The active sites of cellulases are involved in chiral recognition: a comparison of cellobiohydrolase 1 and endoglucanase 1

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Received 14 May 1996; revised version received 14 June 1996

Abstract The cellulases cellobiohydrolase 1 (CBH 1) and endoglucanase 1 (EG 1) from the fungus *Trichoderma reesei* are closely related with 40% sequence identity and very similar in structure. In CBH 1 the active site is enclosed by long loops and some antiparallel β-strands forming a 40 Å long tunnel, whereas in EG 1 part of those loops are missing so that the enzyme has a more common active site groove. Both enzymes were immobilized on silica and these materials were used as chiral stationary phases for chromatographic separation of the enantiomers of two chiral drugs, propranolol and alprenolol. The CBH 1 phase showed much better resolution than did the EG 1 phase, suggesting that the tunnel structure of the protein may play an important role in the chiral separation. The chiral compounds were found to be competitive inhibitors of both enzymes when *p*-nitrophenyl lactoside (*p*NPL) was used as substrate. (*S*)-enantiomers showed stronger inhibitory effects and also longer retention time on the stationary phases than the (*R*)-enantiomers. The consistency between kinetic data and retention on the stationary phases clearly shows that the enzymatically active sites of CBH 1 and EG 1 are involved in chiral recognition.

Key words: Cellulase; Endoglucanase 1; Cellobiohydrolase 1; *Trichoderma reesei*; Chiral stationary phase; Chiral recognition; Catalytic site

1. Introduction

The filamentous fungus *Trichoderma reesei* secretes a very efficient cellulolytic system which contains two exoglucanases (cellobiohydrolase (CBH) 1 and 2), at least four endoglucanases (EG 1, EG 2, EG 3 and EG 5) and one β-D-glucosidase [1,2]. All of these enzymes can act synergistically to degrade crystalline cellulose efficiently. CBH 1 is probably the key enzyme in fungal cellulose degradation. It comprises about 60% of the total cellulolytic proteins of *T. reesei* and is also abundant in other cellulolytic fungi. It has also been shown that deletion of the *CBH 1* gene has a drastic effect on the cellulolysis of *T. reesei* [3]. Most fungal cellulases have a common structural organization where the main part of the enzyme, the catalytic core, is connected through a heavily glycosylated linker region to a small cellulose-binding domain (CBD) [4]. This gives the whole molecule an elongated tadpole shape (180 Å in the case of CBH 1) [5]. The three-dimensional structure of the binding domain has been solved in only one case, but there are good reasons to believe that the others are all folded in the same way on the basis of sequence identity [2,5]. The crystal structure of the CBH 1 catalytic domain reveals a large domain (434 residues) with the dimensions $60 \times 50 \times 40$ Å. About one-third of the domain is folded into a 'β-sandwich' composed of two anti-parallel β-sheets. The rest of the protein consists of loops that connect the β-strands. Some extensive loops stabilised by disulphide bridges, together with the concave 'β-sandwich' form a 40 Å long tunnel where the active site is located [6].

The catalytic domains of CBH 1 and EG 1 show significant amino acid homology with 45% identity [7] and accordingly have similar 3-D structures [6,8]. The EG 1 catalytic domain (371 residues) has the same dimensions as CBH 1 and also has the two large anti-parallel β-sheets forming a 'β-sandwich'. The sequence alignment of CBH 1 and EG 1 showed that four deletions in EG 1 mapped to the tunnel-forming loops in CBH 1. As a result, EG 1 has its active site in a deep but open groove rather than a tunnel (Fig. 1).

CBH 1 has been found to be an excellent chiral selector and its properties were recently reviewed [9]. It can be coupled to a solid support (silica or continuous bed) [10–12] to form a chiral stationary phase (CSP) or used in a carrier-free solution in capillary electrophoresis [13]. A rather broad spectrum of racemic solutes can be resolved, but CBH 1 functions best with amino alcohols such as the drugs used to block β-adrenergic receptors, so called β-blockers. In an attempt to localize the chiral site of CBH 1, we found that both the binding and catalytic domains contained enantioselective sites for propranolol, a β-blocker, but that the major enantioselectivity for propranolol and other solutes could be ascribed to the catalytic domain [14].

In view of the fact that the 3-D structures of the catalytic domains of both CBH 1 and EG 1 have been solved, and since previous data indicated that the active site of CBH 1 was involved in the chiral recognition, we have undertaken this study to further define the chiral site of the proteins.

2. Materials and methods

2.1. Chemicals

Rac-, (*R*)-, (*S*)-propranolol chloride and *para*-nitrophenyl-β-D-lactoside (*p*NPL) were from Sigma Chemical Company (St. Louis, MO). (*R*)- and (*S*)-alprenolol chloride were provided from Astra Hässle (Göteborg, Sweden). All other chemicals used were of analytical grade. The water used was Millipore purified. Spherical diol-silica with a particle diameter of 10 μm, pore size 300 Å, area 60 m^2/g and containing 5 $\mu mol/m^2$ of diol was obtained from Perstorp Biolytica (Lund, Sweden).

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Abbreviations: EG 1, endoglucanase 1; CBH 1, cellobiohydrolase 1; *p*NPL, *p*-nitro-phenyl-β-D-lactoside; CBD, cellulose-binding domain; CSP, chiral stationary phase

PII S0014-5793(96)00685-0

2.2. Experimental apparatus

Microtiter plates (96-well) were from Bibby Sterrilin Ltd. (Stone, Staffs, UK). Twelve-channel 50–300 μl pipette, 12-channel 5–50 μl pipette, 12×8 tips pipette tip racks, 8-well reservoirs for multichannel pipetting and Lab systems Multiskan MCC/340 (version 2.33) were from Lab systems (Helsinki, Finland).

A Beckman 114M Solvent Delivery Module high-performance liquid chromatographic pump (Beckman Instruments, Inc., Altex Scientific Operations, CA) and a 1 μl cell LDC/Milton Roy Spectromonitor D, variable wavelength detector (LDC/Milton Roy, Riviera Beach, FL) connected to a Model BD40 recorder (Kipp and Zonen, The Netherlands) were used. The injector was a Rheodyne (Australia) model with a volume of 20 μl. The columns were made of stainless steel (Skandinaviska GeneTec AB, Kungsbacka, Sweden). A water bath type 02 PT 923 (Hero, Barkerød, Denmark) was used to thermostat the column at 21°C. The pH meter was a model E 623 equipped with a combined pH glass electrode (Metro, Wheres, Switzerland). The spectrophotometer was a model Shimadzu UV-160A (Kyoto, Japan).

2.3. Experiment

2.3.1. Purification of the proteins. Concentrated culture filtrate from the fungus *Trichoderma reesei* strain QM 9414 was obtained from ALKO Ltd., Research Laboratories (Helsinki, Finland). CBH I and EG 1 were purified from the concentrated culture filtrate as described in [1,15]. The purity of the proteins was examined by 10% SDS-PAGE and they were regarded as pure when only one band could be seen in the gel.

2.3.2. Inhibition experiment. The rate of production of *p*-nitrophenol (*p*NP) from *p*NPL was determined with CBH 1 and EG 1 as catalysts in the presence of the enantiomers of alprenolol and propranolol as inhibitors. Microtiter plates (96-well) were used and the concentration of *p*NP was measured at 414 nm ($\varepsilon = 16590\ M^{-1}\ cm^{-1}$) using a Multiscan MCC/340 microtiter plate reader. The conditions were the following for CBH 1: *p*NPL concentration 0.2–5 mM, enzyme concentration 2 μM, 0.1 M sodium acetate buffer, pH 5.0, temperature 20°C, (*R*)- and (*S*)-forms of propranolol and alprenolol 0–1.25 mM. In the case of EG 1, K_M and k_{cat} were determined at: *p*NPL concentration 0.36–9 mM, EG 1 concentration 0.2 μM, other conditions as above. The inhibition experiments with EG 1 were performed at: *p*NPL concentration 0.5–3 mM, EG 1 concentration 0.2 μM, and inhibitor concentrations 0.31–1.25 mM. The incubation time was 15 min in all cases and the reaction was stopped by adding to the reaction mixture an equal volume of 0.5 M Na_2CO_3.

Inhibition studies were also done at pH 6.8. Here the experiments were carried out in test tubes. Conditions: *p*NPL 0.4–3 mM, CBH 1 concentration 2 μM, sodium phosphate buffer, pH 6.8 ($I = 0.1$), (*R*)-alprenolol (0.83 mM) and (*S*)-alprenolol (0.2 mM), incubation time 1 h, otherwise as above. The *p*NP formed was measured at 410 nm in a spectrophotometer.

2.4. Blanks (without enzymes) served as background controls

2.4.1. Preparation of CBH 1 and EG 1 columns. CBH 1 and EG 1 were immobilized on silica and the columns (100×2.1 mm ID) were prepared as in [15].

2.4.2. Calculation of kinetic parameters (K_M, k_{cat} and K_i). Kinetic parameters K_M, k_{cat} and K_i (inhibition constant) were derived by non-linear regression analysis using the 'Ultrafit' program (Biosoft, UK). The program-predefined equations for Michaelis-Menten Kinetics and Competitive Inhibition were used as well as the functions for Robust Weighting and Simple Weighting.

3. Results

The activity of CBH 1 and EG 1 on a soluble substrate, *p*-nitro-phenyl-β-D-lactoside (*p*NPL), was measured in the absence and in the presence of the (*R*)- and (*S*)-enantiomers of two β-blockers, propranolol and alprenolol, respectively. Both enzymes were inhibited by the drugs and the inhibition was competitive according to the Eadie-Hofstee plots (Fig. 2). The kinetic parameters, K_M and k_{cat}, and K_i, derived from the experiments through non-linear regression are shown in Table 1.

The inhibition constant is a direct measure of the inhibitory effect. The lower the K_i value, the stronger is the inhibition, i.e., the stronger is the binding of the compounds. From Table 1 we see the following. (1) The (*S*)-enantiomers of both compounds are in all cases stronger inhibitors than are the corresponding (*R*)-enantiomers. This is valid for both CBH 1 and EG 1. (2) The (*S*)- and (*R*)-forms of propranolol are stronger inhibitors than are the corresponding forms of alprenolol, except for the (*R*)-forms on EG 1, where alprenolol is the stronger inhibitor. (One should note the high standard errors in the experiments with EG 1, which are due to the high K_M value for the enzyme (around 20 mM) compared to the substrate concentrations that could be used (0.4–9 mM) owning to the limited solubility of the substrate.) (3) The inhibition is stronger for CBH 1 than for EG 1. (4) The inhibition is pH dependent and stronger at pH 6.8 than at pH 5.0 (CBH 1 and alprenolol).

CBH 1 and EG 1 chiral stationary phases were used for the separation of racemic alprenolol and propranolol. Table 2 shows the chromatographic data. With CBH 1-CSP the results are completely consistent with the inhibition experiments, i.e. the stronger the inhibitor, the longer it is retained on the column. The difference in K_i between the (*R*)- and (*S*)-enantiomers is reflected in a good separation of both *rac*-alprenolol and *rac*-propranolol at both pH 5.0 and 7.0. Also retention and separation increase with the pH, but the differences are larger than expected from the inhibition results. For example, the K_i value of (*R*)-alprenolol at pH 6.8 is only slightly higher than that of (*S*)-alprenolol at pH 5.0, but the (*R*)-form is retained much longer at pH 7.0 than is the (*S*)-form at pH 5.0.

With EG 1-CSP we see similar trends as with CBH 1. Propranolol is retained longer than alprenolol and the retention increases with pH. But on EG 1-CSP there is almost no separation of the enantiomers as could be expected from the inhibition results. For example, the ratio between the K_i values for the (*R*)- and (*S*)-forms of propranolol on EG 1 is almost the same as on CBH 1, whereas there is good separation only on the CBH 1-CSP (Fig. 3). The retention of both forms of propranolol is fairly long also on the EG 1-CSP.

Table 1
Comparison of inhibition experiments on CBH 1 and EG 1 by propranolol and alprenolol

Parameters	CBH 1	EG 1
pH 5.0		
K_M (mM)	1.21 ± 0.080	20.6 ± 1.5
k_{cat} (s^{-1})	0.127 ± 0.0034	8.20 ± 0.046
K_i (mM)		
(*R*)-propranolol	0.49 ± 0.0093	7.09 ± 2.6
(*S*)-propranolol	0.10 ± 0.012	1.49 ± 0.15
(*R*)-alprenolol	1.00 ± 0.072	6.04 ± 1.2
(*S*)-alprenolol	0.26 ± 0.032	3.77 ± 0.77
pH 6.8		
K_M (mM)	0.48 ± 0.038	
k_{cat} (s^{-1})	0.0060 ± 0.00013	
K_i (mM)		
(*R*)-alprenolol	0.31 ± 0.040	
(*S*)-alprenolol	0.01 ± 0.0050	

Experimental conditions are described in detail in Section 2.3.2.

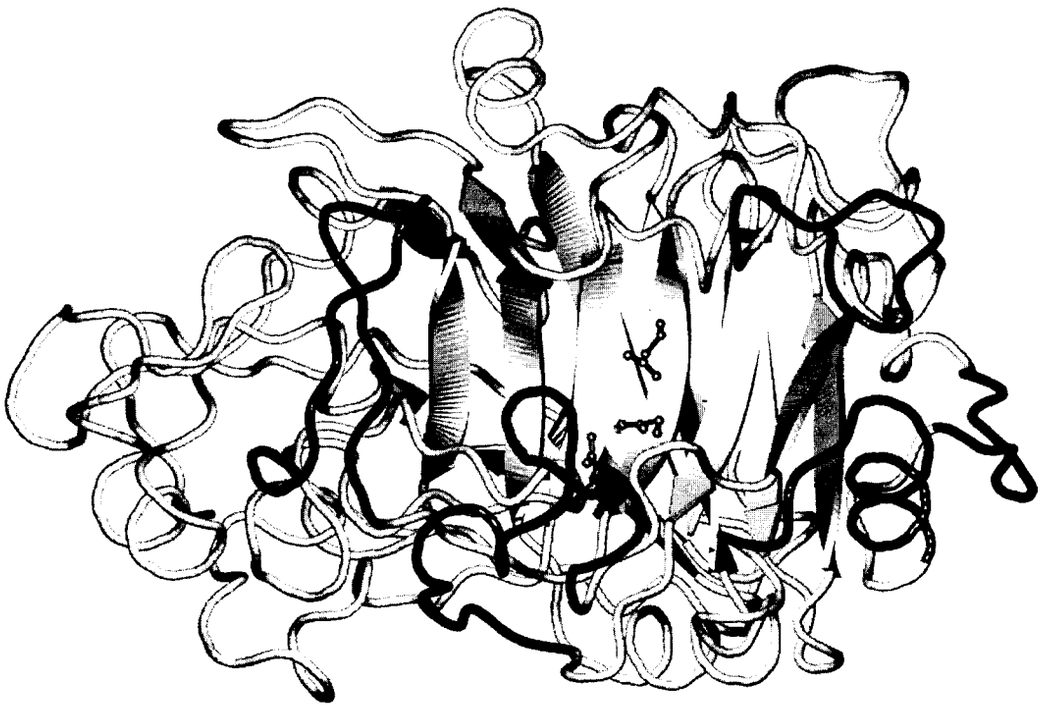

Fig. 1. Structure of CBH 1. Schematic drawing of the CBH 1 Cα backbone. The view is roughly orthogonal to the inner β-sheet of the sandwich. β-strands are shown as arrows and helices/loops as rattlers. Loop regions that are absent in the related EG 1 are indicated by darker colours. The three catalytic residures are drawn as ball-and-stick models. They all lie in the third β-strand from the right in the picture: Glu^{217} (top), Asp^{214} (middle) and Glu^{212} (bottom). The picture was created using the molecular graphics program O[18] and provided by Dr. Christina Divne (Uppsala University).

4. Discussion

Both CBH 1 and EG 1 are competitively inhibited by the β-adrenergic blockers alprenolol and propranolol, indicating that these compounds must interact in close vicinity to the binding site of the substrate *p*NPL. With CBH 1 there is a clear correlation between the strength of the inhibition and the retention time on the column (Tables 1 and 2), which leads to our proposal that the main site for binding of these compounds and discrimination between the enantiomers overlaps with the active site. This is further supported by earlier findings showing that the separation of alprenolol was abolished in the presence of cellobiose or glucose in the mobile phase and that the presence of carboxymethyl cellulose impaired the resolution [12]. Cellobiose and glucose are also inhibitors of the activity of CBH 1 towards *p*NPL, glucose at high concentrations whereas cellobiose is a strong inhibitor with a K_i value around 20 μM (data not shown). Similar phenomena were also found with CBH 2-CSP, where the capacity for chiral recognition was weakened in the presence of glucose and cellobiose in the mobile phase (unpublished results).

On CBH 1 both (*R*)- and (*S*)-alprenolol had lower K_i values, i.e. stronger binding, at pH 6.8 than at pH 5.0, indicating that electrostatic interactions play an important role for binding at the active site. Possible candidates for electrostatic interaction at the active site in the tunnel of CBH 1 are the three carboxylic residues that were proposed to be responsible for hydrolysis, Glu^{212}, Asp^{214} and Glu^{217} [6]. The glutamates 212 and 217 are positioned on opposite sides of a hypothetical glycosidic bond, Glu^{212} 'below' and Glu^{217} 'above', with Asp^{214} on the side. Based on the negative potential that these residues should confer and their configurations, we suggest them to be the primary counterparts for electrostatic interaction with the positively charged β-blockers. Both alprenolol and propranolol are positively charged below their pKa (pH 9.5) and the net charge is practically constant in the pH range used, so the observed increase in binding with pH is presumably caused by an increase in the negative potential at the

Table 2
Comparison of chromatography on CBH 1 and EG 1 phases

Parameters	CBH 1 (pH 5.0)	(pH 7.0)	EG 1 (pH 5.0)	(pH 7.0)
k'				
(*R*)-propranolol	2.52	50.17	5.48	24.6
(*S*)-propranolol	8.18	292.4	5.96	28.8
(*R*)-alprenolol	0.96	24.0	1.83	8.02
(*S*)-alprenolol	5.72	233.8	1.91	8.91
α				
Propranolol	3.24	5.83	1.09	1.17
Alprenolol	5.95	9.74	1.04	1.11

Solid phases: CBH 1, silica; or EG 1, silica.
Mobile phase: 0.01 M sodium acetate buffer, pH 5.0, or 0.01 M sodium phosphate buffer, pH 7.0.
Flow rate: 0.2 ml/min.
Column dimensions: 100×2.1 mm (ID).
To obtain the retention times expressed as capacity factors, k', the (*R*)- and (*S*)-enantiomers were injected separately. The selectivity factors, α, as a measure of the separation efficiency, is defined as $\alpha = k'_S/k'_R$) [15].

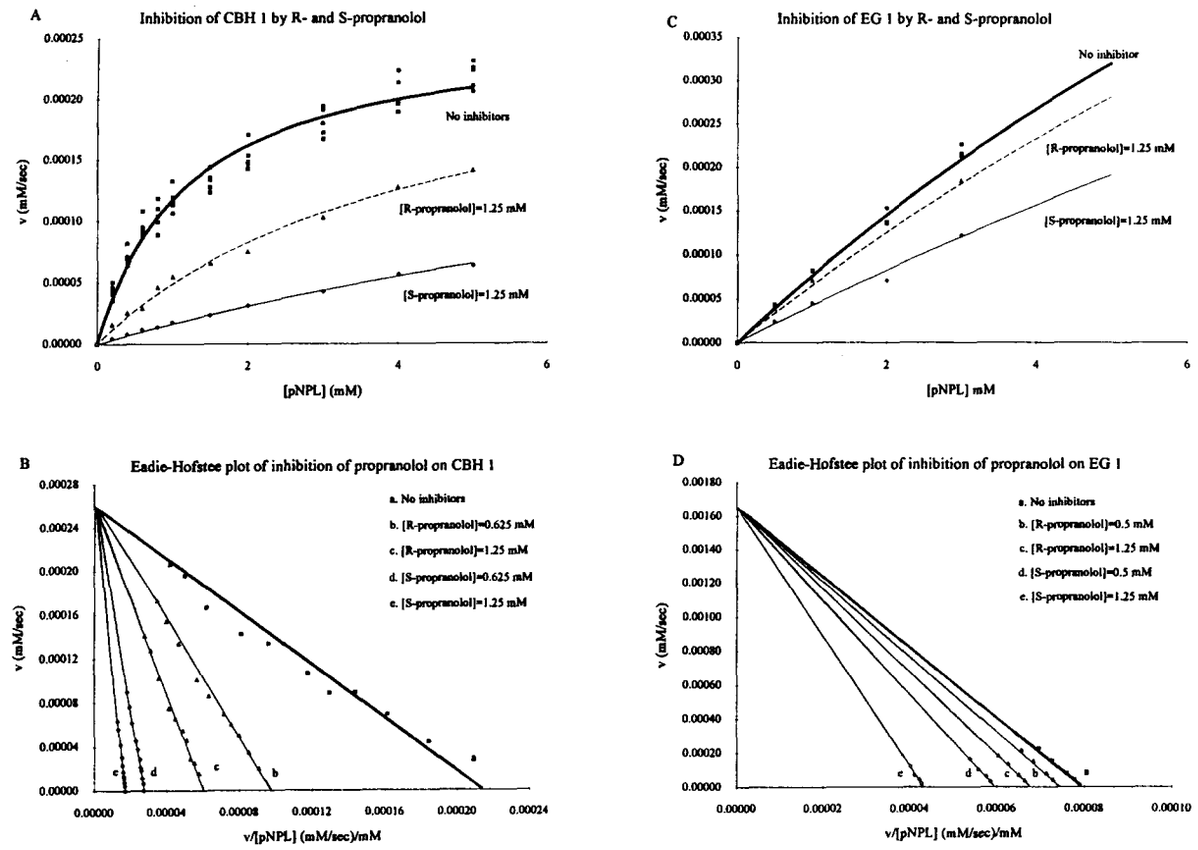

Fig. 2. Competitive inhibition of CBH 1 and EG 1 by R- and S-propranolol. (A,C) Reaction rate (v) versus concentration of the substrate p-nitro-phenyl lactoside ([pNPL]). (B,D) Eadie-Hofstee plot. The release of p-nitrophenol by CBH 1 and EG 1 at 20°C in 0.1 M sodium acetate was measured as described in Section 2 in the presence of no inhibitor (■), R-propranolol (△), or S-propranolol (◇). The lines show the best fit obtained from the non-linear (A,C) or linear (B,D) regression analysis.

active site. At least one of the carboxylic groups involved in the binding thus has a strongly perturbed pKa, probably Glu^{217} the potential proton donor. The protonation states of the catalytic carboxylic groups in CBH 1 are not known, but they are so close that they are likely to affect each other so that the protonation of the whole system is spread over a broader pH range. Yet another residue is likely to affect the charge at the active site, namely, His^{228}, which is close to and probably forms a hydrogen bond to Asp^{214}. At low pH it would be positively charged and thus repel the positively charged amino group of the β-blockers. Again the protonation state is not known. Free histidine has a pKa of 6.0, but here it is likely to be shifted upward because of the close contact with Asp^{214}. This would further broaden the pH range where the active site potential changes. Interestingly, it has been shown by micro-calorimetry that the enthalpy difference between binding of (*S*)- and (*R*)-alprenolol corresponds to the enthalpy for ionisation of one histidine residue [16].

Micro-calorimetric studies have also shown that the binding of alprenolol to CBH 1 increases with temperature [16] and so does the retention of propranolol on CBH 1-CSP [17]. The entropy term is thus the major factor in the binding energy which shows that hydrophobic interaction is also important. This is demonstrated here by the fact that propranolol, with its two-ring naphthyl group, is a stronger inhibitor than alprenolol with a single aromatic ring. In the tunnel of CBH 1 there are four tryptophans and the glycosyl unit binding subsites on each side of the catalytic carboxylates are both made up of tryptophan residues, Trp^{367} and Trp^{376}. They are both at a suitable distance for interaction with the aromatic part of the β-blockers. We suggest that the adrenergic β-blockers bind primarily through their amino group to the catalytic carboxylates, and through the aromatic group to either of the tryptophans 367 or 376. Recent X-ray crystallographic data indicate that the naphthyl group of (*S*)-propranolol stacks to Trp^{376} and that the amino group of the compound interacts with Glu^{212} and Glu^{217} in the active site of the enzyme (unpublished result).

There was no linear correlation between inhibition and retention with increasing pH. The K_i value of (*R*)-alprenolol is slightly higher at pH 6.8 (0.31) than that of (*S*)-alprenolol at pH 5.0 (0.26) (Table 1), but the (*R*)-form is retained almost 5 times longer at pH 7.0 than is the (*S*)-form at pH 5.0 (Table 2). This indicates that the retention must involve also other sites of interaction in addition to the specific binding at the active site and that the influence of these non-specific sites increases with pH. The binding could be inside the tunnel without interfering with the binding of the substrate, as well as on the outside of the protein. It is possible that the non-specific binding is caused by a general electrostatic interaction with the whole protein. In a previous work on the CBH 1-CSP by Marle et al [10] the pH dependence of the retention was investigated in detail, not only for β-blockers but also for other charged compounds including acids and ampholytes and

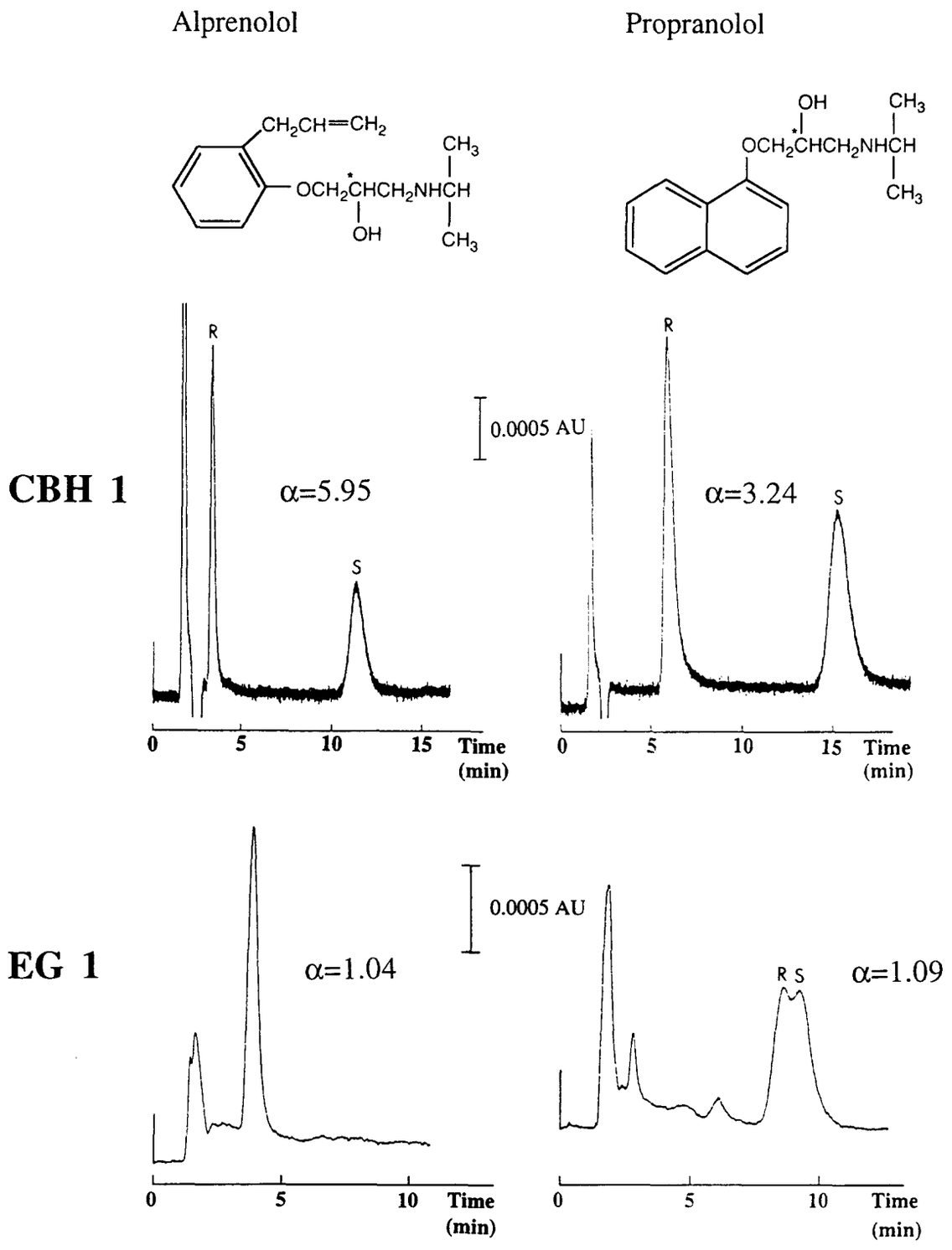

Fig. 3. Chromatography of *rac*-propranolol and *rac*-alprenolol on CBH 1 and EG 1 columns. Solid phases: CBH 1-CSP and EG 1-CSP, respectively. Mobile phase: 0.01 M sodium acetate buffer, pH 5.0. Flow rate: 0.2 ml/min. Temperature: 21°C. Column dimensions: 100×2.1 mm ID. The asymmetric carbon is marked with an asterisk (*).

a few uncharged compounds. All of the charged compounds were influenced by the pH over a broad range from pH 2.2 to 8.1. CBH 1 has a low isoelectric point at around 3.9, above which it is negatively charged, and the surface net charge is rather high as judged from its behaviour in electrophoresis and ion-exchange chromatography. It is thus possible that the CBH 1-CSP to some extent acts as a weak cation exchanger. This unspecific mode of action is, however, not likely to provide a substantial chiral selectivity for the CSP. We propose that specific binding inside the unique tunnel structure of CBH 1 is required for chiral recognition.

Although the discussion above may explain the reason for

the binding of the β-blockers it does not provide the basis for the discrimination of the enantiomers. There are several possibilities as to how this could be achieved. (1) Due to the strong binding the two enantiomers adopt the same conformation and thus the hydroxyl groups on the asymmetric carbon would point in different directions and would interact differently with the protein. (2) Steric hindrance from the hydroxyl group forces one of the enantiomers to adopt a conformation that fits less well. (3) The enantiomers may bind in opposite directions in the tunnel. (4) The more retained (*S*)-enantiomer may adopt a conformation which mimics part of the hydrogen bonding configuration of a glycosyl ring, a conformation that the (*R*)-enantiomer cannot adopt. To get a detailed understanding of the mechanism we probably need the crystallographic structure of a β-blocker–CBH 1 complex combined with molecular dynamic simulation.

The inhibition results (Table 1) show that the β-blockers also bind at the active site of EG 1. The residues we suggested as most important for the interactions in CBH 1, the catalytic carboxylates, the histidine and the two tryptophan residues are also present in EG 1 [8] which therefore should fulfil the prerequisites for chiral discrimination discussed above for CBH 1. There is actually a difference in the K_i values of the enantiomers of both alprenolol and propranolol. The (*S*)-enantiomers are in both cases stronger inhibitors showing that they have higher affinity for the active site than the corresponding (*R*)-form. Nevertheless, very low enantioselectivity was obtained on the EG 1-CSP. One explanation for this may be that a certain binding strength to the active site is required for the discrimination. The affinity of the compounds for the active site of EG 1 was only around one-tenth of that for CBH 1 (Table 1), and thus the specific interaction may be too weak to give a substantial impact on the total binding.

In spite of the weaker inhibition of EG 1, the retention at pH 5.0 is not so very different on the two CSPs. The retention of the (*R*)-enantiomers were even shorter on the CBH 1-CSP (Table 2). The retention on the EG 1-CSP must therefore be governed to a larger extent by non-specific interactions outside of the active site. A general electrostatic attraction could be involved, as discussed for CBH 1. EG 1 is also an acidic protein (pI 4.5) with a rather high net charge.

The main difference between the proteins is that EG 1 lacks part of the extended loops that enclose the active site of CBH 1. In EG 1 the active site lies in a deep groove rather than in a tunnel. The presence of the outer wall in CBH 1 is the most probable explanation for why it is much more efficient in separating the enantiomers. Electrostatic interactions between the compounds and the acidic residues is the main binding force, as mentioned above, but hydrogen-bonding and hydrophobic interactions are probably involved as well [10]. More potential contact points will be provided by the extra amino acids in the tunnel loops of CBH 1, at the same time as they will restrict the access to the active site so that a better fit is required for binding. Furthermore, the binding within a tunnel would exclude more water molecules from contact with the compounds and thus increase the entropic part of the binding. In a groove, part of the molecules would still be exposed to the solvent. The water molecules may form a local hydrophilic environment which could weaken the hydrophobic interactions between the compounds and the residues in the active sites.

5. Conclusion

The β-adrenergic blocking agents act as competitive inhibitors of CBH 1 and EG 1, indicating that the active sites are involved in the chiral recognition. The different chiral selectivity of CBH 1 and EG 1 shows that the tunnel structure of the protein is of vital importance for the chiral recognition.

Acknowledgements: This work is supported by the Swedish Natural Science Research Council and Swedish Research Council for Engineering Sciences. Dr. Ståhlberg was also supported by the Swedish National Board for Industrial and Technical Development (NUTEK). We thank Dr. Christina Divne for the figure of structure of CBH 1 and Dr. David Eaker for the linguistic revision.

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