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Microcalorimetric studies on the complex formation between cellobiohydrolase I (CBH I) from *Trichoderma reesei* and the (R)- and (S)-enantiomers of the β-receptor blocking agent alprenolol

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Abstract

The thermodynamic quantities for the complex formation between the enantiomers of the β-blocking drug alprenolol and cellobiohydrolase I (CBH I), that earlier has been used as a chiral selector for aminoalcohols, revealed positive ΔH^0 — values in all cases implying an entropy driven process. Association constants (K_a) for cellulase and the (R)- and (S)-enantiomers of alprenolol were determined by isothermal titration microcalorimetry and the inhibition constants (K_i) by enzyme inhibition experiments. Both inhibition experiments and microcalorimetry revealed that the affinity between the enantiomers of alprenolol and CBH I was higher in sodium phosphate buffer than in potassium phosphate buffer. This result was in agreement with previously reported liquid chromatographic separations of enantiomers using a chiral stationary phase based on CBH I immobilized to silica particles. The best fit of the isothermal titration data corresponded to a 1:1 binding isotherm.

Keywords: Calorimetry; Association constant; R- and S-alprenolol; Cellobiohydrolase I; Complex formation

1. Introduction

Different kinds of proteins, such as α-AGP [1], HSA [2], BSA [3], chymotrypsin [4], cellulases [5–11], have been employed as chiral selectors and tools, for instance in bioanalysis. The cellulase, cellobiohydrolase I (CBH I) immobilised to silica has successfully been used as a chiral stationary phase in liquid chromatographic enantiomer separations of basic drugs [5–10,12]. It has further been applied as a chiral additive in the background electrolyte in capillary electrophoresis [13,14] and as a mobile phase additive in liquid chromatography [14]. CBH I has also been used in capillary affinity gel electrophoresis [15].

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The cellulase (CBH I) is a protein with a molecular weight of 60 kD. CBH I constitutes about 60% of all cellulose degrading proteins produced by the strongly cellulolytic filamentous fungus *Trichoderma reesei*. CBH I is also well spread among other cellulolytic fungi and it is good reason to believe that it is the key enzyme in fungal cellulose degradation [16]. It has the typical molecular organization of a fungal cellulase with a catalytic domain comprising the main part of the protein connected to a small cellulose binding domain via an extended linker giving an elongated molecule [17]. The most prominent part of the catalytic domain is a 50 Å long tunnel where the active site is located [18].

Recent results show that the active site and the chiral recognition site overlap [9]. Moreover, site-directed mutagenesis of three carboxylic amino acid residues in the tunnel showed that two of these were strongly involved in both the enzymatic catalysis and in the chiral recognition mechanisms [10].

Control of retention and enantioselectivity of the protein based stationary phases is accomplished by the composition of the buffer (pH, cation and kind and amount of organic solvent) [6,12].

In a chromatographic study, an unexpected temperature effect on the separation characteristics of *rac*-propranolol on a CBH I-silica column was obtained [19]. Elevated column temperatures gave rise to an increase in separation factor. In the same study, microcalorimetric titration revealed that the interaction between the enantiomers of propranolol and CBH I was characterised by positive enthalpy changes thus indicating an entropy driven separation process. Interestingly, the enantiomer with the highest affinity had the highest positive enthalpy change, which was verified in a later thermodynamic study [20] based on isotherms of the two propranolol enantiomers, determined by frontal analysis (nonlinear chromatography) on a CBH I-silica column. The biLangmuir isotherm model fitted best to the data indicating two different adsorption sites on the stationary phase (CBH I-silica), interpreted as stereoselective and non-stereoselective sites, respectively. The non-stereoselective sites could emanate both from the silica and the chiral selector while the selective ones are located only on the protein. In another study using the biLangmuir isotherm model, it was suggested that the observed enantioselectivity was mainly due to differences in monolayer capacities of the stereoselective sites and the (R)- and (S)-enantiomers had the same binding constants to these sites [21].

In a recent chromatographic study an increase in the retention and in enantioselectivity of two β-blocking agents was obtained when exchanging potassium for sodium [12] in the mobile phase buffer. An increase of the ionic strength of the buffer resulted in shorter retention and maintained selectivity indicating some kind of an ion-exchange process presumably involving the carboxyl groups discussed above.

As an extension of these studies we now report on determinations of thermodynamic quantities (K_a, $\Delta H^{0\prime}$, $\Delta S^{\circ\prime}$) pertaining to the interaction between (R)- and (S)-alprenolol and CBH I at several temperatures using a novel titration calorimeter based on the heat conduction principle.

Both Na- and K-phosphate buffer were used to get more information on the above mentioned difference in chromatographic behaviour. Furthermore, complementary enzymatic inhibition experiments were performed.

2. Materials and methods

2.1. Isothermal titration microcalorimetry

Thermodynamic quantities were determined by isothermal titration calorimetry using a small vessel (0.3 cm^3) twin heat conduction instrument optimised for high sensitivity and fast response [22]. The ligand (R)- or (S)-alprenolol) was injected in 4 μl aliquots at 6 min intervals using a motor driven Hamilton syringe. In the titrations of (R)-alprenolol, the injected ligand concentration was 15 mM and the CBH I concentration was 1.5 mM and in the titrations of the (S)-form, the ligand concentration was 8 mM and the CBH I concentration was 1 mM. The calorimetric signal was deconvoluted using a time constant of 26 s [22]. Results ($\Delta H^{0\prime}$, K_a) were calculated from the binding isotherms according to Marquardt [23], using a l:l binding model. CBH I was purified from *T. reesei* (strain QM9414) culture filtrate as previously described [24]. (R)- and (S)-alprenolol monohydrogentartrate were kindly supplied by Astra Hässle AB (Mölndal, Sweden). Chemicals were dissolved in 0.10 M Na- or K-phosphate buffer at pH 6.8. All buffer salts were of analytical grade.

2.2. The determination of the inhibition constants by enzyme kinetics

The CBH I activity was determined by measuring the production of *p*-nitrophenol (*p*NP) from the hydrolysis of *para*-nitrophenyl-lactopyranoside (*p*NPL) by CBH I in the absence or presence of (R)- or (S)-alprenolol as inhibitors. The experimental conditions were as follows: *p*NPL 0.125–5 mM, CBH I, 2 μM in 0.10 M Na-phosphate or K-phosphate pH 6.8 and (R)- and (S)- alprenolol, 0.25 or 0.05 mM, respectively. The incubation was performed in a water bath at 25°C for 60 min. The reaction was stopped by adding 0.5 M Na_2CO_3 and the concentration of *p*NP was determined by UV absorbance at 410 nm (ε_{410}=16590 M^{-1} cm^{-1}). Blanks without enzymes were background controls.

The kinetic parameters K_M, k_{cat} and K_i (inhibition constant) were derived by non-linear regression analysis using the software Simfit (W.G. Bardsley, Manchester, UK).

3. Results and discussion

Fig. 1 shows the calorimetric signal during a titration of CBH I with (S)-alprenolol at 25°C. Although the baseline noise increased due to the deconvolution process, it was still within 1 μW. Titrations were made at 6 min intervals but baseline separation can be achieved at intervals down to 2 min (cf. Fig. 1). Integrated peak areas are shown in Fig. 2, together with the calculated 1:1 binding isotherm. An earlier chromatographic study showed that there is another selective site for propranolol located at the cellulose binding domain of the CBH I [7]. However, it was not possible to separate other β-blockers due to the very low affinities to this site (displayed as very short retention times). The titration data could be fitted better to a 1:1 isotherm model than to a 1:2 model. As far as alprenolol is concerned our data indicate that the free protein contributes with the selective site and consequently the nonselective sites observed in chromatography originate mainly from the silica. In this particular experiment shown in Fig. 2, the association constant (K_a) was found to be 37×10^3 M^{-1} and the accompanying enthalpy change ($\Delta H^{0\prime}$) was 25.2 kJ mol^{-1}.

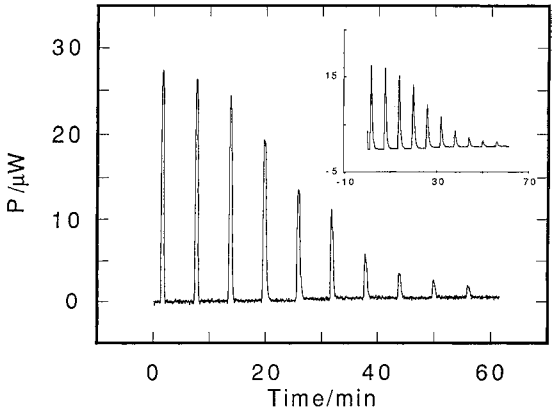

Fig. 1. Record from a calorimetric titration experiment where 8 mM (S)-alprenolol solution is added to 10 consecutive 4 μl injections to 180 μl 1 mM CBH I. The thermogram is corrected for the thermal inertia of the calorimeter using a time constant of 26 s. The small graph shows the uncorrected curve.

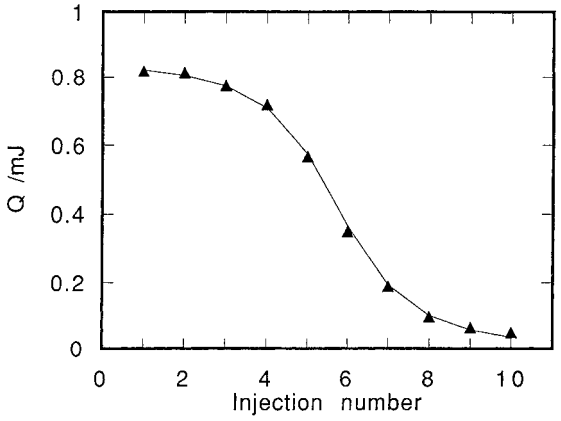

Fig. 2. Titration curve. Comparison of measured reaction heats (▲) to the calculated 1:1 binding isotherm (—).

Thermodynamic quantities ($\Delta G^{0\prime}$, $\Delta H^{0\prime}$, $\Delta S^{0\prime}$) for the complex formation between (R)- and (S)-alprenolol and CBH I in potassium phosphate buffer, are shown in Table 1 and Fig. 3. Each value was calculated from a series of five to six titration experiments.

Values of $\Delta H^{0\prime}$ were in all cases positive (although $\Delta H^{0\prime}$ is expected to change sign at about 40°C for (R)-alprenolol). The binding processes were thus entropy driven, presumably in part as a consequence of dehydration of the both the enantiomer and the binding site on the protein. This assumption was supported by the large negative heat capacity changes

Table 1
Thermodynamic parameters related to the binding of (R)- and (S)-alprenolol to CBH I in K-buffer at 288.15, 298.15 and 308.15 K[a]

Temperature/K	$\Delta H^{0\prime}$/kJ mol^{-1}	$K_a\times10^{-3}$/mol l^{-1}	$\Delta G^{0\prime}$/kJ mol^{-1}	$\Delta S^{0\prime}$/J K^{-1} mol^{-1}
(S)-alprenolol				
288.15	28.2±0.7 (6)	38±5	−25.2±0.4	185±3
298.15	25.1±0.4 (5)	39±6	−26.2±0.4	172±2
308.15	19.0±0.4 (5)	43±9	−27.3±0.6	150±3
(R)-alprenolol				
288.15	11.6±0.2 (5)	7.0±0.7	−21.2±0.3	114±2
298.15	8.1±0.1 (5)	7.6±0.4	−22.1±0.2	102±1
308.15	2.7±0.3 (5)	11.3±2.0	−23.9±0.5	86±1

[a] All errors are given as twice the standard deviation of the mean (2 SEM); values in brackets denote the number of experiments; association constants (K_a) are calculated assuming a 1:1 complex.

(ΔC_p) calculated for (R)- and (S)-alprenolol (−455 and −462 J (mol K)$^{-1}$, respectively). Since ΔC_p values for both enantiomers were almost equal, the difference in $\Delta S^{0\prime}$ cannot be explained by differences in the extent of dehydratization.

The difference in thermodynamic quantities are reflected in the association constants (K_a). (S)-alprenolol bound stronger ($K_a=39\pm6\times10^3$ M^{-1} at 298.15 K) to CBH I than the (R)-form ($K_a=7.6\pm0.4\times10^3$ M^{-1} at 298.15 K). The enantioselectivity

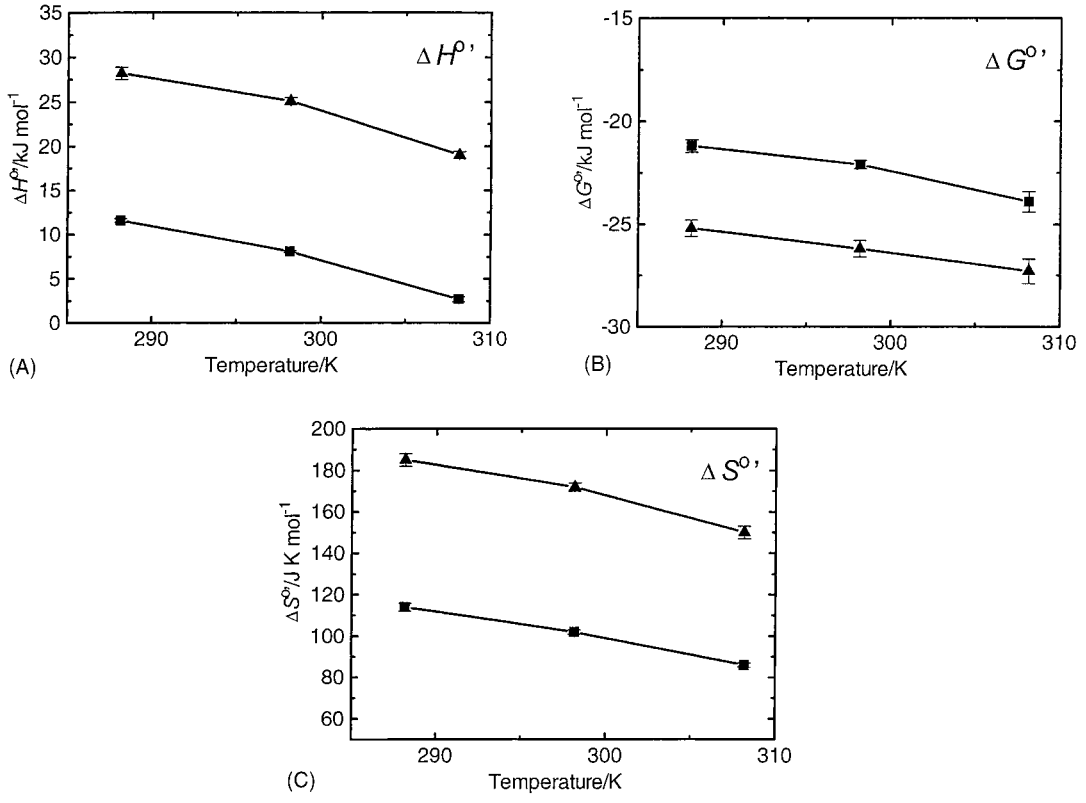

Fig. 3. A–C Thermodynamic functions ($\Delta H^{0\prime}$, $\Delta G^{0\prime}$ and $\Delta S^{0\prime}$) pertaining to the complexation of (S)-alprenolol (▲) and (R)-alprenolol (■) with CBH I. Results were calculated from isothermal titration experiments at three temperatures (288.15, 298.15 and 308.15 K). All errors are given as twice the standard deviation of the mean (2SEM).

Table 2
Thermodynamic parameters related to the binding of (R)- and (S)-alprenolol to CBH I in K- and Na-buffer at 298.15 K[a]

Counter-ion	$\Delta H^{0\prime}$/kJ mol^{-1}	$K_a \times 10^{-3}$/mol l^{-1}	$\Delta G^{0\prime}$/kJ mol^{-1}	$\Delta S^{0\prime}$/J K^{-1} mol^{-1}
(S)-alprenolol				
K	27.3±0.9 (5)	40±3	−26.2±0.2	180±4
Na	23.5±0.7 (5)	57±7	−27.1±0.2	170±2
(R)-alprenolol				
K	7.4±0.5 (5)	6.1±0.6	−21.6±0.3	97±2
Na	7.0±0.4 (6)	6.9±0.9	−21.9±0.3	97±2

[a] All errors are given as twice the standard deviation of the mean (2 SEM); values in brackets denote the number of experiments; association constants (K_a) are calculated assuming a 1:1 complex.

of alprenolol is thus simply explained by the five-fold difference in association constants and it does not seem to be in agreement with the earlier findings concerning propranolol [21], where the enantioselectivity was explained by differences in adsorption site capacities of the cellulase-silica phase. However, the experimental conditions were not the same as in our study.

It is noteworthy that the stronger binding (S)-enantiomer also had the highest positive $\Delta H^{0\prime}$. However, the reason for this effect could not be elucidated by the results from these experiments.

Table 2 displays the results obtained upon exchanging potassium for sodium ion in the buffer used as titration medium. Note that results shown in Tables 1 and 2 emanate from different experimental series. As can be seen from these tables, the accuracy between the different series is good.

A significant increase in the affinity constant was obtained for (S)-alprenolol when exchanging potassium for sodium [21] ions in the buffer used in the titrations, while the constant of the (R)-enantiomer only rose slightly. These observations are in accordance with the chromatographic results (see discussion above) [12].

The association constants, measured as inverted inhibition constants, K_i, are found in Table 3. It has earlier been reported [9] that β-receptor blocking agents act as competitive inhibitors and thus bind to a site that overlaps with the substrate binding site. The good agreement between the inverted inhibition constants, that measure a specific interaction between the ligand and protein, and the constants determined from microcalorimetry, which measure all interactions, gives a further indication that the binding of alprenolol to CBH I is dominated by a single site.

4. Conclusions

Both the results from inhibition studies and microcalorimetry show the expected differences in affinity constants between the protein and the enantiomers ((R)- and (S)-alprenolol) in free solution. There are good agreements between the constants emanating from the two methods. The titrations indicated a 1:1 binding isotherm between each enantiomer and CBH I. The affinity constant between the (S)-enantiomer and CBH I is higher in sodium- than in potassium phosphate buffer used as solvent. Furthermore, both enzyme kinetics and microcalorimetry are good complements in exploring retention behaviours in liquid chromatography using CBH I based chiral stationary phases.

Table 3
Inversed inhibition constants for (R)- and (S)-alprenolol in K- and Na-phosphate buffers[a]

Cation	$K_i^{-1} \times 10^{-3}$/mol l^{-1}
(S)-alprenolol	
Potassium	37±3.0
Sodium	45±2.9
(R)-alprenolol	
Potassium	4.4±0.58
Sodium	6.7±1.8

[a] The computational of the experiments gave K_M=0.36± 0.012 mM and k_{cat}=0.0060±0.00035 s^{-1} (sodium phosphate); K_M=0.37±0.019 mM and k_{cat}=0.0047±0.00014 s^{-1} (potassium phosphate).

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