Thermodynamic Study of an Unusual Chiral Separation. Propranolol Enantiomers on an Immobilized Cellulase

Torgny Fornstedt,† Peter Sajonz, and Georges Guiochon*

Contribution from the Department of Chemistry, University of Tennessee, Knoxville, Tennessee, 37996-1600, and Division of Chemical and Analytical Sciences, and Oak Ridge National Laboratory, Oak Ridge, Tennessee, 37831

Received September 3, 1996[⊗]

Abstract: The thermodynamics of interaction of (*R*)- and (*S*)-propranolol between an acetic acid buffer (pH = 4.7 and 5.5) and the protein cellobiohydrolase I immobilized on silica gel was studied between 5 and 45 °C. The equilibrium data were fitted to a biLangmuir adsorption isotherm with excellent agreement. One of the two Langmuir contributions is the same for both enantiomers and accounts for the nonspecific interactions between these compounds and most sites on the surfaces (type-I, nonselective sites). It has a large saturation capacity. The second contribution accounts for the chiral selective interactions (type-II sites). It has a lower monolayer capacity than the first. The interaction enthalpy and entropy on type-I sites are −1.1 kcal/mol and +0.1 cal/(mol K), respectively. For type-II sites, they are −1.9 kcal/mol and −2.6 cal/(mol K), respectively, for (*R*)-propranolol and +1.6 kcal/mol and + 11.6 cal/(mol K), respectively, for (*S*)-propranolol at pH = 5.5. This explains why at this pH the retention time of the less-retained *R* enantiomer decreases with increasing temperature, while the retention time of the *S* enantiomer increases, causing a large increase of the separation factor when the temperature is raised from 5 to 45 °C. The saturation capacity of the chiral contributions depends strongly on the pH, and the retention times of both enantiomers decrease with increasing temperature at pH = 4.7.

Introduction

A wide variety of stationary phases able to perform chiral separations has been described in the recent past.[1] This activity attests to the importance of the problem, the number of different specific cases, and the impossibility for any given chromatographic system to separate enantiomeric pairs belonging to more than a few structural families.[1] Further progress in this area requires a better understanding of the chiral recognition mechanisms involved. From this point of view, we can distinguish two types of chiral phases, those which are derived from natural products (e.g., cellulose) and in which a high proportion of the carbon atoms have a well-defined chirality and those which contain a relatively low proportion of such centers. In the former type, the whole solution is a chiral environment, most molecular interactions are chiral and they need not be strong. In the latter type, chiral selective interactions are rarer; to achieve separation they must be strong, and it is commonly agreed that the formation of a three-point complex interaction is needed to achieve chiral separations. These surfaces are heterogeneous. Therefore, it is possible to isolate the chiral interactions and to study them. Such phases are usually made by bonding a certain group to a silica surface. This group can originate from a specifically designed small molecule[2] or from a large and complex one (e.g., a protein).[3] The present work deals only with this second type of chiral phases.

Among the most successful bonded phases are those obtained with certain proteins. Properties and applications of immobilized proteins have been recently reviewed.[1,3] Their main advantages over cellulose derivatives[4,5] are a better enantioselectivity and the use of aqueous mobile phases, a strong advantage for bioanalytical applications because it makes the sample pretreatment easier.[6] Their drawbacks are a poor efficiency and the strong peak tailing observed under analytical conditions, causing a decrease of the peak height and, hence, of the detection sensitivity.[6,7] A better understanding of the mechanism of chiral recognition could allow an acceleration of mass transfers and, hence, an improvement of the column efficiency.

The separation of the enantiomers of β-receptor antagonists (a group of amino alcohols) is important because these enantiomers have often considerably different pharmacological and metabolic behaviors.[6−9] Among the proteins used as chiral selectors, cellobiohydrolase I (CBH I) gives probably the best results with this group of drugs. It affords large separation factors for almost all enantiomeric pairs of β-receptor antagonists.[10,11] However, it is limited to the separation of basic chiral drugs and lacks enantioselectivity for almost all acidic chiral drugs (warfarin is a rare exception).[10,11] Furthermore, columns packed with CBH I bonded silica have a lower efficiency and exhibit more strongly tailing peaks than most protein-based columns. The detection limits of compounds eluted from the CBH I column are quite poor.[6,7]

CBH I is a cellulase enzyme. It catalyzes the degradation of cellulose into cellobiose. Its molecule consists of nearly 500 amino acid residues, structurally organized into three parts: a

† Permanent address: Analytical Pharmaceutical Chemistry, Uppsala University BMC, Box 574, S-751 23 Uppsala, Sweden.

[⊗] Abstract published in *Advance ACS Abstracts,* January 1, 1997.

(1) Allenmark, S. *Chromatographic Enantioseparation*; Ellis Horwood, Chichester, 1991; p 129.
(2) Welch, C. *J. Chromatogr., A.* **1994**, *666*, 3.
(3) Allenmark, S.; Andersson, S. *J. Chromatogr., A.* **1994**, *666*, 167.
(4) Okamoto, Y.; Kaida, Y. *J. Chromatogr., A.* **1994**, *666*, 403
(5) Oliveros, L.; López, P.; Minguillón, C.; Franco, P. *J. Liq. Chromatogr.* **1995**, *18*, 1521.
(6) Johansson, M.; Sjöberg, P.; Hesselgren, A.-M.; Salmonson, T. *Chirality* **1995**, *7*, 290.
(7) Fornstedt, T.; Hesselgren, A.-M.; Johansson, M. Manuscript in preparation.
(8) Howe, R.; Shanks, R. G. *Nature* **1966**, *210*, 1336.
(9) Benveniste, H.; Hansen, A. J.; Ottosen, N. S. *J. Neurochem.* **1989**, *52*, 1741.
(10) Marle, I.; Erlandsson, P.; Hansson, L.; Isaksson, R.; Pettersson, C.; Pettersson, G. *J. Chromatogr.* **1991**, *586*, 233.
(11) Isaksson, R.; Pettersson, C.; Pettersson, G.; Jönsson, S.; Ståhlberg, J.; Hermansson, J.; Marle, I. *Trends Anal. Chem.* **1994**, *13*, 431.

S0002-7863(96)03145-9 CCC: $14.00

large core, a small cellulose-binding domain, and a flexible connecting arm.[10−12] The molecule has been enzymatically cleaved into two fragments.[13] Nearly all of the chiral selectivity originates from the core. A small contribution comes from the arm but only at pH values above ca. 7.[13] This supports the assumption that the major chiral adsorption site is the enzymatically active one (i.e., the catalytic domain of the core). This site contains several β-sheets and α-helical segments arranged to form an extended flattened tunnel (ca. 40 Å long), into which the cellulose chain can be threaded and cleaved.[12] The free carboxylic groups of the peptide chain and the amine groups of the β-receptor antagonists are all ionized in the pH range where chiral separations take place. Thus, chiral recognition could involve two strong ion pair bindings. The third interaction point could be a part of the hydrophobic surface.[14]

An unusual effect of temperature on the separation of *rac*-propranolol on CBH I has been reported recently.[15] The retention factor of the more retained (*S*)-propranolol increases with increasing temperature, from 10 to 40 °C, while the retention factor of (*R*)-propranolol decreases.[15] Few similar observations have been made in chiral chromatography. Gilpin *et al*. examined the influence of temperature on the separation of D- and L-tryptophan on immobilized BSA.[16] The plot of ln k' versus $^1/_T$ is linear for the D and nonlinear for the L isomer. The retention time of L-tryptophan increases with increasing temperature from 5 °C to approximately 22 °C, at which temperature it goes through a maximum and then decreases when the temperature increases further, up to 40 °C. Thus, the enantioselectivity is maximum around 20−24 °C. Pirkle found unusual temperature effects in his study of the separation of spirolactam enantiomers on a column packed with chemically bonded (*R*)-*N*-(3,5-dinitrobenzoyl)phenylglycine.[17] With increasing temperature, the retention factor decreased first, then increased, and finally decreased again. Plots of the logarithm of the separation factor, ln α, versus $^1/_T$ were linear, indicating that, whatever the cause of this unusual behavior, it affects both enantiomers equally and that chiral recognition might not be involved. Thermodynamic functions were derived from the equilibrium data of Tröger's base on cellulose microcrystalline triacetate, using ethanol as the mobile phase.[18] Both adsorption enthalpy and entropy were negative and increased with increasing temperature.

It has been shown earlier how to separate the nonchiral and the chiral contributions to the retention in analytical chiral chromatography.[19] This requires the determination of the adsorption isotherms of both enantiomers, followed by the separation of the nonchiral and the chiral contributions to these isotherms. When the density of chiral sites is low enough, this separation is possible with a reasonable accuracy.[19] This method was applied with success to the study of the chiral retention mechanisms of the enantiomers of amino acid derivatives on bovine serum albumin (BSA).[19−21] The approach consisting in modeling equilibrium isotherms or retention factors with one single association constant,[15,22,23] as if there were one single retention mechanism involved, is incorrect.

The thermodynamic functions of the retention mechanism in reversed phase, achiral chromatography have been determined in many cases.[24−27] Usually, both enthalpy and entropy of adsorption are negative,[24,25] but positive adsorption entropies were observed at high water content of the mobile phase.[26,27] For a series of alkylbenzenes eluted on silica beads coated with poly(dimethylsiloxane) formamide, the enthalpy of transfer was exothermic ($\Delta H° < 0$) while the entropy of transfer was positive.[28] Except for the retention of C_{60} and C_{70} on (dinitrobenzoyl)phenylglycine and 2-((2,4,5,7-tetranitro-9-fluorenylidene)amino)oxy)propionic acid,[29] we are unaware of any clear, demonstrated endothermic behavior in chiral or achiral chromatography. The observations made in chiral[15−17] and achiral chromatography[26] and discussed above are most likely the result of the superimposition of an endothermic and an exothermic mechanism.

Immobilized protein phases used in chiral chromatography behave as heterogenous surfaces.[19−21,30] Retention is explained by a mixed mechanism involving both chiral and nonchiral interactions. Often, the mass transfer kinetics also is heterogeneous, being much slower for the chiral adsorption mechanism than for the nonchiral one.[30] The investigation of the complex thermal behavior reported above requires the determination of isotherm data in a sufficiently wide temperature range and the separation of each isotherm into its chiral and achiral contributions. As previously done for *N*-benzoyl-D- and -L-alanine on immobilized BSA, the thermodynamic functions of the chiral recognition mechanism can be derived with reasonable accuracy.[31] Forgetting to take into account the nonchiral contribution, on the other hand, can lead only to erroneous conclusions.[15,22]

The present work has several goals. The first is to determine the enthalpy and entropy of the chiral and nonchiral retention mechanisms for propranolol on immobilized CBH I and to use this information to derive some conclusions regarding these mechanisms. The second goal is to illustrate why the conclusions of studies based on raw data which lump together the effects of the chiral and nonchiral interactions are wrong and what is the possible extent of these errors.

Theory

We review the general model of equilibrium behavior on a heterogeneous surface of the type used here and the models of adsorption isotherms available for this study.

I. Model of Adsorption Behavior. Chiral protein phases are made by bonding to a silica surface certain protein molecules which contain at least one chiral-binding domain (often a cavity or tunnel[19−21,30]). The surface of these adsorbents contains two different types of adsorption sites, type-I and type-II sites (see below), and must be considered as heterogeneous. The validity

(12) Divne, C.; Ståhlberg, J.; Reinikainen, T.; Ruohonen, L.; Pettersson, G.; Knowles, J. K. C.; Teeri, T. T.; Jones, T. A. *Science* **1994**, *265*, 524.
(13) Marle, I.; Jönsson, S.; Isaksson, R.; Pettersson, C.; Pettersson, G. *J. Chromatogr.* **1993**, *648*, 333.
(14) Vandenbosch, C.; Massart, D. L.; Lindner, W. *Anal. Chim. Acta* **1992**, *270*, 1.
(15) Jönsson, S.; Schön, A.; Isaksson, R.; Pettersson, C.; Pettersson, G. *Chirality* **1992**, *4*, 505.
(16) Gilpin, R.; Ehtesham, S.; Gregory, R. *Anal. Chem.* **1991**, *63*, 2825.
(17) Pirkle, W. *J. Chromatogr.* **1991**, *558*, 1.
(18) Jacobson, S.; Golshan-Shirazi, S.; Guiochon, G. *J. Am. Chem. Soc.* **1990**, *112*, 6492.
(19) Seidel-Morgenstern, A.; Guiochon, G. *J. Chromatogr.* **1993**, *631*, 37.
(20) Jacobson, S.; Golshan-Shirazi, S.; Guiochon, G. *Chromatographia* **1991**, *31*, 323.
(21) Jacobson, S.; Guiochon, G. *J. Chromatogr.* **1992**, *600*, 37.
(22) Yang, J.; Hage, D. S. *J. Chromatogr., A* **1996**, *725*, 273.
(23) Loun, B.; Hage, D. S. *Anal. Chem.* **1994**, *66*, 3814.
(24) Yamamoto, F.; Rokushika, S.; Hatano, H. *J. Chromatogr. Sci.* **1989**, *27*, 704.
(25) Cole, L.; Dorsey, J. *Anal. Chem.* **1992**, *64*, 1317.
(26) Cole, L.; Dorsey, J.; Dill, K. *Anal. Chem.* **1992**, *64*, 1324.
(27) Grushka, E.; Colin, H.; Guiochon, G. *J. Chromatogr.* **1982**, *248*, 325
(28) Sjöberg, M.; Silveston, R.; Kronberg, B. *Langmuir* **1993**, *9*, 973.
(29) Diack, M.; Compton, R. N.; Guiochon, G. *J. Chromatogr.* **1993**, *639*, 129.
(30) Fornstedt, T.; Zhong, G.; Bensetiti, Z.; Guiochon, G. *Anal. Chem.* **1996**, *68*, 2370.
(31) Jacobson, S.; Golshan-Shirazi, S.; Guiochon, G. *J. Chromatogr.* **1990**, *522*, 23.

of such a surface model has been ascertained by earlier studies which have successfully used this model to describe the adsorption equilibrium behavior of enantiomers on chiral phases obtained by protein immobilization.[19−21,30−32]

On type-I sites take place all possible low-energy molecular interactions between analyte molecules and atoms or groups of atoms belonging to the adsorbent surface. These interactions can originate from the nonchiral parts of the protein molecule and/or from the adsorbent (silica) matrix. They include hydrophobic interactions and London dispersive interactions as well as isolated polar interactions involving Debye or Keesom forces and even single hydrogen bond interactions. These interactions are those responsible for retention on conventional non-enantioselective phases (e.g., in reversed phase chromatography on chemically bonded silicas). The number of possible interactions of this type is extremely large, and although their individual energies are modest, they contribute quite significantly to the overall retention because of their number. It has been shown that such interactions exist for CBH I immobilized on silica.[30] These interactions explain the retention of molecules which cannot interact with the enantioselective sites (e.g., molecules which cannot enter the cavity of an immobilized protein because they are too big). The kinetics of interaction on type-I sites (i.e., of adsorption/desorption) is fast. This explains why, although this type of sites is certainly not homogeneous, it is possible to assume that it is so. Finally, a Langmuir isotherm accounts well for the adsorption behavior in the range of (low) concentrations which is useful for chiral separations (see below) because the activity coefficient in solution remains constant within this range. The validity of this model is confirmed by many side comments found in papers by experts in chiral separation. For example, Booth and Wainer[33] invoke "... solute stationary phase interactions governing general retention". This is exactly what type-I sites do in our surface model.

On type-II sites take place the selective interactions responsible for enantiomeric separations. The requirement that at least three interaction points are necessary between the enantiomer and the chiral selector for chiral recognition is generally accepted[34,35] One of these interactions can be steric and thus provided by a surface such as the silica wall (e.g., Pirkle phases[2]) or by a hydrophobic zone on the protein surface[14] for immobilized protein phases. The other two interactions needed involve usually the formation of hydrogen bonds or of very strong polar interactions. For CBH I, it is suspected that the three-point interaction involves the formation of at least two ion pairs.[12] Type-II sites are much less numerous than type-I sites. Thus, their saturation capacity is lower than that of type-I sites, and in spite of a much higher adsorption energy, their contribution to overall retention is comparable to that of type-I sites. Because type-II sites are few, they are scattered on the surface and adsorbate−adsorbate interactions are unlikely. The conditions required for a Langmuir isotherm (localized adsorption, no adsorbate−adsorbate interactions) are satisfied, and this isotherm model accounts well for the chiral adsorption behavior. Because their adsorption energy is high and they are relatively few, type-II sites are saturated at relatively low concentrations. At higher concentrations, the ability to perform enantiomeric separations vanishes. Because of the high interaction energy and of the steric requirements, mass transfer is slower on type-II than on type-I sites.[30]

The widely different behaviors of the two types of sites explain the many unusual properties which have been previously reported for this type of chiral phase. These properties are essentially related to heterogeneous thermodynamic and kinetic behaviors. Conversely, they also allow the use of chiral phases as simple, illustrative models of heterogeneous surfaces which have a well-known composition.

Finally, we should emphasize that the situation may become more complicated when ligands as complex as proteins are used as the chiral selectors. More than one chiral recognition site may exist on the protein molecule. These different chiral recognition sites are bound to have different thermodynamic and kinetic properties. Then, a multiLangmuir isotherm would become necessary to account for the adsorption equilibrium. The identification of the parameters of such an isotherm (i.e., of two more parameters per additional enantioselective site on the protein) would be most difficult, requiring accurate data in a wide enough range of concentration. It is also possible that these different sites have opposite stereoselectivity, causing the extent of separation to decrease with increasing concentration and, possibly, the elution order to reverse. Such a complex situation has not been described yet, to the best of our knowledge.

II. Adsorption Isotherms. At constant temperature, the concentrations of a component in the mobile and the stationary phase at equilibrium are related by the isotherm equation. The Langmuir isotherm equation is the simplest model of nonlinear isotherm. It accounts well for the adsorption of single components on homogeneous surfaces at low concentrations (so that the activity coefficient in solution remains nearly constant). A reduced form of this isotherm is

$$\theta = \frac{q^*}{q_s} = \frac{(a/q_s)C}{1 + bC} = \frac{bC}{1 + bC} = \frac{\Gamma}{1 + \Gamma} \qquad (1)$$

In this equation, θ is the fraction of monolayer coverage of the surface, q^* is the stationary phase concentration at equilibrium with the mobile phase concentration C, a is the initial slope of the isotherm, equal to the equilibrium constant of adsorption which is related to the adsorption energy (see later), $a/b = q_s$ is the monolayer capacity or specific saturation capacity of the stationary phase, b is a numerical coefficient, and Γ is a dimensionless concentration. The column saturation capacity is the product of q_s by the amount of stationary phase in the column. Thus, q_s depends on the total number of adsorption sites on the surface of the stationary phase. The dimensionless product $\Gamma = bC$ characterizes the deviation of the isotherm from linear behavior. If Γ is negligible compared to unity, the isotherm behaves as if it were linear. If Γ is larger (e.g., if $\Gamma = 1$, $\theta = 0.5$ (eq 1)), then the isotherm is no longer linear; an important curvature of the isotherm is experienced. The determination of accurate estimates of the isotherm parameters requires that measurements be performed in a range of values of Γ significant compared to unity.

Since chiral stationary phases have two different types of adsorption sites, as explained previously, the simplest isotherm model accounting for adsorption on these heterogenous surfaces is a biLangmuir adsorption isotherm. Each term of this model accounts for the contribution of one type of sites. This model has been used successfully to describe the adsorption equilibrium behavior of enantiomers on chiral protein stationary phases.[19−21,30−32] The equation is written

$$q^* = q^*_1 + q^*_2 = \frac{q_{1,s} b_1 C}{1 + b_1 C} + \frac{q_{2,s} b_2 C}{1 + b_2 C} \qquad (2)$$

(32) Guiochon, G.; Golshan-Shirazi, S.; Katti, A. M. *Fundamentals of Preparative and Nonlinear Chromatography*; Academic Press: Boston, MA, 1994; Chapter X.
(33) Booth, T. D.; Wainer, I. W. *J. Chromatogr.* **1996**, *737*, 157.
(34) Dalgliesh, C. E. *J. Chem. Soc.* **1952**, 3940.
(35) Pirkle, W. H.; Finn, J. *J. Org. Chem.* **1981**, *46*, 2935.

where $q_{1,s}$ and $q_{2,s}$ are the monolayer capacities of type-I and type-II adsorption sites, respectively. The coefficients b_1 and b_2 are related to the adsorption energy on the two types of sites. Because type-I sites are not enantioselective, the values of the coefficients $q_{1,s}$ and b_1 are the same for the two optical isomers, while the coefficients $q_{2,s}$ and b_2 are different. Since enantioselective adsorption sites are fewer than nonselective ones, the saturation capacity of the latter is larger while, as explained previously, the energy of adsorption is higher for type-II sites. Otherwise these sites would not contribute significantly to the retention. So, $q_{2,s}$ is usually is much smaller than $q_{1,s}$ and b_2 is much larger than b_1. It turns out that a_1 and a_2 are often of a comparable magnitude.

From eq 2, we derive the general expression of the retention factor on an enantioselective phase under conditions of infinite dilution

$$k' = F(q_{1,s}b_1 + q_{2,s}b_2) = F(a_1 + a_2) = k'_1 + k'_2 \quad (3)$$

The retention factor k', is the sum of two contributions, originating from type-I and type-II sites, respectively. Thus, the enantioselectivity α_{es}, which is conventionally taken as the ratio of the retention factors of the two enantiomers,[10,11] is an empirical factor without physical significance. From eq 3, we obtain, assuming that the *S* enantiomer is the more retained,

$$\alpha_{es} = \frac{a_1 + a_{2,S}}{a_1 + a_{2,R}} \quad (4)$$

In a study of chiral recognition mechanisms, the separation factor of the two enantiomers on the type-II sites or true enantioselectivity separation factor, $\alpha = a_{2,S}/a_{2,R}$, is the relevant parameter. Because the contribution of the nonchiral type-I sites to the retention factor (eq 3) is often of the same order of magnitude than the contribution of the chiral type-II sites, the empirical factor is significantly lower than the true one, therefore leading to serious underestimates of the importance of the degree of enantioselectivity actually achieved with the chiral phase. Admittedly, α_{es} is the more important parameter for practical purposes. This explains why the design of highly selective chiral phases involves the dense bonding of highly selective chiral groups or molecules to a surface giving low nonselective retention.

Obviously, it is not possible to derive the coefficients $a_{2,R}$ and $a_{2,S}$ from experimental determinations performed under linear conditions. Measurements of isotherm data have to be carried out in a range of concentrations within which the isotherm is nonlinear, and the experimental data obtained have to be properly modeled. For the reasons explained above, eq 2 will be adequate in most cases (for the type of chiral phases studied here).

III. Thermodynamic Functions of Adsorption. The standard molar Gibbs free energy of adsorption, $\Delta G°$, is the difference between the molar standard Gibbs free energy of the adsorbed and unabsorbed molecules, at constant temperature. Under equilibrium, the standard molar Gibbs free energy of adsorption[31,36] at temperature T (K) can be written as

$$\Delta G° = -RT \ln K = -RT \ln a \quad (5)$$

where R is the universal gas constant and $K = a = q/C$ is the thermodynamic equilibrium constant.[19] Since the a coefficients of the biLangmuir isotherm equation are equal to the equilibrium constants for the corresponding adsorption processes, the classical thermodynamic functions of the two types of sites can be derived from the temperature dependence of the corresponding equilibrium constants. From the Gibbs−Helmholtz equation,[36] we can derive the temperature dependence of the equilibrium constants (van't Hoff equation)

$$\frac{\partial(\ln a)}{\partial\left(\frac{1}{T}\right)} = -\frac{\Delta H°}{R} \quad (6)$$

This equation allows the determination of the standard molar enthalpy of adsorption, $\Delta H°$, from the slope of the temperature dependence of the logarithm of the equilibrium constant. Because k' is easy to measure on chromatograms, a simplified version of the van't Hoff equation is often used in analytical chromatography,

$$\ln k' = \ln F - \frac{\Delta H°}{RT} + \frac{\Delta S°}{R} \quad (7)$$

The adsorption enthalpy and entropy are derived from the slope, $-\Delta H°/R$, and the intercept, $(\Delta S°/R + \ln F)$, of a plot of $\ln k'$ versus $1/T$. This plot is linear if $\Delta H°$ and $\Delta S°$ do not depend significantly on the temperature within the range used in the measurements, which is the usual case. This approach is generally used in chiral chromatography.[22] A very important restriction, however, is that the surface must be homogenous. Otherwise, the contributions of the different mechanisms which contribute to the surface inhomogeneity are assembled in a combined term whose magnitude is a function of temperature. This is the general case in chiral chromatography (see eq 3) and, in particular, for the chiral chemically bonded phases studied here. In such a case, this approach does not give a correct estimate of the thermodynamic functions of the chiral interactions.

The alternative approach detailed here consists in determining $\Delta G°$ from eq 5 and the value of the equilibrium constant of adsorption obtained from the equilibrium isotherm data and in deriving $\Delta H°$ from eq 6 and the temperature dependence of the same constant, b. Then, the entropy is obtained from the fundamental equation

$$\Delta S° = -\frac{\Delta G° - \Delta H°}{T} \quad (8)$$

Since the equilibrium constants are obtained separately for type-I and type-II sites (b_1 and b_2 in eq 2), the thermodynamic functions $\Delta G°$, $\Delta H°$, and $\Delta S°$ can be determined separately for the two types of sites. This procedure allows the direct determination of the chiral contributions to the isotherms and to the retention factors, of the thermodynamic functions of the chiral and nonchiral retention mechanisms, and of the true chiral separation factor.

Experimental Section

I. Apparatus. The chromatographic system used consisted of a HP 1090 liquid chromatograph (Hewlett-Packard, Palo Alto, CA) equipped with a diode-array UV detector, a computer data acquisition system, and a multisolvent delivery system. The column was placed in a laboratory-assembled column jacket. The temperatures of the column and of the mobile phase reservoirs of the chromatograph were controlled using a M20 LAUDA circulating water-bath (Lauda, Königshofen, Germany).

II. Chemicals. (*R*)-(+)-Propranolol and (*S*)-(−)-Propranolol (Sigma, St. Louis, MO) were of 99% purity. They were used without further purification. (*S*)-(−)-Propranolol is the active β-blocking enantiomer.[15] The buffer salts were anhydrous sodium acetate >99.5% and acetic acid >99.5% (Fluka, Buchs, Switzerland). The water used was HPLC

(36) Atkins, P. W. *Physical Chemistry*; Oxford University Press: Oxford, 1995.

grade water from Fisher (Fisher Scientific, Pittsburgh, PA). After dissolving the buffer salts, the stock solutions were filtered with 0.2 μM CA filters (Nalgene Company, Rochester, NY).

III. Column and Immobilization of the Stationary Phase. The silica particles containing the immobilized protein were packed in a 100 × 4.6 mm stainless steel column. The protein cellobiohydrolase I was immobilized as described previously.[11,30] The concentration of CBH I immobilized on the silica support was determined by measuring at 280 nm the UV absorbance of the solution before and after its reaction with the aldehyde silica. Protein (50.7 mg) bonded per gram of diol silica was used. The amount of protein in the column (46.2 mg) was derived from this concentration and from the weight of dry silica that the column contained.

IV. Mobile Phase. Solutions of an acetic buffer at two different pH values, 4.7 and 5.5, were used as the mobile phase. The mobile phase at pH = 4.7 was an acetic acid buffer containing 0.02 M sodium acetate and 0.02 M acetic acid. Its ionic strength was $I = 0.02$. Its pH was measured at pH = 4.66 with a calibrated American pH II pHmeter (Baxter Scientific, Stone Mountain, GA). The mobile phase at pH = 5.5 was an acetic acid buffer containing 0.020 M sodium acetate and 3.15 mM acetic acid. Its ionic strength was $I = 0.02$ and its pH was measured to be 5.47.

The mobile phase flow rate was 0.80 mL/min. It was measured for each individual chromatographic run and accounted for in the calculations (with two significant digits).

V. Procedures. The adsorption isotherms of the two propranolol enantiomers were determined by frontal analysis in the staircase mode (i.e., by increasing stepwise the concentration of the solute in the eluent percolating through the column and recording the detector signal). The solute concentration in the eluent is adjusted by using the solvent delivery system of the chromatograph in the gradient mode. Pure mobile phase is used as solvent A, and a solution of one enantiomer is used as solvent B. A step gradient is programmed. Because measurements must be made in a broad concentration range, three different bulk concentrations of each enantiomer at each temperature were used successively as solvent B. Measurements were made for mobile phase concentrations ranging from 0.25 μM to 1.70 mM, an approximately 7000-fold dynamic range. The UV detector absorbance was recorded at 220 and 230 nm, depending on the concentration.

The column hold-up volume was derived from the time of the water disturbance peak. It was found to be 1.24 mL ($t_o = 1.55$ min at a flow rate of 0.80 mL/min) in the preliminary study (see elution profiles in Figures 1−3) and 1.18 mL during the remaining part of the study (acquisition of the frontal analysis data). The hold-up time was found not to change with temperature (up to 45 °C). All frontal analysis data were corrected for the dead volume of the whole instrument and for the column hold-up volume. This correction includes also the volumes of the mixing chamber of the HP 1090 system and of the steel capillaries between mixing chamber and column and column and detector. This total correction volume was determined to be 1.79 mL.

The best values of the parameters of the biLangmuir isotherm (eq 2) were calculated using the software PCNONLIN 4.2 Scientific Consulting (Apex, NC).

Results and Discussion

Almost all pairs of β-blocker enantiomers are separated on immobilized CBH I, often with high selectivities.[10,11] The *S* enantiomer is always the more retained component.[10,11] The mobile phase is an aqueous buffer solution. Small amounts of organic solvents, such as 2-propanol, can be used as modifiers. The retention times of both β-blocker enantiomers increase with decreasing concentration of the organic modifier and with decreasing concentration of the buffer (i.e., with decreasing ionic strength). The *S* enantiomer retention time increases rapidly with increasing mobile phase pH while the *R* enantiomer retention time increases more slowly. Thus, the enantioselectivity of the stationary phase increases markedly with increasing mobile phase pH. In the pH range used in this study (4.7 to 5.5), the net charge of the protein is negative (pI = 3.9) whereas the amine group of propranolol is protonated, which gives a positive total charge for this solute (pK_a = 9.5).

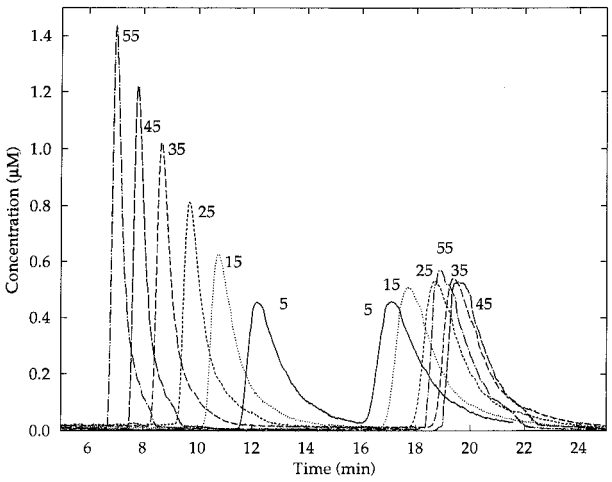

Figure 1. Elution profiles of (*R*)- and (*S*)-propranolol at different temperatures, in analytical concentrations. (*S*)-Propranolol is the more retained enantiomer and the one showing the unusual temperature dependence at this mobile phase pH value. Conditions: column, 100 mm × 4.6 mm; stationary phase, immobilized CBH I on silica; eluent, acetic buffer at pH = 5.47, $I = 0.02$; mobile phase flow rate, 0.8 mL/min; sample, 10 μL of a 0.05 mM solution of (*R*)- and (*S*)-propranolol, each, in the acetic buffer.

Influence of Temperature on the Chiral Separation. The unusual temperature effect previously reported[15] is illustrated by the chromatograms of *rac*-propranolol obtained at different temperatures and overlaid in Figure 1. In this figure, the retention time of the maximum of the *S* enantiomer peak in a pH = 5.5 buffer increases with increasing temperature from 5 to 45 °C, while the behavior of the peak of the less retained R enantiomer is normal. The retention time of the *S* enantiomer increases with increasing temperature between 5 and 45 °C, decreases between 45 and 55 °C, and is still higher at 55 °C than at 25 °C. As explained by eq 7, this temperature dependence corresponds to an endothermic slope of the van't Hoff plot of the S isomer, the R isomer exhibiting the conventional exothermic adsorption behavior. The earlier authors did not, unfortunately, report the van't Hoff plot of the S enantiomer at pH = 5.5. All the other plots published had positive slopes, indicating exothermic behavior [ref 15, Figures 3 and 4]. Complementary microcalorimetric experiments made with the free, unbound protein showed endothermic enthalpy changes for both enantiomers. The (*R*)-propranolol had a complexation enthalpy of +3.3 kJ/mol, the (*S*)-propranolol a complexation enthalpy of +8.6 kJ/mol.[15] It was suggested that the discrepancy between calorimetric and chromatographic results was due to differences in the complexation enthalpy of the free and the immobilized protein.[15]

To understand better the origin of this unusual temperature effect, a more detailed investigation was undergone. The van't Hoff plots were acquired at different mobile phase pH. The adsorption isotherms of both enantiomers were acquired at different temperatures at pH = 5.5, and the thermodynamic functions of both retention mechanisms were determined.

Dependence of the Thermodynamics Functions on the Mobile Phase pH. Figures 2 and 3 show the van't Hoff plots for the experimental retention factors (i.e., $k' = F(a_1 + a_2)$) of the *R* and *S* enantiomers at pH = 4.7 and 5.5, respectively. All four plots are linear within the temperature range studied, except for the point at 55 °C and pH = 5.5 for the *S* enantiomer. In the former case (pH = 4.7), the global adsorption processes of both enantiomers are exothermic (Figure 2). In the latter case

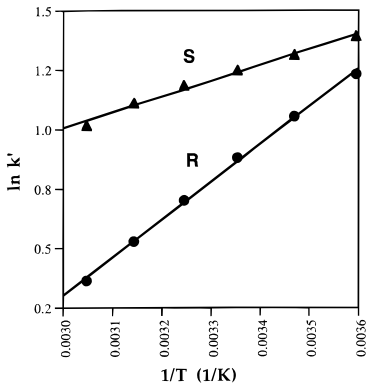

Figure 2. Effect on temperature on the peak capacity factors at analytical concentrations of (*R*)- and (*S*)-propranolol at an acetic buffer mobile phase of pH value 4.7. Symbols, experimental data; lines, best linear fit. Same experimental conditions as for Figure 1 except for the mobile phase; acetic buffer pH = 4.66, $I = 0.02$.

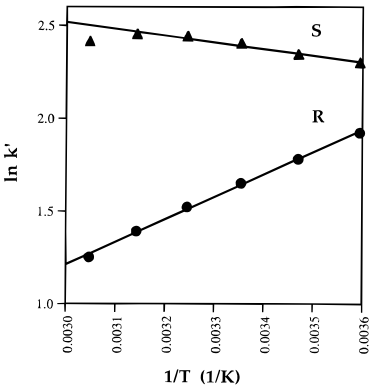

Figure 3. Effect on temperature on the peak capacity factors at analytical concentrations of (*R*)- and (*S*)-propranolol at an acetic buffer mobile phase of pH value 5.47. Symbols, experimental data; lines, best linear fit. Same experimental conditions as for Figure 1.

(pH = 5.5), the global adsorption of (*R*)-propranolol is still exothermic but that of (*S*)-propranolol is endothermic (Figure 3). Figures 2 and 3 show also that the enantioselective separation factor (α_{es}) increases with increasing temperature, significantly in the former case (pH = 4.7), rapidly in the latter one (pH = 5.5).

The influence of the pH on the temperature dependence of the adsorption behavior of (*S*)-propranolol is exceptional. An influence of the pH on the conformation of CBH I has been suggested by circular dichroism studies,[10] but this result has not been confirmed by X-ray crystallographic studies performed on samples of the protein crystallized at different pH values.[12]

Adsorption Isotherm Data. In previous work,[30] the equilibrium between the propranolol enantiomers and immobilized CBH I has been found consistent with the two type of sites model of interaction described earlier in this paper. The equilibrium isotherm was well described by the biLangmuir model (eq 2). This result was also consistent with our previous works on immobilized proteins as chiral selectors.[19−21,31,32] Since the surface of the adsorbent is heterogenous,[30] it is incorrect to derive the thermodynamic functions directly from the van't Hoff plots of the retention factors (Figures 2 and 3) which include the contributions of both types of sites (eq 3). Instead, we must resolve nonchiral and chiral contributions to the total retention and study separately the thermodynamic functions of the chiral interactions of the two enantiomers.[31] This cannot be done by using only the results of analytical (i.e., linear) chromatography.[31,32]

The acquisition and modeling of isotherm data require some caution when complex solutions are used as the mobile phase, as was done in this case. The concentrations and valencies of the buffer components which serve as counterions or co-ions of the solute(s) influence the activity coefficients of all the dissolved ions.[37,38] They also affect the equilibrium constants.[37,38] Finally, under nonlinear conditions, the presence of additives influences the equilibrium isotherm.[38−40] This effect arises through the change in the ionic strength of the solution. It may also arise through competition, in which case system peaks would be observed. No system peaks were observed with the buffer components of the mobile phase used in this study, however, the same as used to acquire the profiles shown in Figure 1. This phase contains only 3.15 mM of undissociated acetic acid, 20.0 mM of acetate ions and 20.0 mM of sodium ions. These concentrations should be compared with the maximum concentration of solute used in the frontal analysis runs (1.70 mM). The adsorption strength of the undissociated acid is small compared to that of propranolol.

Frontal analysis was carried out using the staircase method which is simple and accurate.[32] Since the measurements of adsorption data must be made in a broad concentration range, three frontal analysis staircase runs were acquired successively at each temperature and for each enantiomer. Twenty-three data points were acquired between 0.25 μM to 1.70 mM, at each temperature between 5 and 45 °C. The highest temperature used in the preliminary study (55 °C) was avoided because exposure of the column at a high temperature during the time required for the measurements (over 8 h) was felt to cause considerable risk of damaging the column.

Figures 4 and 5 show the adsorption isotherm data (symbols) obtained for (*R*)- and (*S*)-propranolol, respectively. The main figures show the data in the intermediate concentration range (concentration below 0.1 mM). The insets in the upper left corners show enlargement of the data in the low concentration range (below 5.0 μM), and the insets in the lower right corners show enlargement of the data in the high concentration range (up to 1.7 mM). These data were fitted to different isotherm models, using as nonlinear estimation method the Gauss−Newton algorithm with the Levenberg modification as implemented in the software PCNONLIN 4.2. In this regression, the experimental data were given a weight equal to $1/q_{pred}$, where q_{pred} is the stationary phase concentration predicted by the model. The Langmuir model gives a poor fit and cannot account for the data obtained. The biLangmuir model fitted best to the data. The best values obtained for the biLangmuir coefficients are reported in Table 1. The lines in Figures 4 and 5 show the isotherms derived from these coefficients. Comparison of the lines and the symbols demonstrates that the biLangmuir model fits excellently to the experimental data in the whole concentration range.

In Figure 4, we see that the higher the temperature, the lower the amount of (*R*)-propranolol adsorbed at equilibrium, as expected for an exothermic adsorption process and in agreement with the results obtained with the (*R*)-propranolol retention factor data (Figure 3). The isotherm at the top of Figure 4 is the one obtained at the lowest temperature studied (5 °C, solid line). The one at the bottom was recorded at the highest temperature (45 °C, extra-long dashed line).

The results shown in Figure 5 are more complex. In the high concentration part of the main figure and in the lower right corner inset, the isotherms of (*S*)-propranolol are in the same

(37) Poppe, H. *J. Chromatogr. A* **1993**, *656*, 19.
(38) Fornstedt, T.; Guiochon, G. *Anal. Chem.* **1994**, *66*, 2686.
(39) El-Fallah, M. Z.; Guiochon, G. *J. Chromatogr.* **1990**, *522*, 1.
(40) El-Fallah, M. Z.; Guiochon, G. *Anal. Chem.* **1991**, *63*, 2244.

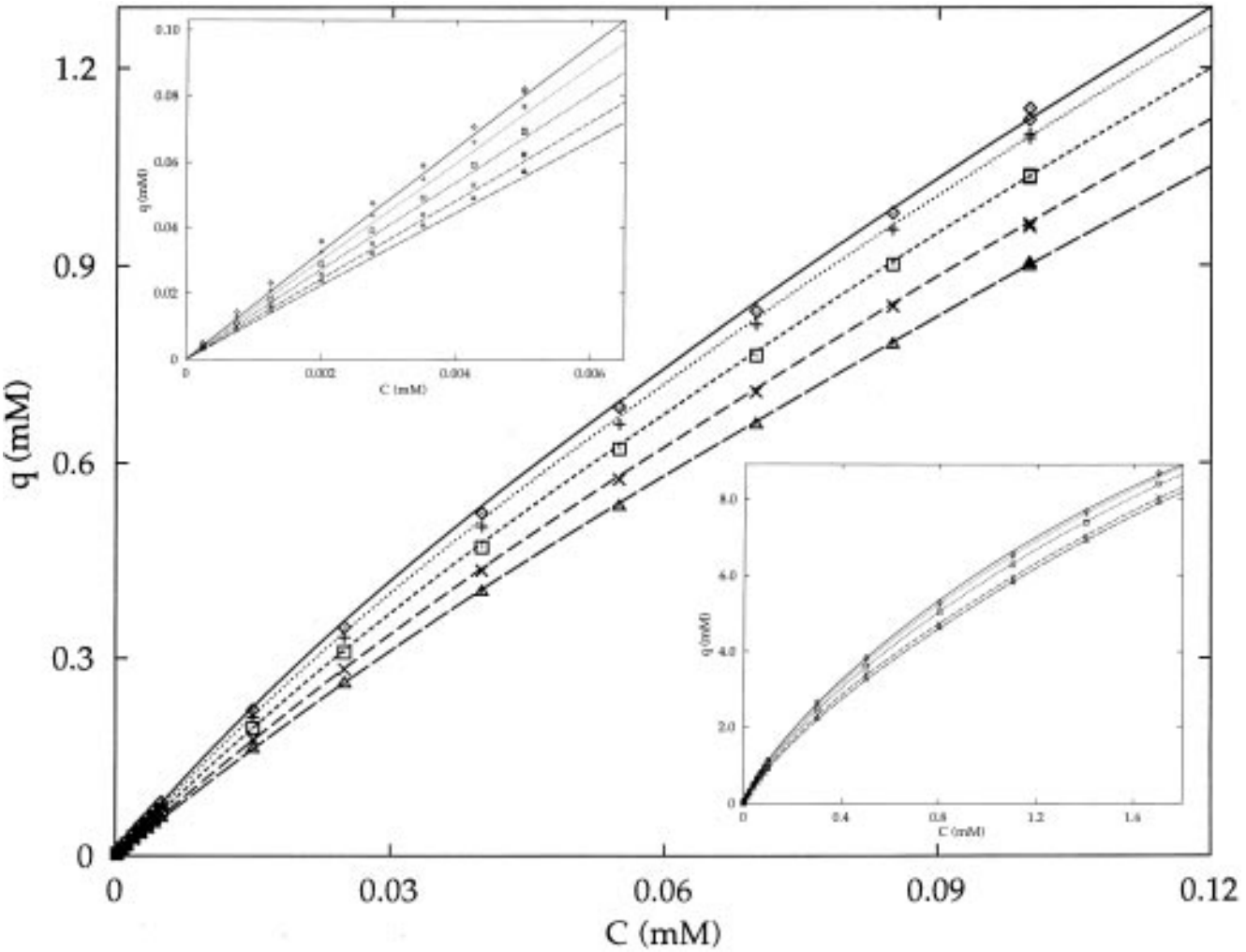

Figure 4. Single-component equilibrium isotherms for (*R*)-propranolol at increasing temperatures. Same experimental conditions as for Figure 1. Symbols, experimental data; lines, calculated data using the best biLangmuir isotherms (Table 1). The figure shows the medium concentration range where the highest mobile phase concentration is 0.1 mM. The inset in the upper left corner shows the low-concentration range (below 5.0 μM), and the inset at the lower right corner the high-concentration range (up to 1.7 mM). Temperatures in K: (solid line) 278.15; (dotted line) 288.15; (dashed line) 298.15; (long-dashed-line) 308.15; (extra-long dashed line) 318.15.

order as those of (*R*)-propranolol in Figure 4. The amount adsorbed at equilibrium decreases with increasing temperature. This is in apparent contradiction with the results obtained previously for the band profiles (Figure 1) and the van't Hoff plot (Figure 3), obtained under the same experimental conditions as the data in Figure 5. However, the picture provided by the curves in the left part of the main figure and in the upper left corner inset (low concentration range) is quite different. The initial slopes of the isotherms are in the opposite order, the top isotherm being the one acquired at the highest temperature (45 °C, extra-long dashed line) and the bottom isotherm being acquired at the lowest temperature studied (5 °C, solid line). This unexpected result is in agreement with the band profiles in Figure 1 and the van't Hoff plot of the retention factors in Figure 3. More careful examination of the main Figure 5 shows that there is a transition range (around 0.025 mM) in which a reversal of the order of the isotherms takes place. At lower concentrations, the isotherms are in an order corresponding to an endothermic enthalpy of adsorption, the amount adsorbed at equilibrium at constant mobile phase concentration increasing with increasing temperature. At higher concentrations, the isotherms are in the order corresponding to an exothermic enthalpy of adsorption, the amount adsorbed at equilibrium with a constant mobile phase concentration decreasing with increasing temperature.

The values of the isotherm coefficients a_i, b_i, and q_s of the isotherms of the two enantiomers on both types of sites (eq 3) are reported in Table 1 for each temperature. The *a* and *b* terms for the type-I sites are closely similar for the *R* and the *S* enantiomers. This is consistent with the assumptions of the model and validates our assumption that the type-I sites are the nonchiral adsorption sites. The column saturation capacities of these sites are nearly the same for the two enantiomers. There is a slight tendency for it to increase with increasing temperature, but this result is marginally significant, given the precision of the data. This suggests that there are no important changes in the conformation of the protein in the temperature range studied which would affect the nonchiral type-I sites. The *b* coefficient decreases, albeit moderately, with increasing temperature. On average, the adsorption equilibrium constant for the nonchiral interactions, *b*, decreases by 33% for the two enantiomers when the temperature increases from 5 to 45 °C.

The column saturation capacities q_s for (*R*)- and (*S*)-propranolol on the enantioselective type-II sites are close and nearly 30 times lower than the saturation capacity of the nonselective sites. These capacities depend little, if any, on the column temperature (Table 1). This indicates that there are no serious changes in the conformation of CBH I which could affect the saturation capacity of the enantioselective type-II sites. This result contrasts with the one obtained for the enantiomers

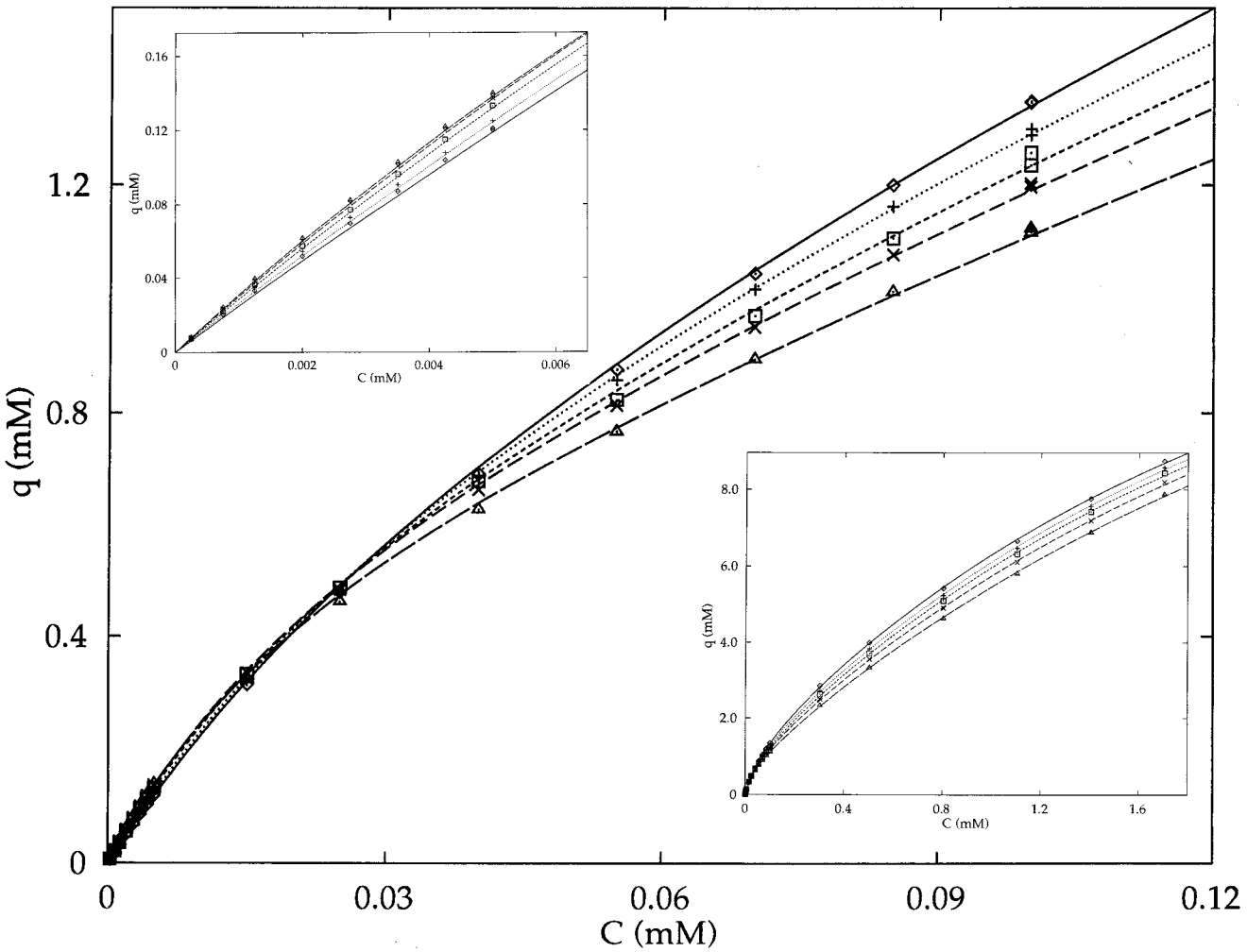

Figure 5. Single-component equilibrium isotherms for (*S*)-propranolol at increasing temperatures. All other conditions are as for Figure 4.

Table 1. BiLangmuir Isotherm Parameters

site	isomer	T (K)	a	RSD[a] (%)	b (mM^{-1})	RSD (%)	q_s (mM)
I	*R*	278.1	7.78	(3.4)	0.382	(7.0)	20.4
		288.1	7.19	(3.5)	0.330	(7.4)	21.8
		298.1	6.62	(3.1)	0.287	(6.7)	23.1
		308.1	5.59	(4.2)	0.210	(10.5)	26.6
		318.1	5.73	(3.2)	0.220	(7.9)	26.0
I	*S*	278.1	7.23	(2.3)	0.347	(5.5)	20.8
		288.1	6.95	(1.7)	0.320	(4.6)	21.7
		298.1	6.95	(1.7)	0.323	(4.8)	21.5
		308.1	6.57	(1.5)	0.297	(4.3)	22.1
		318.1	6.14	(1.4)	0.266	(4.7)	23.1
II	*R*	278.1	8.79	(3.8)	13.652	(14.1)	0.64
		288.1	8.08	(3.0)	10.295	(12.7)	0.78
		298.1	7.14	(2.3)	8.215	(10.2)	0.87
		308.1	6.68	(2.6)	5.979	(10.5)	1.12
		318.1	5.58	(2.4)	6.385	(10.1)	0.87
II	*S*	278.1	18.05	(2.0)	18.137	(5.9)	1.0
		288.1	19.89	(1.7)	22.286	(4.6)	0.89
		298.1	22.30	(2.1)	29.790	(4.9)	0.75
		308.1	24.30	(1.8)	33.970	(4.0)	0.72
		318.1	25.80	(2.1)	40.254	(4.2)	0.64
I[b]	*R, S*	298.1	4.79		0.209		22.9
II[b]	*R*	298.1	2.52		10.300		0.245
II	S	298.1	5.41		8.510		0.636

[a] RSD = relative standard deviation. [b] Previous results, same stationary phase at pH = 4.7, different column.[30]

of some aminoacid derivatives on BSA, a case in which there was a clear decrease of the saturation capacity on the chiral type-II sites with increasing temperature.[31]

The values of the *a* and *b* coefficients for the enantioselective type-II sites differ profoundly for the *R* and the *S* enantiomer. They vary in opposite directions with increasing temperature (Table 1). The *b* coefficient of the (*R*)-propranolol decreases approximately 2-fold when temperature increases from 5 to 45 °C while for the *S* enantiomer the *b* term increases approximately 2-fold. Thus, the phenomenon observed earlier and described in Figures 1 and 3 is to be ascribed to the opposite signs of the temperature dependence of the equilibrium constant of the *R* and *S* enantiomers with the enantioselective sites. In a similar study made with BSA as the selector for the separation of enantiomers of aminoacid derivatives, it was reported that the *b* terms of the nonselective and the two selective Langmuir contributions all decreased with increasing temperature.[31] Since the column saturation capacity, q_s, hardly varies with temperature in the present case, changes in the *a* term originate solely from changes in the *b* term. The relative importance of the temperature dependence of the *b* terms associated with the nonselective and the *R* selective Langmuir terms is comparable to that previously reported for BSA used as a chiral selector.[31] This explains the importance of careful temperature control for the obtention of reproducible results in chiral chromatography.

The equilibrium isotherms of the two propranolol enantiomers with CBH I at pH = 4.67 and ionic strength = 0.05 are also well accounted for by the biLangmuir model.[30] The values of the coefficients of the three Langmuir isotherm contributions to the global isotherms are reported at the bottom of Table 1. These values are close to those reported here at the same temperature (298.1 K), except for two values involving the

Table 2. Other biLangmuir Isotherm Parameters

ref	stationary phase	solute	isomer	b_1 (mM^{-1})	b_2 (mM^{-1})	$q_{s,1}$ (mM)	$q_{s,2}$ (mM)	a_1	a_2
this work	CBH I (25 °C)	propranolol	*R*	0.287	8.21	23.1	0.87	6.62	2.14
			S	0.323	29.8	21.5	0.75	6.95	22.3
31	resolvosil BSA	*N*-benzoylalanine	L	0.151	4.18	18.9	1.15	2.85	4.82
			D	0.163	7.74	17.4	1.32	2.83	10.2
41	Chiralcel OJ	methyl mandelate	D	0.0118	0.084	549	25.4	6.50	2.14
			L	0.0118	0.170	549	25.4	6.50	4.31
42	Chiralcel OJ	ketoprofen	*R*	0.0048	0.0514	598	85.3	2.89	4.40
			S	0.0048	0.0793	598	85.3	2.89	6.79

[a] True chiral selectivity, $a_{2,j}/a_{2,i}$.

Table 3. Enantioselectivity and Chiral Contribution to Retention

ref	phase	α_{es} (apparent)[a]	α (true)[b]	relative chiral contribution (%)[c] *R*	*S*
this work	protein (at 25 °C)	2.1	3.1	51.9	76.2
30	protein	1.4	2.1	34.5	53.0
31	protein	1.7	2.1	62.8	78.3
42	cellulose	1.25	2.0	24.7	39.9
43	cellulose	1.3	1.5	60.4	70.1

[a] $\alpha_{es} = (a_1 + a_{2,S})/(a_1 + a_{2,R})$, where a is the first coefficient of the Langmuir isotherm. [b] $\alpha = a_{2,S}/a_{2,R}$. [c] Relative chiral contribution to the retention factors of enantiomers: for the *R* enantiomer, $100a_{2,R}/(a_1 + a_{2,R})$; for the S enantiomer, $100a_{2,S}/(a_1 + a_{2,S})$.

enantioselective isotherms, b for the *S* isomer and q_s for the *R* isomer. They are both approximately 3.5 times smaller at the lower pH. This suggests that the retention mechanism of β-blockers (an especially of the *S* enantiomer) on immobilized CBH I is extremely sensitive to the pH. This explains why the exceptional temperature dependence of the retention factor of the S isomer is observed at pH = 5.5 (Figures 1 and 3) and not at pH = 4.7 (Figure 2). At the former pH, the contribution of adsorption on the enantioselective sites accounts for nearly three quarters (76.2%) of the retention factor while under the latter it accounts for about half (53%; cf., Table 3 and below).

The biLangmuir parameters obtained for *N*-benzoylalanine on BSA at 30 °C,[31] for methyl mandelate and for ketoprofen on a cellulose derivative (Chiralcel OJ) at 25 °C are given in Table 2.[41,42] This allows a comparison of the characteristics of the retention mechanisms involved. An important difference is in the mobile phases used, aqueous buffers in the case of the proteins, hexane modified by a low concentration of a polar solvent with Chiralcel OJ. The biLangmuir parameters obtained for both the nonchiral and the chiral parameters on BSA (Table 2) and on CBH I at pH 5.5 and 4.7 (Table 1) are comparable (ratios of the order of 1 to 2). In the case of methyl mandelate and ketoprofen adsorbed on Chiralcel OJ, the coefficients a_i remain of the same order of magnitude as those of the protein phases, but the coefficients b_i and $q_{s,i}$ differ considerably (Table 2). The saturation capacity of both types of sites on Chiralcel is 25−30 times larger for methyl mandelate, while the saturation capacity of the chiral sites for ketoprofen is nearly 80 times larger than on the immobilized proteins. The b terms are correspondingly much smaller, which was expected for the type-II sites. Table 3 compares the conventional or apparent (eq 4) and the true ($\alpha = a_{2,S}/a_{2,R}$) enantioselectivities for the different immobilized proteins and the cellulose derivative compared in Tables 1 and 2. The difference is important. It is related to the differences between the relative contribution of the enantioselective and the nonchiral retention mechanisms, as illustrated in Table 3. The proteins appear to be more selective than cellulose.

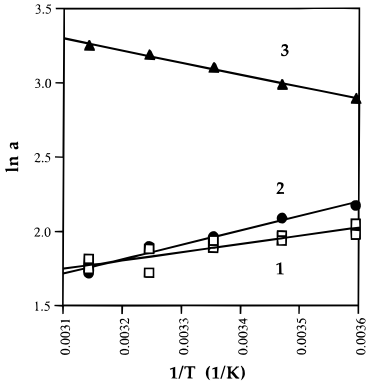

Figure 6. Van't Hoff plot for the determination of the enthalpy of adsorption. Symbols, experimental data; lines, best linear fit. Experimental conditions are those for Figure 1. (1) (*R*)- and (*S*)-Propranolol on the nonchiral type-I sites, (2) (*R*)-propranolol on the enantioselective type-II sites, and (3) (*S*)-propranolol on the type-II sites.

These results illustrate the differences mentioned above regarding the two kinds of chiral phases, those that have a high (e.g., cellulose derivatives) and a low (e.g., immobilized proteins) proportion of enantioselective groups. The difference in the density of these chiral groups in the two kind of phases is reflected by the difference in the monolayer capacities of their type-II sites. Their difference in interaction energy, reflected by the much higher values of b_2 for the immobilized proteins than for the cellulose derivatives, compensates for the lower site-II density, resulting even in a higher selectivity for the protein phases. This higher interaction energy, in turn, is explained by the location of the chiral sites, in a cavity or tunnel structure, in which molecular interactions are strong, steric constraints are high, and mass transfer are slow.[19−21,30]

For preparative purposes the saturation capacity of the chiral sites is important. Obviously, the much smaller monolayer capacity of the protein phases (on the average 67 times lower than that of the cellulose derivative) makes these phases poorly useful for preparative purposes. For analytical applications, however, a small chiral monolayer capacity is much less of a problem (except in trace analysis).

Thermodynamic Functions from Nonlinear Adsorption Data. Figure 6 shows a plot of the logarithm of the a coefficients (Table 1) versus the reverse of the absolute temperature (symbols). Lines 1, 2, and 3 correspond to the adsorption of both enantiomers on the nonselective type-I sites, of the *R* enantiomer on the enantioselective sites, and of the *S* enantiomer on these same sites, respectively. The standard molar enthalpy of adsorption, $\Delta H°$, was derived from the slope of the best straight lines (cf., eq 6) obtained by linear regression (solid lines). It is noteworthy that the set of data points in Figure 6 is consistent with a linear behavior, although the nonselective

(41) Charton, F.; Jacobson, S.; Guiochon, G. *J. Chromatogr.* **1993**, *630*, 21.
(42) Charton, F.; Bailly, M.; Guiochon, G. *J. Chromatogr., A.* **1994**, *687*, 13.

Table 4. Thermodynamic Adsorption Parameters Derived from Nonlinear Data

site	isomer	T (K)	$\Delta G°$ (kcal/mol)	$\Delta H°$ (kcal/mol)	$\Delta S°$ (cal/mol K)
1	*R*	278.1	−1.13	−1.10	+0.14
		288.1	−1.13	−1.10	+0.12
		298.1	−1.12	−1.10	+0.08
		308.1	−1.05	−1.10	−0.13
		318.1	−1.10	−1.10	+0.03
	S	278.1	−1.09	−1.10	−0.01
		288.1	-1.11	−1.10	+0.05
		298.1	−1.15	−1.10	+0.18
		308.1	−1.15	−1.10	+0.19
		318.1	−1.15	−1.10	+0.16
2	*R*	278.1	−1.20	−1.92	−2.60
		288.1	−1.20	−1.92	−2.52
		298.1	−1.16	−1.92	−2.55
		308.1	−1.16	−1.92	−2.47
		318.1	−1.09	−1.92	−2.63
2	*S*	278.1	−1.60	+1.61	+11.55
		288.1	−1.71	+1.61	+11.54
		298.1	−1.84	+1.61	+11.58
		308.1	−1.95	+1.61	+11.57
		318.1	−2.05	+1.61	+11.53

Table 5. Thermodynamic Adsorption Parameters Derived from Linear Data

pH	isomer	T (K)	$\Delta G°$l (kcal/mol)	$\Delta H°$l (kcal/mol)	$\Delta S°$l (cal/(molK))
4.7	*R*	278.1	−0.94	−3.16	−8.00
		288.1	−0.86	−3.16	−8.00
		298.1	−0.78	−3.16	−8.00
		308.1	−0.70	−3.16	−8.00
		318.1	−0.62	−3.16	−8.00
	S	278.1	−1.03	−1.31	−1.05
		288.1	−1.01	−1.31	−1.05
		298.1	−1.00	−1.31	−1.05
		308.1	−0.99	−1.31	−1.05
		318.1	−0.98	−1.31	−1.05
5.5	*R*	278.1	−1.31	−2.40	−3.90
		288.1	−1.27	−2.40	−3.90
		298.1	−1.23	−2.40	−3.90
		308.1	−1.20	−2.40	−3.90
		318.1	−1.16	−2.40	−3.90
	S	278.1	−1.52	+0.72	+8.04
		288.1	−1.60	+0.72	+8.04
		298.1	−1.68	+0.72	+8.04
		308.1	−1.76	+0.72	+8.04
		318.1	−1.84	+0.72	+8.04

sites include a broad variety of interactions and have a wide energy distribution, each one with its own temperature dependence. The adsorption enthalpy of each enantiomer on the nonselective sites is −1.10 kcal/mol, those of (*R*)- and (*S*)-propranolol on the enantioselective sites are −1.92 and + 1.61 kcal/mol, respectively (Table 4).

The Gibbs free energy, $\Delta G°$, and the entropy, $\Delta S°$, of adsorption of the two enantiomers on both types of sites were derived from eqs 5 and 8, respectively. They are reported in Table 4. The values of the Gibbs free energy obtained for both enantiomers on the nonselective type-I sites are very close to each other, -1.11 ± 0.03 and -1.13 ± 0.03 kcal/mol for the *R* and *S* enantiomers, respectively. This result was expected and validates the model. On the enantioselective sites, the Gibbs free energy for the *R* enantiomer is -1.16 ± 0.04 kcal/mol. For the *S* enantiomer, it decreases by 28% (to −2.05 kcal/mol) when temperature increases from 5 to 45 °C.

Like the adsorption enthalpy, the adsorption entropy is nearly independent of the temperature in all cases. The values obtained on the nonselective sites are very close, 0.05 and 0.11 cal/(mol K) for the *R* and the *S* enantiomers, respectively, in agreement with the assumption of the isotherm model. They are hardly different from 0. On the enantioselective sites, the adsorption entropy is -2.55 ± 0.06 cal/(mol K) for the *R* enantiomer and $+11.55 \pm 0.02$ cal/(mol K) for the *S* enantiomer. This high value of the adsorption entropy for the *S* enantiomer allows an endothermic retention mechanism to take place, the negative term $T\Delta S°$ outweighing the positive value of $\Delta H°$.

Thermodynamic Functions from Linear Adsorption Data. It is useful at this stage to illustrate and explain the errors arising from neglecting to account for the contribution of the underlying achiral retention mechanism. The thermodynamic functions were derived, as conventional in chromatography, from the lumped retention data acquired in the linear mode and shown in Figures 2 (pH = 4.7) and 3 (pH = 5.5), using eq 7 to evaluate $\Delta H°$ from the slope and $\Delta S°$ from the intercept of the plots of $\ln k'$ versus $^1/_T$. The $\Delta G°$ was then derived from the inversion of the conventional eq 8.

Comparison of the data in Table 4 and in the lower part of Table 5 (pH = 5.5) illustrates the magnitude of the error made. The $\Delta H°$ is −2.40 kcal/mol for the R enantiomer in Table 5 while for the same experimental conditions the adsorption enthalpies on type-I and type-II sites are −1.10 and −1.92 kcal/mol, respectively, an average value corresponding to the relative weight of the chiral and nonchiral contributions to retention (Table 3). For the *S* enantiomer, $\Delta H°$ derived from the linear data is +0.72 kcal/mol (Table 5), suggesting a slightly endothermic behavior. This value is a complex average (see Table 3) between the exothermic contribution of adsorption on type-I sites ($\Delta H° = -1.10$ kcal/mol) and the endothermic contribution of type-II sites ($\Delta H° = +1.61$ kcal/mol) in Table 4. Similar observations could be made for the adsorption enthalpies at pH = 4.7.

The errors made in the determination of the entropies are still larger, probably in part because of the inaccuracy of the estimate of the phase ratio in eq 7. For the R enantiomer at pH = 5.5, $\Delta S°$ is −3.90 cal/(mol K) in Table 5. By contrast, the values derived from the nonlinear adsorption data are +0.05 and −2.55 cal/(mol K), for the type-I and type-II sites, respectively. For the S enantiomer, $\Delta S°$ derived from the linear data is +8.04 cal/(mol K) (Table 5). The values derived from the nonlinear adsorption data were +0.11 and +11.55 cal/(mol K) on the type-I and type-II sites, respectively. One major conclusion of an analysis of the dependence of the retention factor on the temperature based on the use of chromatographic data acquired under linear conditions would be the loss of the peculiarity of the enantioselective mechanism, its negative adsorption energy and large adsorption entropy.

Enantioselective Retention Mechanism. The values obtained for the adsorption entropies of the two enantiomers studied suggest that the adsorption of (*S*)-propranolol on the chiral sites perturbs the ordered structure of the water molecules surrounding the solute and the protein molecules. The adsorption of the *S* enantiomer on type-I sites or of the *R* enantiomer on either type of site perturbs this structure much less strongly, if at all. Note that while the difference between the adsorption entropies of the two enantiomers is large, $\Delta(\Delta S°)$ is positive, the adsorption entropy of the *S* enantiomer being the larger of the two. Therefore, the situation is opposite to the one reported by Berthod *et al.*[46] and we cannot derive any conclusion regarding whether the *R* or the *S* enantiomer does not penetrate into the protein tunnel. If they do, they must shed several water molecules. The difference in their behaviors could explain why the mass transfer kinetics of the *S* enantiomer appears to be

much slower than that of the *R* isomer,[30] as shown by its broader, tailing peak (Figure 1).

There are two different sources of ordered water molecules around a protein molecule.[43,44] First, most proteins in aqueous solutions are surrounded by a tightly bound hydration layer in which water molecules are more highly ordered and less mobile than in the bulk water.[43] These water molecules are bound to the ionized or polar groups of the protein. This hydration layer contains about 0.3 g of water per 1 g of protein, which is equivalent to nearly two water molecules per amino acid residue.[44] This ordered layer is perturbed when a molecule of propranolol binds to a type-II site. It displaces several water molecules in the process. The perturbation is more important when two ionized carboxylic groups are involved as seems probable. A second source of ordered water molecules is in the regions of the solution involved in hydrophobic interactions. Close to nonpolar regions of any solute molecule, water molecules are more tightly held together and organized than in the bulk.[44] When a nonpolar part of a molecule dissolved in an aqueous solution comes into close interaction with a nonpolar region of the protein surface, the ordered water molecules adjacent to the two surfaces are expelled, and a less ordered water structure is obtained.[44] It seems likely that the large entropy increase associated with adsorption of the *S* enantiomer on the enantioselective sites of the protein is explained by a better fit of this molecule to the protein structure than that for the *R* enantiomer. The positive enthalpy could be explained by a change in the conformation of either the protein or the *S* enantiomer which would allow the expulsion of a larger number of water molecules.

Conclusions

The results reported in this work demonstrate that retention data acquired in the linear domain are insufficient to provide a good understanding of the chiral mechanism of separation observed in analytical chromatography. It is incorrect to derive thermodynamic parameters directly from retention data acquired under analytical conditions because the retention factor results from a mixed mechanism.[33] The Van't Hoff plots of the analytical retention factor do not give thermodynamic functions which can be ascribed to any single retention mechanism, nonchiral or chiral. Thermodynamic data must be acquired in the nonlinear region of the isotherm. They must be properly modeled. This allows the separate determination of the contributions of the chiral and nonchiral interactions to the retention factor.

The unusual temperature dependence of the retention factor of (*S*)-propranolol is allowed by a high entropy of interaction of this compound with the immobilized protein which compensates for a positive interaction enthalpy. At constant mobile phase concentration, the extent of adsorption on the chiral sites increases rapidly with increasing pH, while a pH change has little effect on the nonchiral adsorption in the range studied. At low pH (pH = 4.7), the chiral mechanism contributes moderately to retention and the consequences of the thermodynamic property of the chiral retention mechanism are not seen. At high pH (pH = 5.5), the chiral mechanism controls retention. At constant mobile phase concentration, the amount adsorbed increases with increasing temperature, until the chiral sites become saturated. It is expected that the temperature effect observed (Figures 1 and 3) would take place, albeit moderately, at pH between 4.9 and 5.3 and would become dramatic at any pH exceeding 5.3. Obviously, because the type-II sites are much fewer and have a higher adsorption energy than the type-I sites, they are overloaded at low concentrations. Then, the properties of the nonselective sites control the overall retention behavior and chiral separations become impossible.

The unusual thermodynamic characteristics of the enantioselective retention mechanism studied here are almost certainly due to a considerable decrease of the degree of organization of the water molecules around the protein chiral adsorption site (i.e., in its inner tunnel). No other phenomena could produce such a high molar interaction entropy. That the interaction enthalpy is positive suggests that the disorganization of the water molecules is associated with the weakening of their hydrogen bondings and, possibly, with some bending or torsion of several bonds in the propranolol and/or the protein molecule to adopt the conformation which frees the optimum number of water molecules. This could also explain the slow and heterogenous kinetics of retention previously demonstrated.[30]

Finally, our work shows that serious errors are made when calculating thermodynamic parameters from the retention factors of analytical-sized peaks if the adsorbent is heterogenous. A mixed retention mechanism then takes place and must be accounted for. Otherwise, the data reported are meaningless. The contributions of the different retention mechanisms can be separated properly in favorable circumstances. Because the interaction energies with the two types of sites are quite different, this is possible for chemically bonded phases. In other cases, (e.g., with cellulose microcrystalline triacetate), this was not possible.[45]

Acknowledgment. This work was supported in part by grant CHE-9201663 from the National Science Foundation and by the cooperative agreement between the University of Tennessee and the Oak Ridge National Laboratory. We acknowledge the support of Maureen S. Smith in solving our computational problems. We thank Roland Isaksson and Göran Pettersson (Uppsala University, BMC) for the generous gift of the stationary phase material and the chemicals used in the immobilization procedure and for useful discussions. T.F. is grateful for the financial support awarded to him by Astra Hässle AB (Mölndal, Sweden) and by the Swedish Academy of Pharmaceutical Sciences (The Göran Schill Memorial Foundation).

JA9631458

(43) Creighton, T. *Proteins: structures and molecular properties*; W. H. Freeman and Company: New York, 1993; Chapter 7.

(44) Lewin, S. *Displacement of water and its control of biochemical reactions*; Academic Press: London and New York, 1974.

(45) Seidel-Morgenstern, A.; Guiochon, G. *Chem. Eng. Sci.* **1993**, *48*, 2787.

(46) Berthod, A.; Li, W.; Armstrong, D. W. *Anal. Chem.* **1992**, *64*, 873.