1. Introduction

The separation of enantiomers remains of great interest. Numerous chiral stationary phases (CSP) have been recently described [1]. While the development of analytical or preparative separations of enantiomers is important, the study of the retention mechanisms involved in these separations presents a fundamental importance for a better understanding of the complex liquid–solid equilibria involved in RPLC because all the molecular interactions of the two enantiomers are identical, except for those that involve the very chiral center. A study of the differences between the data obtained for the two enantiomers allows a better understanding of these chiral interactions [2]. An important class of CSPs consists of proteins immobilized on silica. The study of the mechanism of chiral separation on these CSPs affords an original approach for the understanding of the properties of the important functional sites on these proteins. Recent studies of the separation of β-blockers on cellobiohydrolase I (Cel7A) illustrate this point [3–6].

Cel7A from *Trichoderma reesei* has a molecular mass of about 60 kDa and a pI of 3.9 [7]. Cel7A degrades cellulose, giving β-cellobiose as the product [8]. The enzymatic active site involves both acidic and hydrophobic amino-acid residues [9,10]. Chromatographic and enzyme kinetic studies have shown that the chiral active site for the separation of β-blockers overlaps with the enzymatic site [11–13]. A X-ray crystallography study confirmed the model of interaction between a β-blocker, (*S*)-propranolol, and the active site [14]. The interactions between Cel7A immobilized on silica and different β-blockers have been thoroughly investigated by nonlinear chromatography [3–6]. A two-type of sites model, with a bi-Langmuir equilibrium isotherm, accounts very well for the adsorption behavior of several β-blockers at finite concentrations. The parameters of one of the two types of sites are very similar for the two enantiomers (nonselective sites) whereas those of the other type (enantioselective sites) differ significantly. These studies demonstrated also that the retention of the most retained (*S*)-enantiomer increases rapidly

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with increasing pH. This was explained by a large increase in the interaction energy at the selective sites [5,6,15]. Recently, we derived the distribution of interaction energies between the immobilized Cel7A and the two enantiomers of alprenolol and propranolol [15]. These two distributions are identical at low energies but differ significantly at high energies, the distribution of the (*S*)-enantiomer having a larger mode, located at a higher energy than the distribution of the (*R*)-enantiomer. This last result is consistent with the modeling of the adsorption equilibrium data with a bi-Langmuir model and with the existence on the surface of the CSP of two different types of interaction sites. In order to validate the procedure used to determine the energy distribution, these results must be compared to those of direct measurements of the difference between the complexation energies of the two enantiomers.

Hedeland et al. have previously measured the interaction energies between (*R*)- and (*S*)-alprenolol and Cel7A [16]. They used isothermal titration calorimetry (ITC) in potassium and sodium phosphate buffers at pH 6.8. They found that both enantiomers have positive complexation enthalpies and entropies with Cel7A but, unfortunately, they did not determine the influence of the solution pH on these thermodynamic functions. Yet, independent investigations have shown this influence to be strong [5,6]. This makes unreasonable any comparison between the results of Hedeland et al. [16] and the extrapolation of these independent measurements.

The goal of this study was to measure by ITC the thermodynamic characteristics of the interactions between the two enantiomers of alprenolol and Cel7A in solution, to investigate the influence of the pH on these properties, and to compare the results thus obtained with those of previous chromatographic studies [6,15] and with those of Hedeland et al. [16]. It was also to compare the difference between the average energies of the high-energy modes of the distribution obtained for the two enantiomers and reported previously with the difference between the complexation energies measured for these compounds in solution, at different values of the pH.

2. Experimental

2.1. Equipment

A Beckman Φ45 pH-meter (Beckman, Fullerton, CA, USA) was used to measure the pH of the buffers. To elute the protein from the purification column, a Pharmacia LKB Pump P-1 (Pharmacia, Sweden, EU) was used. A stirred ultrafiltration cell—Amicon® 8050 (Millipore Co., Bedford, MA, USA) was used to desalt/concentrate the protein solution after elution. The buffers for ITC were filtered through a 0.22 μm PES Millex® GP filter (Millipore, Carrigtwohill, Ireland, EU). The purity was checked by a Gelelectrophoresis Fisher FB-VE 16-1 system (Fisher Scientific, Pittsburgh, PA, USA). The protein was freeze-dried in a Virtis apparatus (The VirtisCompany, Inc., Gardiner, NY, USA). The ITC data were obtained with VP-ITC MicroCalorimeter (MicroCal, Northampton, MA, USA). The nonlinear regression was performed by Microcal™ Origin™ Version 5.0 (MicroCal).

2.2. Chemicals

Sodium acetate anhydrous (>99%) from Sigma (St. Louis, MO, USA), acetic acid glacial from Fisher (Fair Lawn, NJ, USA), Phosphoric acid (85%) and sodium hydroxide from Mallinkrodt (Paris, KY, USA), and Nanopure II water from Sybron (Branstead, NJ, USA) were used to prepare the buffers. (*S*)-alprenolol hydrogen tartarate and (*R*)-alprenolol phosphate were from AstraZeneca (Mölndal, Sweden, EU) and were kindly provided by Dr. Fornstedt (Uppsala University, Sweden, EU). Bio-Rad Protein Assay Dye Reagen Concentrate was from Bio-Rad Laboratories (Hercules, CA, USA). Q-Sepharose™ fast flow and Q-Sepharose™ high performance anionic exchange media were from Amersham Biosciences (Uppsala, Sweden, EU). SDS–PAGE Molecular Weight Standards, Broad Range, was from Bio-Rad Laboratories (Hercules). Cel7A Econase CEP was from AB Enzymes Oy (Rajamäki, Finland, EU) and was a kind gift from Dr. Vehmaanperä (Roal Oy, Rajamäki, Finland, EU).

2.3. Procedures: protein purification

The procedure involved three successive steps.

2.3.1. First step: preparative LC

The crude protein mixture was dissolved in a sodium acetate (NaAc) buffer (pH 4.2 with ionic strength $I = 0.025$ M) and applied to a Fast Flow column (25 mL) using a flow rate of 3 mL min^{-1}. The column was washed with 100 mL of the same buffer. A gradient was then applied, ranging from $I = 0.025$ M to 0.3 M (pH 4.2), in order to elute the protein. After the gradient was completed, solutions of $I = 0.3$ M and 1 M of NaAc (pH 4.2) were used to wash the column and finally, a solution at $I = 0.025$ M to equilibrate it. Fractions of 5 mL each were collected and analyzed for their protein content using BioRad® (BR). From each test tube, 30 μL was added to a solution containing 100 μL BR and 400 μL water. All fractions containing the protein were pooled together for further purification (see second step, below).

2.3.2. Second step: desalting

After completion of this first step of purification, the pooled fractions of the protein solution were concentrated and desalted by ultrafiltration. The desalting ratio was in a factor of approximately 100 times. The desalted/concentrated protein solution was diluted in a solution of NaAc at pH = 4.0 and $I = 0.025$ M, for further purification.

2.3.3. Third step: preparative HPLC

A high performance chromatographic column (volume 75 mL) was used for the last purification step. The procedure

was basically the same as the one used with the first column, but the pH was slightly lower, pH 4.0, and the flow rate was also lower, 2 mL min^{-1}. The volume of solution used for the washing was 200 mL.

After completion of the third purification step of Cel7A, the fractions were collected, desalted by a factor exceeding a thousand times (see second purification step, above), and freeze-dried. SDS–PAGE 10% was used to determine the purity of Cel7A after freeze-drying. One single band was observed, close to the band of bovine serum albumin, corresponding to a formula mass of 66 kg mol^{-1} in the molecular weight standard.

2.4. Procedures: isothermal titration microcalorimetry

(*S*)-alprenolol hydrogen tartarate and (*R*)-alprenolol phosphate with concentration of 3 mM were dissolved in their respective buffers, sodium acetate at pH 5.5 and 5.8, and sodium phosphate pH 6.8, all at a ionic strength I = 0.1 M. The sample cells of the VP-ITC apparatus have an actual volume of 1.8 mL, with a working volume of 1.4 mL. The cell was filled with Cel7A solutions at a concentration of 0.2 mM. The concentration used was chosen as a compromise between the need of having a high enough signal, allowing the possibility of recording a sufficient number of data points, and the need not to shift the equilibrium by causing significant changes of the ionic strength and the pH of the solution. The ligands (*S*)- and (*R*)-alprenolol were added by 9 μL increments to the sample cell. During all the titrations, the temperature was hold constant at 25 °C.

3. Results and discussion

Fig. 1a shows the calorimetric signal recorded during a titration of Cel7A with (*S*)-alprenolol in a sodium acetate buffer, at pH 5.8 and I = 0.10 M, at 25 °C. The area of each peak in this figure is equal to the amount of heat evolved by the complexation reaction between the Cel7A in the calorimeter cell and the reagent added. The successive additions of the titration reagent were made at a 3 min interval, the shortest value giving baseline separation of the peaks. The amounts of heat evolved during the titration are plotted versus the molar ratio in Fig. 1b. This plot is compared to the one calculated for the best 1 μL binding isotherm. This particular experiment gave a value of the association constant equal to 25 mM^{-1} and a complexation enthalpy ΔH° = 4.9 kCal mol^{-1}.

The association constants, K_a, and the thermodynamic constants, $\Delta H^{\circ\prime}$ and $\Delta S^{\circ\prime}$, for the complex formation between (*S*)-alprenolol and Cel7A at different pH values are reported in Table 1. The value obtained for (*R*)-alprenolol at pH 6.8 is also included. Measurements were also performed for this enantiomer at lower pH (5.5 and 5.8). However, the signal observed was very low, the noise important, and the titration curves were too scattered and too shallow to allow a sufficient precision on the amounts of heat evolved. Accordingly,

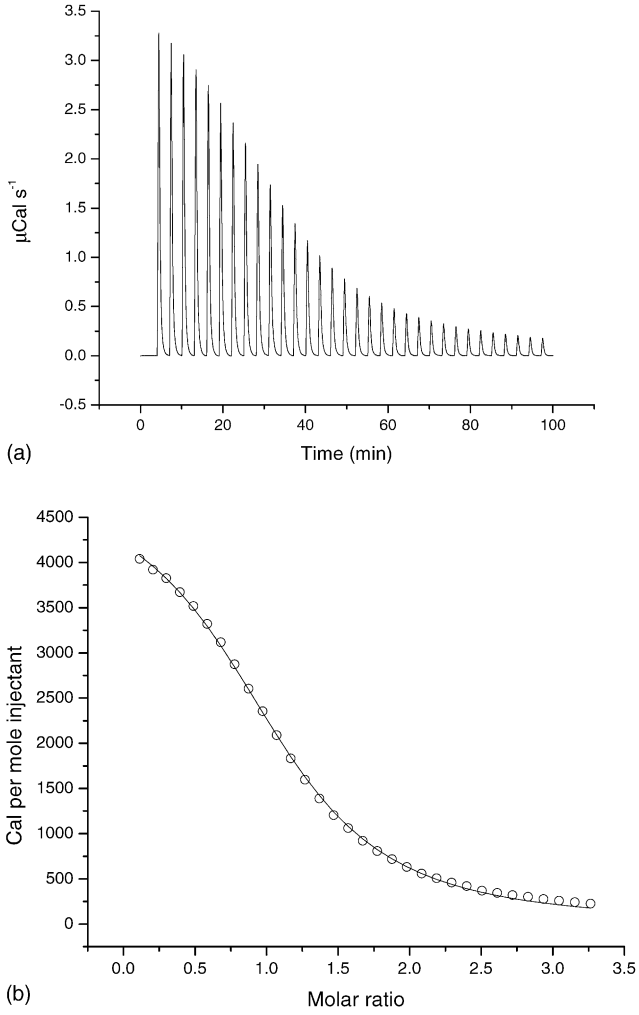

Fig. 1. ITC titration data describing the complex formation of (*S*)-alprenolol and Cel7A at 25 °C in a sodium acetate buffer at pH 5.8. (a) Differential power signal recorded during calorimetric titration of the cell content (1.4 mL) of a 0.20 mM solution of Cel7A by 32 consecutive additions of 9 μL aliquots of a 3 mM (*S*)-alprenolol solution. (b) Titration curve derived from the data recorded in part (a) by integration with respect to time and normalization to the number of mole of ligand added. Comparison of measured reaction heat (◯) to the calculated 1:1 binding isotherm (-).

the nonlinear regression program either gave values with a very low precision or even did not converge at all. Thus, no values are reported for these two low pH in Table 1. For each enantiomer, three titration experiments were performed at pH 6.8 and 5.8. For (*S*)-alprenolol, five titrations were performed at pH 5.5.

It is obvious from Table 1 that all the thermodynamic parameters of the complexation (K_a, $\Delta H^{\circ\prime}$, $\Delta S^{\circ\prime}$) increase with increasing pH and that they are higher for the (*S*)-alprenolol than for the (*R*)-alprenolol. The values obtained for $\Delta H^{\circ\prime}$ were positive in all cases confirming that the complexation is endothermal. This same endothermal behavior was also observed for (*R*)-alprenolol at pH values below 6.8, although no quantitative value could be measured, the signal being too low, as explained above. This indicates a low energy of

Table 1
Thermodynamic values

Analyte	pH	K (mM^{-1})	R.S.D.* (%)	ΔH (kCal mol^{-1})	R.S.D.* (%)	ΔS (Cal mol^{-1} K^{-1})
(*S*)-alprenolol	5.5	17	7.8	4.2	1.7	34
	5.8	26	1.3	4.9	0.41	36
	6.8	52	24	9.3	9.0	53
	6.8[a]	57	6.1	5.6	1.5	41
	5.0	8.6[b]	2.7			
		8.8[c]				
	5.5	21[b]	1.9			
		19[c]				
	6.0	42[b]	1.9			
		38[c]				
(*R*)-alprenolol	6.8	5.4	7.8	2.0	1.4	24
	6.8[a]	6.9	6.5	1.7	2.9	23
	5.0	8.2[b]	13			
		8.8[c]				
	5.5	6.1[b]	13			
		6.2[c]				
	6.0	6.5[b]	9.9			
		6.2[c]				

R.S.D.*: relative standard deviation of the mean.
[a] Values from ref. 16.
[b] Value from ref. 6.
[c] Value from ref. 15.

interaction. The binding process is entropically driven in the whole pH range considered, a phenomenon most probably explained by the loss of water molecules during the formation of the complex [17]. The formation of hydrophobic interactions is supported by the negative change in heat capacity ($\Delta C_p = \Delta\Delta H/\Delta T$) for both enantiomers that was reported in ref. 16. The reason for the entropically driven process is probably that the retention mechanism involves the inclusion of the enantiomers in a cavity. In this key and lock type of interactions, one enantiomer fits very well to the cavity of the protein whereas the other does not. X-ray crystallography have shown that the (*S*)-enantiomer fits very well to the enzymatic site of Cel7A [14]. However, to our knowledge, there is no publications on X-ray crystallography with the (*R*)-enantiomer, an indication of problems encountered in attempts to crystallize the corresponding complex.

Results of similar calorimetric measurements performed at pH 6.8 had been previously published [16]. The values reported for the association constants are in good agreement with our results; 57 and 6.9 mM^{-1} for the (*S*)- and the (*R*)-enantiomer, respectively (cf. Table 1). Also, the enthalpy change and the entropy for (*R*)-alprenolol are relatively similar, 1.7 kCal mol^{-1} and 23 Cal mol^{-1} K^{-1}. For (*S*)-alprenolol, however the difference, as compared to ref. 14, is high for $\Delta H^{\circ\prime}$ and $\Delta S^{\circ\prime}$, 5.6 kCal mol^{-1} and 41 Cal mol^{-1} K^{-1} were reported. Because $\Delta S^{\circ\prime}$ is derived from K_a and from $\Delta H^{\circ\prime}$, itself obtained from the dependence of K_a on the temperature (through $\Delta S = R(\ln K_a + \Delta H/RT)$) and because both complexation enthalpy and entropy are positive, an underestimated complexation enthalpy is consistent with an underestimated complexation entropy. The reason for the differences in the estimated value of $\Delta H^{\circ\prime}$ could originate from differences in the purity of the sample of protein used and probably also from the experimental set-up (choice of concentrations used, etc.).

The values of the thermodynamic parameters of the reaction of complexation of the two enantiomers of alprenolol obtained by ITC are consistent with the values of these same parameters derived previously using nonlinear chromatography [6,15]. In the case of the experiments reported in this work, the protein was dissolved in an aqueous buffer. By contrast, in chromatography, the protein was immobilized on silica. For (*S*)-alprenolol, the equilibrium constants of the selective site are 19–21 and 38–42 mM^{-1} at pH 5.5 and 6.0, respectively [6,15]. The former value (at pH 5.5) is in excellent agreement with the ITC result at the same pH. Interpolating the values obtained at different pH values in the earlier work [6,15] and in the present one gives also an excellent agreement at pH 5.8 (cf. Fig. 2). In the present work, because ITC measures the total amount of heat generated by the complexation reaction, it was not possible to separate the contributions of the nonselective interaction sites at the protein from the contribution of the enantioselective sites. The titrations were carried out at low concentrations. Making systematic measurements in a wide range of reagent concentrations would have been possible but too long and would have exceeded our possibilities. Measurements by nonlinear chromatography are much faster. The large difference between the values of the thermodynamic parameters of the complexation reaction for the two enantiomers demonstrates that enantioselective interactions take place between the protein and the (*S*)-enantiomer. Similar enantioselective interactions remain possible with the (*R*)-enantiomer but, if they do take place at all, they are far weaker (see Table 1).

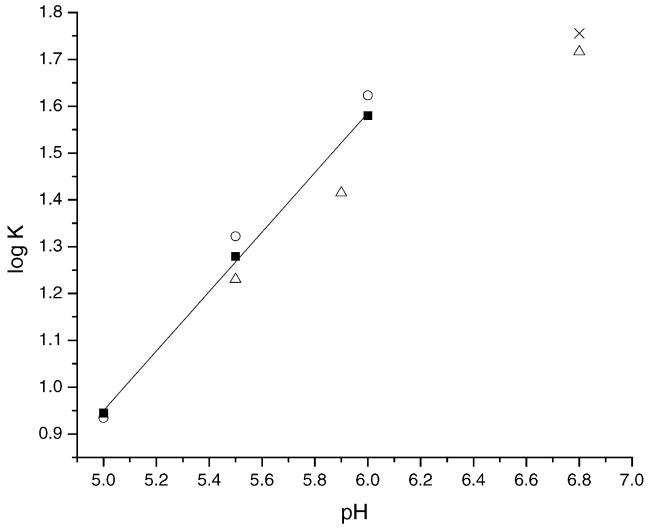

Fig. 2. Plot of the dependence of log K_a on pH. Symbols: experimental data, from ITC data, present study (△), and from ref. 16 (×), constants obtained by fitting the bi-Langmuir model (◯), and AED (■). Line: best linear fit to the AED data.

Finally, the adsorption data obtained in nonlinear chromatography were used to determine the distribution of adsorption affinities, i.e., of the adsorption constants [15]. The distributions obtained for (*S*)-alprenolol and (*R*)-alprenolol are nearly identical at low values, i.e., at high concentrations, the range in which the low binding-energy sites that are the nonselective sites become populated. In the high binding energy range, the two distributions exhibit a second affinity energy mode for which marked differences are observed. This mode is much larger for (*S*)-propranolol and takes place at a markedly higher value of the equilibrium constant than for (*R*)-alprenolol. The ratio of the two average equilibrium constants at pH 5.0 is 1.0; it is 3.1 at pH 5.5 and 6.1 at pH 6.0 [15]. A linear extrapolation of these results to pH 6.8 would give a ratio of 10. The value measured in this study is 9.7, an excellent agreement. The value derived from ref. 16 is 8.3, still in good agreement with the results of this extrapolation. However, we know how uncertain a method extrapolation is when used to compare data sets. In this specific case, we must be particularly cautious because different buffers had to be used at pH 5.0–6.0 (sodium acetate buffer) and at pH 6.8 (sodium phosphate buffer). Protein structures and chiral recognition mechanisms can be affected by the nature of the counter-anion (acetate versus phosphate). Acetate and phosphate are known to have different positions in the Hofmeister series, i.e., phosphate ions precipitate proteins more effectively than acetate ions and the protein is thus more prone to inactivation in phosphate buffers [18].

4. Conclusion

The molecular interactions of both enantiomers of alprenolol with Cel7A are endothermic in the pH range 5.5–6.8. The formation of the chiral recognition complex is thus entropically driven. The values of the equilibrium constants for the (*S*)-enantiomer obtained by ITC agree with the values obtained for the equilibrium constants of the enantioselective interactions found by nonlinear chromatography. For (*R*)-alprenolol the signal is very low at low pH and the shallow titration curve derived from the data suggests a low value of the corresponding equilibrium constant—a value still lower than the one obtained by chromatography. Whether this is correct or whether there are multiple nonselective sites on the protein could not be determined in this study but it was not its purpose.

The close agreement between the results obtained by ITC and those derived from the adsorption energy distribution (AED) confirms the validity of the expectation-maximization method used to derive the AED from equilibrium adsorption data.

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