Characterization of Solute Binding at Human Serum Albumin Site II and its Geometry Using a Biochromatographic Approach

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ABSTRACT Chiral recognition mechanism relationships for binding at site II on human serum albumin (HSA) were investigated using D, L dansyl amino acids. Sodium phosphate salt was used as a solute-HSA interaction modifier. A new model was developed using a biochromatographic approach to describe the variation in the transfer equilibrium constant with the salt concentration, i.e., the nature of the interactions. The solute binding was divided into two salt concentration ranges *c*. For the low *c* values, below 0.03 M, the nonstereoselective interactions constituted the preponderant contribution to the variation in the solute binding with the salt concentration. For the high *c* values, above 0.03 M, the solute binding was governed by the hydrophobic effect and the stereoselective interactions. The different contributions implied in the binding process provided an estimation of both the surface charge density (σ/F) and the surface area of the site II binding cavity accessible to solvent, which were found to be equal to around 10.10^{-7} mol/m^2 and 2 nm^2. As well, the excess of sodium ions excluded by the solute transfer from the surface area of the pocket were about -0.7 for dansyl norvaline and -0.8 for dansyl tryptophan.

INTRODUCTION

Human serum albumin (HSA) is the major soluble constituent of the circulatory system, implicated in colloid osmotic blood pressure and the transport of drugs and other small molecules (Fehske et al., 1981). This is a globular protein (molecular mass $\approx$ 66,000) consisting of a single chain of 585 amino acid residues, which is formed into subdomains by paired 17 disulfide bonds. Only a few specific binding sites are present on HSA (Muller et al., 1994). The most important are sites I and II, which are also called warfarin binding sites and benzodiazepine binding sites (Sudlow et al., 1975). He and Carter (1992) have determined the three-dimensional structure of HSA, which shows that these two binding sites are located in hydrophobic cavities in subdomains IIA and IIIA. Many ligands, such as fenbufen, diazepam, and piroxicam, were found to bind preferentially on the site II binding cavity (Sudlow et al., 1976; Bree et al., 1989). This cavity is accessed through an 8- to 10-Å diameter opening between two helicoidal structures (He and Carter, 1992; Wanwilmolruk et al., 1983). The distribution of hydrophobic and hydrophilic residues in the binding crevice is distinctly asymmetric. The principal nonpolar residues are sequestered into the hydrophobic cavity inside the protein core and the polar residues onto the surface (He and Carter, 1992). Many previous investigations of ligand binding to the HSA site II cavity have been reported in the literature. These studies have been based on a variety of experimental techniques including equilibrium dialysis, fluorescence, circular dichroism, crystallography, and biochromatography. Maruyama et al. (1993) have studied the mechanistic aspects of suprofen binding to site II using dialysis and spectroscopic techniques. Thermodynamic analysis and proton relaxation rate measurements have indicated that the hydrophobic side chain of suprofen was deeply inserted in the hydrophobic crevice, whereas the carboxyl group interacted with the cationic residue at the surface of HSA. Similar behavior was observed for the binding of caprofen (Kohita et al., 1994) or sulindac (Russeva et al., 1994) to HSA.

Affinity chromatography with protein immobilized on the support is especially suited for studying drug-protein interactions. A number of previous reports have examined the mechanisms of the compound binding on various protein stationary phases. Allenmark et al. (1984) described the molecular interactions that were implicated in the retention behavior of different solutes on immobilized bovine serum albumin (BSA). More recently, Schill et al. (1986) investigated the binding and stereoselectivity properties of the alpha1-glycoprotein (AGP) column. The thermodynamic processes involved in the binding and separation of warfarine enantiomers on the HSA column were characterized by Loun and Hage (1994) using frontal analysis. The stereochemical aspects of benzodiazepine binding to HSA were defined using a quantitative structure-enantioselective retention relationship (QSERR) (Kaliszan et al., 1992). Numerical simulations of the chromatographic process were applied by Vidal-Madjar et al. (1988) to determine the equilibrium isotherm of phenylbutazone with HSA immobilized on diol-silica. Recently, a review of the use of HSA in biochromatography to examine the solute-protein interactions was published by Hage and Tweed (1997).

In earlier reports, our group, using affinity chromatography, studied the binding of negatively charged test molecules, i.e., the dansyl amino acids on the immobilized HSA. It has been previously shown by Sudlow et al. (1975, 1976) that the L dansyl tryptophan and L dansyl norvaline molecules have a single high-affinity binding region on HSA that

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is known to be located at site II. Studies on the enthalpy-entropy compensation temperature have demonstrated that the D and L enantiomers of these two dansyl amino acids have the same binding location on HSA, i.e., site II (Peyrin et al., 1998b,c). The role of both the structural behavior of the site II binding crevice and the hydrophobic effect on the retention mechanism of solutes has been demonstrated using temperature studies and differential scanning calorimetry (Peyrin et al., 1997). More recently, in order to gain more insight into the complex problem area of the mechanism of retention and chiral recognition, a study of the surface tension effect of sucrose on the solute retention factor was carried out by varying the salting-out agent concentration in the mobile phase (Peyrin et al., 1998a). It was demonstrated that the compound retention decrease accompanying the sucrose concentration increase was governed by a restriction of the binding cavity surface area accessible to the salting-out agent due to the increased surface tension effects. By assuming the binding cavity to be a sphere and using a model that takes into account the curvature dependence of surface energy, we were able to attribute this behavior to a reduction in the curvature radius of the site II pocket (Peyrin et al., 1998a). A binding mode of dansyl amino acids on the HSA site II cavity was described in which the compound hydrophobic groups occupied the nonpolar interior of the cavity and the carboxylate and sulfonalimido groups interacted with the cationic and polar residues of the cavity rim, forming electrostatic and hydrogen bonds (Peyrin et al., 1998b,c,d).

To extend our investigation to the molecular aspects of solute-site II cavity binding, the influence of sodium hydrogen phosphate as an ionic strength modifier of bulk solvent on the interaction forces controlling the solute cavity association was investigated using affinity chromatography. This paper presents a new mathematical model based on the respective contributions of the long-range electrostatic and hydrophobic interactions and the short-range interactions for ligand-receptor association. An analysis of the experimental values of the transfer equilibrium constants for D, L dansyl amino acid enantiomers gave an evaluation of this general model of solute-HSA binding.

THEORETICAL CONSIDERATIONS

This theoretical approach considers that all D/L enantiomers bind only to their common single high-affinity region, i.e., site II (see above), without interacting with any nonspecific site on HSA or support material surfaces. In the zonal elution method used in this chromatographic study, the amount of solute injected into the column must be low enough to consider the retention time proportional to the original slope of the adsorption isotherm. In this case, the retention factor is a direct measure of the number-average association equilibrium constant (global binding constant) (Hage and Tweed, 1997). Only the protein high-affinity sites are generally involved (Sebille et al., 1990); Vidal-Madjar et al. (1988) also demonstrated that the global binding constant value measured by zonal elution is very close (difference $<4\%$) to the value of the high-affinity binding constant (characteristic of the specific interaction) determined using a multisite equilibrium model. As well, in a high performance liquid chromatography system, the nonspecific regions significantly contributing to the undesirable solute retention are those able to engage in strong energy interactions with the compound. These interactions are represented mainly by the electrostatic interactions between the analyte and the unreacted negatively charged silanol groups of the support material (Huang et al., 1996; Wirth et al., 1997). This silanophilic effect is of a great importance when the analyzed solute is positively charged (Marle et al., 1991; Thompson et al., 1995; Huang et al., 1996). However, in our system, all the dansyl amino acids ($2.36 < pKa1 < 2.38$ and $pKa2 = 4.56$) were negatively charged at the mobile phase, $pH = 6.0$, of the study. This implied that the contribution of the nonspecific interactions between the silica and the analyte to the total Gibbs free energy of the solute transfer had to be severely limited. Moreover, it has been previously observed that the nonspecific binding of the unprotonated species at the pH used in their studies, i.e., for warfarin (Tweed et al., 1997) or phenylbutazone (Vidal-Madjar et al., 1988) to the support silica in the HSA columns, is negligible. On the basis of these considerations, it is reasonable to consider that, by introducing a small amount of dansyl amino acid into the column to work in linear elution conditions (see Experimental Methods), the solute retention is the result of the interaction at the high affinity site on the HSA stationary phase. Two kinds of interactions, long- and short-range, are implied in the ligand receptor binding (Leckband et al., 1992; van Oss, 1996).

The interactions dependent on long-range forces consist principally of the hydrophobic effect and electrostatic interactions for two oppositely charged species (Leckband et al., 1992). Thus, the primary Gibbs free energy change of transfer of the solute from the bulk solvent to the HSA cavity could be broken down as follows:

$$\Delta G_I^\circ = \Delta G_{I,H}^\circ + \Delta G_{I,es}^\circ \quad (1)$$

where $\Delta G_{I,H}^\circ$ corresponds to Gibbs free energy change due to the hydrophobic effect and $\Delta G_{I,es}^\circ$ corresponds to the Gibbs free energy change due to the electrostatic interactions.

Hydrophobic interactions between solute and HSA

It has been known for several years that increasing the ionic strength of a bulk solvent increases its surface tension and the energy required for cavity formation (Janado et al., 1995). Thus, there is a loss of solvent entropy in the first hydration shell in the water structure and a reduction in the energy of solute medium-solvation interactions. The sodium ion reacts in the same way as classical osmotropic or salting-out agents, such as polyols or sugars (Back et al., 1979),

which are known to increase the hydrophobic interactions by enhancement of medium surface tension. In a biochromatographic system, if the addition of Na^+ disturbs the surface tension of the bulk solvent (mobile phase), then its concentration in the surface layer of HSA or solute must differ from its concentration in the medium. Considering n as the excess of ion for surface area accessible to the solvent of the part of binding cavity implied in the interaction process and using the Gibbs adsorption isotherm, it was assumed that the relationship between $\Delta G^{\circ}_{I,H}$ n, and the salt concentration c was for a constant surface with a radius curvature r (Peyrin et al., 1998a):

$$\left(\frac{\partial \Delta G^{\circ}_{I,H}}{\partial \ln c}\right)_T = 2n_r RT \tag{2}$$

R is the gas constant and T the absolute temperature. The integration of Eq. 2 gives:

$$(\Delta G^{\circ}_{I,H})_T = 2RT\int n_r \partial \ln c \tag{3}$$

Electrostatic interactions between solute and HSA

Usually, with an increase in salt concentration, the electrostatic interactions between the positively charged residue (Arg 410) and the carboxylate group of dansyl amino acids decrease, implying a restriction of the solute binding on HSA. The Gouy-Chapman theory (Bard and Faulkner, 1981) was applied to enable us to calculate the HSA cavity surface charge density and its dependence upon sodium phosphate salt concentration. The electrostatic contribution to the primary free energy of interaction $\Delta G^{\circ}_{I,es}$ is related to the surface potential φ_o, where z is the charge of the solute being adsorbed and F the Faraday constant:

$$\Delta G^{\circ}_{I,es} = z\mathrm{F}\varphi_o \tag{4}$$

The Gouy-Chapman theory relates the surface potential to the surface charge density σ which has units of charge per area:

$$\sigma = \sqrt{8RT\epsilon\epsilon_o I}\,\sinh(\Delta G^{\circ}_{I,es}/2RT) \tag{5}$$

This relation accounts for a mobile phase of dielectric constant ϵ and ionic strength I (ϵ_o is the permittivity of free space). The ionic strength I of the phosphate buffer-acetonitrile solvent mixture (Peyrin et al., 1999) is given by the well known equation:

$$I = \tfrac{1}{2}\, c_i z_i^2 \tag{6}$$

where z_i is the charge of species i of concentration c_i in the mixture. In our case, Eq. 6 can be rewritten as

$$I = 4c \tag{7}$$

where c is the concentration of sodium phosphate salt.

As $\sinh x \cong x$ under typical chromatographic conditions (Wirth et al., 1997), combining Eqs. 5 and 7 leads to:

$$(\Delta G^{\circ}_{I,es})_T = (2RT\sigma)/(32\ \mathrm{R}T\epsilon\epsilon_o)^{1/2}(c^{1/2}) \tag{8}$$

As the contributions of the free energy are additives, combining Eqs. 3 and 8 gives:

$$(\Delta G^{\circ}_{I})_T = 2RT\left[\int n_r^P \partial \ln c + (\sigma)/(32\ \mathrm{R}T\ \epsilon\epsilon_o)^{1/2}(c^{1/2})\right] \tag{9}$$

Following this first contact step, the solute engages strong specific short-range interactions with the cavity residues (Ross and Subramanian, 1981). These interactions are represented for the dansyl amino acid binding on the site II cavity by (1) van der Waals interactions between the solute apolar groups and the hydrophobic residues (Peyrin et al., 1998b) as the consequence of the intracavitary dehydration process of ligand receptor interface, called intracavitary hydrophobic interaction (van Oss, 1996); and (2) hydrogen bonding between the electron donor group of solute and electron acceptor residues of the cavity rim and/or steric repulsion for solute with large steric bulkiness, called intracavitary nonhydrophobic interaction (Peyrin et al., 1998c,d). The electrostatic interactions were also implied in the specific process in the cavity. It has been demonstrated that electrostatic and hydrophobic interactions were interconnected (van Oss, 1996). When two opposite charges present on solute and cavity residue are neutralized by ion-pairing association, then the dehydration process at the interface ligand-receptor is enhanced by an increase in their hydrophobic character. In a previous work, it was demonstrated that when coulombic interactions between dansyl amino acid and site II cavity diminished by increasing pH, the decrease in the solute affinity for the binding cavity was accompanied by an enhancement of the chiral discrimination (Peyrin et al., 1998b). The solute association process in the cavity interior decreased when the hydrophobic character of the ligand-receptor pair decreased with pH. Thus, the solute interacted more favorably with residues at the cavity rim through strong stereoselective H-bonding (or steric interactions) in relation to the compound. This fact would indicate that the intracavitary hydrophobic interaction is not the preponderant factor in the chiral recognition process and suggests that its variation is principally governed by the other interactions involved in the crevice. Several previous examples of chiral discrimination occurring through H-bonding or steric interactions between solute and chiral selectors have been reported in the literature (Allenmark, 1986; Kaliszan et al., 1992; Loun and Hage, 1994; Armstrong et al., 1994; Thompson et al., 1995).

Intracavitary Gibbs free energy changes between solute and HSA

These contributions are called ξ_{II} constants. The electrostatic forces act exclusively on the intracavitary process by

increasing the dehydration effect at the interface solute-cavity on the basis of the interconnection described below, noted es $\rightarrow$ H. Thus, two additive effects, $\xi_{II,es\rightarrow H}$ and $\xi_{II,H}$ for, respectively, the electrostatic and hydrophobic interactions, are implied in the Gibbs free energy of the intracavitary hydrophobic process:

$$\Delta G^{\circ}_{II,H} = RT[(\xi_{II,es\rightarrow H})/c^{1/2} + (\xi_{II,H})] \quad (10)$$

$\xi_{II,H}$ is expected to be independent of the salt concentration because of the large antipathy between the hydrophobic residues of the cavity interior and Na^+ ions (Dill, 1990). However, in Eq. 10, no allowance was made for the stereoselective interactions (noted $\xi_{II,X}$, X = D or L). It has been shown that when the water release phenomenon increases in the intracavitary process (for example, by increasing electrostatic interactions), the nonhydrophobic stereoselective contributions decrease inversely (Peyrin et al., 1998b). This interdependence between these two aspects of the binding process are represented by the stereoselective constants $\xi_{II,es\rightarrow H,X}$ and $\xi_{II,H,X}$ corresponding, respectively, to $\xi_{II,es\rightarrow H}$ and $\xi_{II,H}$. As $\xi_{II,es\rightarrow H}$ is inversely proportional to $c^{1/2}$ (Eq. 10), then its corresponding stereoselective contribution $\xi_{II,es\rightarrow H,X}$ is expected to be a function of $c^{1/2}$ because these two interactions behave in opposite directions. $\xi_{II,H,X}$ is considered to be independent of c. The Gibbs free energy of chiral recognition process is determined by the following equation:

$$\Delta G^{\circ}_{II,X} = RT[(\xi_{II,es\rightarrow H,X}c^{1/2}) + (\xi_{II,H,X})] \quad (11)$$

Combining Eqs. 10 and 11, the Gibbs free energy of the intracavitary process is obtained:

$$(\Delta G^{\circ}_{II,H,X})_T = RT[(\xi_{II,es\rightarrow H}/c^{1/2}) + (\xi_{II,es\rightarrow H,X}c^{1/2}) + (\xi_{II,H}) + (\xi_{II,H,X})] \quad (12)$$

The total Gibbs free energy change that occurs during the HSA-solute interaction process with Eqs. 12 and 9 is:

$$(\Delta G^{\circ}_{I,II,X})_T = RT\left[2\int n_r \partial \ln c + ((\xi_{I,es} + \xi_{II,es\rightarrow H})/(c^{1/2})) + (\xi_{II,es\rightarrow H,X}c^{1/2}) + (\xi_{II,H}) + (\xi_{II,H,X})\right] \quad (13)$$

where $\xi_{I,es}$ is equal to $(2\sigma)/(32\ RT\ \epsilon\epsilon_o)^{1/2}$ and represents the long-range contribution of electrostatic forces. It is known that the retention factor at temperature *T,* for the enantiomer X denoted $k'_{X,T}$, is related to the change in free energy $(\Delta G^{\circ}_{I,II,X})_T$ incurred during the transfer between the mobile and stationary phases. This relationship is expressed by (Guillaume and Guinchard, 1997):

$$\mathrm{Lnk}'_{X,T} = -(\Delta G^{\circ}_{I,II,X})_T/(RT) + \ln \phi \quad (14)$$

The equilibrium constant $K_{X,T}$ of the solute transfer from bulk solvent to cavity HSA is:

$$k' = \phi K \quad (15)$$

where ϕ represents the phase ratio (volume of the stationary phase divided by volume of the mobile phase). Substitution of Eq. 14 for Eq. 13 leads to:

$$\mathrm{Lnk}'_{X,T} = -\left[2\int n_r \partial \ln c + ((\xi_{I,es} + \xi_{II,es\rightarrow H})/(c^{1/2})) + (\xi_{II,es\rightarrow H,X}c^{1/2}) + (\xi_{II,H}) + (\xi_{II,H,X})\right] + \ln \phi \quad (16)$$

This equation links the variation of $\mathrm{lnk}'_{X,T}$, i.e., the variation of $K_{X,T}$, with c. It has a general shape corresponding to:

$$\mathrm{Lnk}'_{X,T} = (A/c^{1/2}) + (Bc^{1/2}) + (C\ln c) + D \quad (17)$$

with $(A/c^{1/2})$ and $(C\ln c)$ = nonstereoselective contributions, $(Bc^{1/2}$ = stereoselective contribution, and D = constant. As well, for X = D or L, the constant of equilibrium exchange process:

$$HSA_{II}D + L \leftrightarrow HSA_{II}L + D$$

was represented by:

$$\alpha = K_D/K_L \quad (18)$$

This constant is the reflection of the chiral recognition properties of the site II cavity for these compounds. Combining Eqs. 15, 16, and 18 gives:

$$\ln \alpha = [(\xi_{II,H,L} - \xi_{II,H,D})] + [(\xi_{II,es\rightarrow H,L} - \xi_{II,es\rightarrow H,D})c^{1/2}] \quad (19)$$

EXPERIMENTAL METHODS

Apparatus

The HPLC system consisted of a Merck Hitachi pump L7100 (Nogent-sur-Marne, France), an Interchim Rheodyne injection value model 7125 (Montluçon, France) fitted with a 20-μl sample loop and a Merck L 4500 diode array detector. The chiral column (150 $\times$ 4.6 mm), which consists of HSA bound to a 7-μm silica matrix, was supplied by Shandon HPLC (Cergy-Pontoise, France) and used at a controlled temperature of 25°C in an Interchim Crococil oven TM No. 701 (Montluçon, France). After each utilization, the column was stored at 4°C until further use. To study the effect of the flow rate on the retention factor, the retention time values of the dansyl amino acids and dead time marker were measured at 0.4, 0.6, 0.8, 1.0, 1.2, and 1.4 mL/min. The maximum relative difference of the retention factor of these compounds was never greater than 2%, meaning that the k′ values (corresponding to the equilibrium constants) were independent of the flow rate in this range. Thus, the flow rate was maintained constant equal to 1 mL/min throughout the study.

Solvents and samples

HPLC grade acetonitrile (Merck) was used without further purification. Sodium hydrogen phosphate and sodium dihydrogen phosphate were supplied by Prolabo (Paris, France). Water was obtained from an Elgastat option water purification system (Odil, Talant, France) fitted with a reverse osmosis cartridge. D, L dansyl norvaline and D, L dansyl tryptophan were obtained from Sigma Aldrich (Saint Quentin, France). Sodium nitrate (Merck) was used as a dead time marker. The mobile phase consisted of a sodium phosphate salt-acetonitrile (88/12 v/v) at pH 6.0 with salt concentrations varying from 0.001 to 0.1 M. To examine the concentration dependencies of solute retention corresponding to the binding capacity of the immobilized HSA, retention measurements were related to varying amounts of injected solute (20 μl with solute concentration varying from 2 to 50 mg/L). There was no significant change in retention factor values for any of the D/L solutes over this range. Thus, 20 μl of each solute or a mixture of these were injected at a concentration of 20 mg/L where the retention was sample concentration-independent (i.e., linear elution conditions).

RESULTS AND DISCUSSION

To obtain the coefficients of Eqs. 17 and 19, the k′ and α values for the D and L enantiomers were determined for a wide range of salt concentrations (0.001–0.1 M). Sixteen c values were included in this range. All the experiments were repeated three times. The coefficients of variation of the k′ and α values were less than 2.5%, indicating high repeatability and good stability for the chromatographic system. Using a weighted non-linear regression (WNLIN) (Bevington, 1969), the data were fitted to Eqs. 17 and 19. After the WNLIN procedure, the calculated parameters were used to estimate the k′ and α values at different salt concentrations with the measured values. The correlation between predicted and experimental k′ values and α values exhibited slopes equal to 1.02 and 1.03, respectively (ideal is 1.00) with $r^2 > 0.98$. This good correlation between the predicted and experimental values can be considered adequate to verify the theoretical model.

Retention behavior

All the dansyl amino acids exhibited similar variation for lnk′ with c. Fig. 2 represents the experimental curve obtained for the D dansyl tryptophan at $T = 25°C$. It was shown that the solute binding was minimal for a c value equal to around 0.03 M. Similar ionic strength effects were obtained for the retention of N-benzoyl amino acids on immobilized BSA (Allenmark et al., 1984) and for the L tryptophan binding on the HSA stationary phase (Yang et al., 1997).

For the low c values below 0.03 M

In this salt concentration area, when $c \rightarrow 0$, Eq. 17 was reduced to:

$$\mathrm{Lnk'_{X,T}} = (\mathrm{A}/c^{1/2}) + (\mathrm{Cln}c) + \mathrm{D} \qquad (20)$$

because the term $\mathrm{B}c^{1/2} \rightarrow 0$. The theoretical values of Lnk′ obtained from this equation were plotted against c for D dansyl tryptophan (Fig. 1). For the low values, the experimental curve became asymptotic to the theoretical curve re-created using Eq. 20. In this range, the ionic double layer was thick and weakly substantial with a high Debye length. Thus, the electrostatic interactions constituted the preponderant contribution to the variation in the solute binding with salt concentration. When c increased, the $(\mathrm{A}/c^{1/2})$ term decreased, governing a reduction in the solute transfer (Allenmark et al., 1984).

For the high c values above 0.03 M

When $c \rightarrow \infty$, the general equation (Eq. 17) was rewritten as follows

$$\mathrm{Lnk'_{X,T}} = (\mathrm{B}c^{1/2}) + (\mathrm{Cln}c) + \mathrm{D} \qquad (21)$$

because the term $\mathrm{A}/c^{1/2} \rightarrow 0$. The theoretical values of lnk′ corresponding to this equation were plotted against c (Fig.

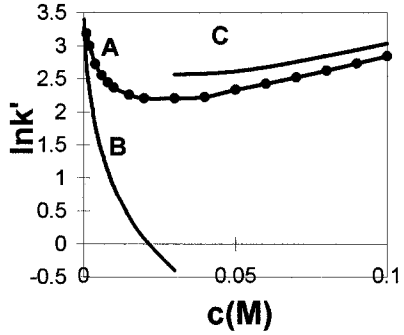

FIGURE 1 Experimental variations in the lnk′ value for D dansyl tryptophan in relation to the salt concentration (*A*) with the theoretical curves recreated from Eqs. 20 (*B*) and 21 (*C*).

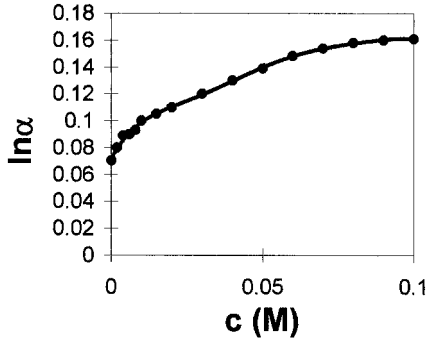

FIGURE 2 Experimental variations in the lnα value for dansyl norvaline in relation to the salt concentration.

1) for D dansyl tryptophan. For the high values, the experimental curve became asymptotic to the curve recreated from Eq. 21. In this area, the ionic layer double was thin and dense with a weak Debye length. Thus, the phosphate salt concentration increase was expected to weakly affect the electrostatic shielding, which was close to saturation. The dominant effects of the salt were on the solvent properties of the bulk solvent (Dill, 1990). The sodium ion was predicted to increase the hydrophobic effect by increasing the surface tension of bulk solvent (Janado et al., 1995). Thus, it can be said that the solute binding variation with c was governed by both the hydrophobic effect (Cln$c$) (Allenmark et al., 1984) and the stereoselective interactions related to the increase in the (B$c^{1/2}$) term. Therefore, the solute transfer was enhanced when c increased.

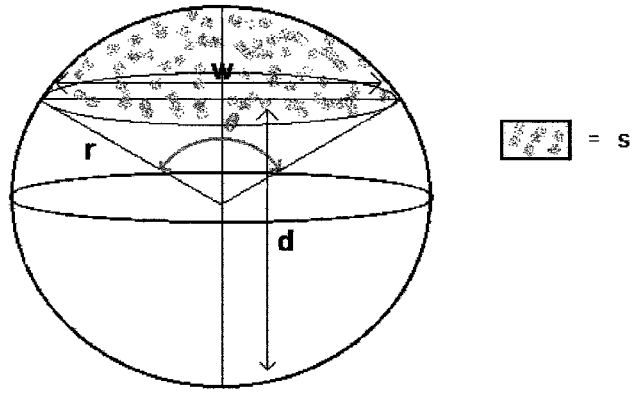

FIGURE 3 Schematic representation of the theoretical binding cavity with a depth of about 16 Å (d), width of 8 Å (w), and curvature radius of 8.5 Å (r).

Chiral recognition behavior

Fig. 2 represents the experimental variation in lnα with c for the D, L dansyl tryptophan enantiomers. The increase in the exchange equilibrium constant with a c increase was attributed to the increasing occurrence of stereoselective H-bonding between the electron donor group of the solute (sulfonalimido group) and the electron acceptor residue of the binding cavity represented by Tyr 411 or steric repulsion due to the size and bulkiness of the groups of dansyl amino acids.

Estimation of the binding features

From parameters A, the surface charge density σ can be calculated. The σ values were obviously found to be independent of the solute because the long-range electrostatic contribution to the Gibbs free energy of transfer was considered to be identical whatever the molecular structure or the configuration of the D/L solutes. The σ/F value, expressed in moL/m^2, was equal to 8.5×10^{-7}, the maximum variation obtained was 4.5%. It is known that site II is a hydrophobic cleft about 16 Å deep (d) and about 8 Å wide (w) (Wanwinolruk et al., 1983) with a radius curvature r of about 8.5 Å with the cationic group (Arg 410) located at the surface (Fig. 3). As a first approximation, it could be assumed that the theoretical spherical surface area accessible to the solute s was equal to $2\pi r(w/2)\Delta\theta$ where $\Delta\theta = 2\text{Arctg}[(w/2)(d - r)]$ was the excluded solid angle (Fig. 4). The corresponding theoretical σ/F value was equal to 7.9×10^{-7} moL/m^2. The difference between the theoretical and experimental values was less than 8%, showing the good reliability of the approximated model. The corresponding s value was found to be around 2 nm^2. This approaches the classical accessible surface area for a ligand receptor cited in the literature. For example, for the dextran-antidextran association (Kabat, 1976), the accessible surface area was assumed to be around 8 nm^2. As well, from the C coefficients of Eq. 16, the excess of sodium ion n_r was determined for the surface area accessible to solvent of the binding cavity implied in the hydrophobic process. Obviously, n_r was independent of the enantiomeric configuration and was equal to -0.7 for dansyl norvaline and -0.8 for dansyl tryptophan.

CONCLUSION

A general HSA-solute binding model was established to investigate the respective contributions of the interactions implied in both the solute transfer and the chiral recognition. The experimental values of transfer equilibrium constants obtained by varying the sodium phosphate salt concentration provided verification of the predictive theory and access to the structural features of the site II cavity. As well, the hydrophobic effect was quantified by determining the number of Na^+ ions excluded from the surface cavity in the course of solute transfer.

Based on these results, it can be noted that the hydrophobic and electrostatic contributions are preponderant in the retention of dansyl amino acids on immobilized HSA. This is in agreement with the findings reported by several authors who demonstrated the hydrophobic and electrostatic nature of the interactions between drugs with an acidic character and HSA (Maruyama et al., 1993; Deschamps-Labat et al., 1997). It can also be observed that the steric effect and hydrogen bonding govern chiral discrimination. Similar observations have been made for the binding of R and S warfarin to the site I of the HSA. Chattopadhyay et al. (1998) have demonstrated that the more the enantiomer (S warfarin) is retained, the more it interacts with the polar residues near the site surface, whereas the less the enantiomer (R warfarin) is retained, the stronger the hydrophobic interactions in the binding crevice.

The general relations between the different components of solute binding and the transfer equilibrium constant could be used to describe chromatographically other ligand-receptor associations found in biological media, such as substrate-enzyme or antigen-antibody.

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