Characterization of discrete classes of binding sites of human serum albumin by application of thermodynamic principles

Saïk URIEN,*†§ Phuc NGUYEN,* Sébastien BERLIOZ,* Francoise BRÉE,* Francis VACHEROT‡ and Jean-Paul TILLEMENT*

*Laboratoire de Pharmacologie, Faculté de Médecine, Université Paris XII, Paris, France, †INSERM, 94010 Créteil, Paris, France, and ‡Laboratoire CRRET, URA CNRS 1813, CJF INSERM No. 9014, Université Paris XII, Paris, France

The binding interactions of four ligands differing in acid–base properties with human serum albumin (HSA) were examined as a function of temperature. Binding to HSA decreased with increasing temperature for all four ligands. The bound and free ligand concentrations obtained at different temperatures were satisfactorily fitted to a model that incorporates the effect of temperature as an independent covariable and that directly allows the estimation of the enthalpic and entropic components of the ligand–albumin interaction, along with the precision of this estimation. Using this analysis, the binding of acidic ligands could be resolved into two classes of saturable sites, with the determination of the corresponding number of sites, whereas interpretation of binding data at each isolated temperature allowed only the determination of one saturable plus one non-saturable class of site. The thermodynamic constants indicate that binding of ionizable ligands to HSA involves electrostatic plus hydrophobic interactions, whereas only hydrophobic interactions are involved in binding to a second low-affinity class of site when present. Binding of non-ionizable ligands resembles that of the second class of low-affinity sites of ionizable ligands.

INTRODUCTION

Human serum albumin (HSA) is a major plasma protein. It interacts with a variety of endogenous and exogenous ligands, such as acidic drugs, neutral compounds and basic drugs, and a role has been recognized for it as a high-affinity carrier for most acidic drugs (Fehske et al., 1981). Ligand binding to HSA can occur by a saturable process, a non-saturable process or a combination of both. Generally, acidic drugs bind to albumin on two classes of saturable site, high-affinity and low-affinity; when the number of binding sites for the low-affinity process is high, the corresponding binding appears to be non-saturable, and the corresponding number of sites and association constant cannot be determined. Binding of basic and neutral compounds generally occurs on one class of site (Tillement et al., 1984).

We now report a detailed analysis of the effect of temperature on the binding to HSA of four ligands differing in acid–base properties. The enthalpic and entropic components of the interaction were directly estimated from binding data obtained at various temperatures for a given ligand. When the protein binding involved two classes of site, the thermodynamics of the reaction for each class was determined. When the second class of site was characterized as a non-saturable process at a given temperature, the information obtained from several temperatures allowed the number of sites and the association constant to be estimated. This analysis incorporating information from all the binding data allows the precision and significance of the parameters to be determined.

MATERIALS AND METHODS

HSA

HSA (Sigma A1887) was dissolved in Sörensen's phosphate buffer (KH_2PO_4, Na_2HPO_4, 0.066 M) and used without further modification in the binding experiments. The HSA preparation was 99% pure according to the manufacturer as checked by agarose electrophoresis. The purity was further confirmed by SDS/PAGE combined with reversed-phase h.p.l.c.

Ligands

Radiolabelled compounds (Figure 1) were obtained from the following manufacturers: 6-[^{14}C]benzoyl benzothiazolin-2-one (BBO; 2.04 GBq/mmol; 98% pure), from Amersham, Gif/Yvette, France; [^{14}C]amino-2-trifluoromethoxy-6-benzothiazole (riluzole; 2.07 GBq/mmol; 99.7% pure), from C.E.A., Gif/Yvette, France; [^{14}C]isopropyl-4-(2,1,3-benzoxadiazol-4-yl)-1,4-dihydro-5-methoxycarbonyl-2,6-dimethyl-3-pyridinecarboxylate (isradipine; 0.935 GBq/mmol; 98% pure), from Sandoz, Basel, Switzerland; 4-nitro-2-[^{14}C]phenoxymethanesulphonanilide (nimesulide; 0.66 GBq/mmol; 98% pure), from Amersham International, Amersham, Bucks., U.K.

Binding experiments

Equilibrium dialysis and measurement of radioactivity were conducted at pH 7.4 as previously described (Urien et al., 1982). Incubation temperatures were maintained using Thermomix circulators (Braun Scientelec). Drugs were used in the following ranges: BBO, 1–102 μM; isradipine, 0.8–32 μM; nimesulide, 0.6–94 μM; riluzole, 4.5–215 μM. HSA concentration was 10 μM (nimesulide, BBO), 40 μM (riluzole) or 75 μM (isradipine).

Data analysis

According to the law of mass action, $K_A = [PL]/[L][P]$, the protein-bound (B = [PL]) and free (F = [L]) ligand concentrations from equilibrium-dialysis experiments are related by the following relationship:

$$B = \sum_{i}^{j}\left(\frac{n_i K_{A_i} F}{1 + F K_{A_i}}\right) P_t \qquad (1)$$

where P_t is the total protein concentration, and n and K_A are

Abbreviations used: HSA, human serum albumin; BBO, benzoyl benzothiazolin-2-one.

§ To whom correspondence should be addressed.

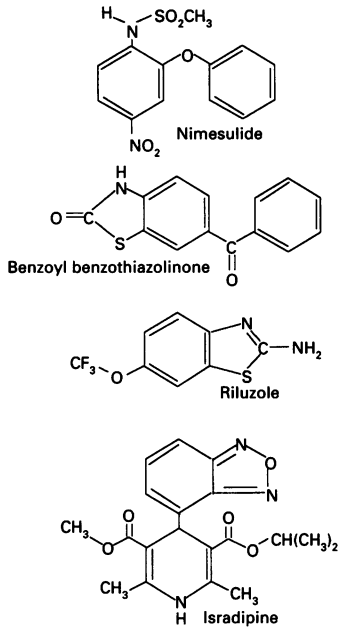

Figure 1 Chemical structures of the ligands

respectively the number of binding sites and association constant. The bound drug concentration is the sum of the 1st to the jth term if there are several (j) classes of binding site.

When the low-affinity binding appears to be non-saturable, eqn. (1) becomes:

$$B = \left(\frac{nK_AF}{1+FK_A}\right)P_t + nK'_AFP_t \quad (2)$$

where nK'_A denotes the total binding constant for the non-saturable process.

The equilibrium constant, K_A, is related to the equilibrium free-energy difference, ΔG^0, between the free and bound states as follows:

$$K_A = e^{-\Delta G^0/RT} \quad (3)$$

where R is the gas constant (8.319 J/mol per K) and T is the temperature in K. The enthalpy (ΔH^0) and entropy (ΔS^0) constants are related to ΔG^0:

$$\Delta G^0 = \Delta H^0 - T\Delta S^0 \quad (4)$$

Then, combining eqns. (3) and (4):

$$K_A = e^{-(\Delta H^0/RT - \Delta S^0/R)} \quad (5)$$

It follows that the observed association constant, K_A, can be expressed in terms of the enthalpy and entropy contributions. These thermodynamic parameters are usually determined from the slope and y intercept of the $\ln(K_A)$ versus $1/T$ plot (Van't Hoff or Ahrrenius plot). Here, these parameters were directly determined from the equilibrium-dialysis data, i.e. by combining eqn. (1) with eqn. (4) providing an integrated model:

$$B = \sum_i^j \left\{\frac{n_i[e^{-(\Delta H_i^0/RT - \Delta S_i^0/R)}]F}{1+Fe^{-(\Delta H_i^0/RT - \Delta S_i^0/R)}}\right\}P_t \quad (6)$$

with $j = 1$ or $j = 2$, one or two classes of binding site.

Then the binding data (F,B) obtained at different temperatures can be analysed together and described in terms of three unknowns, n, ΔH^0 and ΔS^0 for each class of binding site. These were estimated by a non-linear least-squares fit of at least 30 values of (F,B) at three different temperatures.

RESULTS

Table 1 summarizes the effect of temperature variations on the association constant of the binding of ligand to HSA. For all of the ligands there was a relationship between temperature and affinity, a decrease in the extent of binding being observed when temperature increased. In the case of acidic ligands, namely nimesulide (pK_a 6.5) and BBO (pK_a 7.8), two classes of site were observed, saturable and non-saturable (Figure 2). Binding of

Table 1 Binding constants (determination ± S.D.) of various ligands to HSA as a function of temperature

n, number of binding sites; K_A, the corresponding association constant; nK'_A, binding constant for the second class in a non-saturable binding process [see eqn. (2)].

Ligand	T (°C)	First binding class n	First binding class K_A (mM^{-1})	Second binding class nK'_A (mM^{-1})
Nimesulide	20	0.91 ± 0.15	487 ± 84	38.8 ± 7.6
	31	1.22 ± 0.09	246 ± 31.2	8.00 ± 2.60
	42	1.31 ± 0.02	136 ± 4	6.67 ± 0.4
BBO	20	2.26 ± 0.14	171 ± 18	23.2 ± 3.1
	31	2.57 ± 0.24	94.8 ± 11.6	16.6 ± 3.9
	42	2.01 ± 0.30	81.6 ± 14.4	24.7 ± 4.7
Riluzole	20	4.85 ± 0.69	11.5 ± 2.1	
	31	6.66 ± 0.52	5.47 ± 0.54	
	37	7.30 ± 0.79	3.82 ± 0.4	
	42	5.59 ± 0.46	4.31 ± 0.4	
Isradipine	20	1.00 ± 0.11	94.0 ± 13.3	
	31	2.31 ± 0.56	33.2 ± 8.9	
	42	1.96 ± 0.56	30.3 ± 9.7	

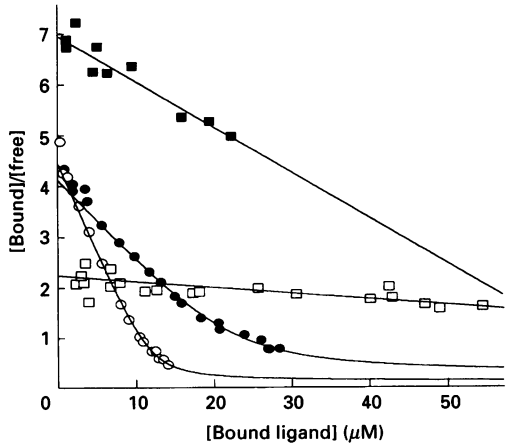

Figure 2 Scatchard plot of the binding of BBO (●), isradipine (■), nimesulide (○) and riluzole (□) at 20 °C and pH 7.4

Curves are drawn according to eqn. (2) using the values in Table 1. Note the curvature of the binding isotherms for BBO and nimesulide.

Table 2 Thermodynamic parameters (±S.D.) determined for ligand–albumin interactions

Ligand	Class of site	n	ΔH^0 (kJ/mol)	ΔS^0 (J/mol per K)	No. of observations
Nimesulide	1st	1.07±0.08	−32.36±2.03	−2.51±6.70*	44
	2nd	1.23±0.36	−7.39±2.91*	+54.01±9.63	
BBO	1st	1.32±0.32	−35.80±4.73	−18.76±15.91*	56
	2nd	3.22±0.25	−12.85±2.66	+41.45±8.16	
Riluzole		6.31±0.34	−29.41±0.46	−24.91±1.88	80
Isradipine		1.24±0.19	−11.35±1.05	+54.43±3.35	30

* Value not significantly different from zero.

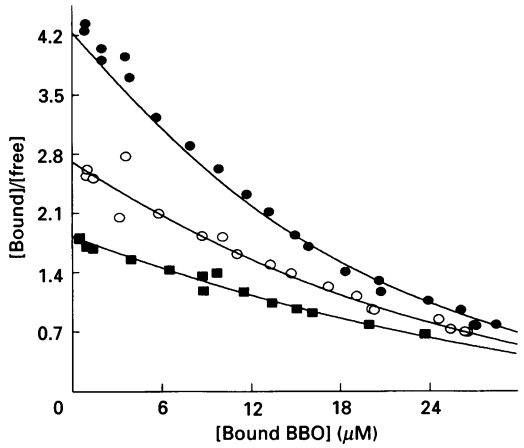

Figure 3 Scatchard plot of the binding of BBO to albumin at pH 7.4 and 42 (■), 31 (○) and 20 (●) °C

Curves are drawn according to eqn. (6) using the values in Table 2. Note the increasing curvature of the binding isotherms when temperature is lowered, showing the different behaviour of the two classes of site as a function of temperature. Albumin concentration was 10 μM in Sörensen's phosphate buffer.

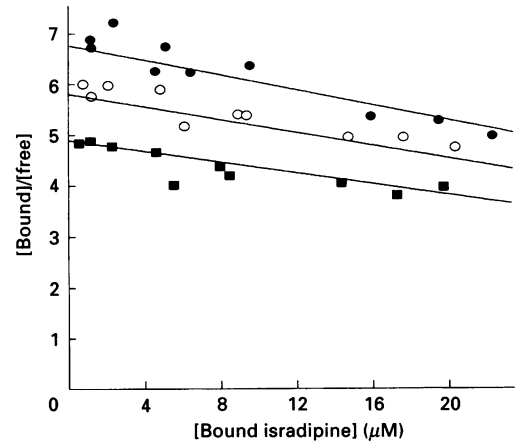

Figure 4 Scatchard plot of the binding of isradipine to albumin at pH 7.4 and 42 (■), 31 (○) and 20 (●) °C

Curves are drawn according to eqn. (6) using the values in Table 2. Note that the binding isotherms are satisfactorily fitted by straight lines, indicating the presence of only one class of site. Albumin concentration was 75 μM in Sörensen's phosphate buffer.

Table 3 Comparison of association constants (mM^{-1}) determined at 20, 31 and 42 °C ('observed') with those calculated using the thermodynamic constants obtained from the integrated model

The differences between observed and calculated values are not significant [analysis of variance, repeated measures, $F(1,18$ d.f.$) = 1.167$, $P = 0.29$, not significant].

Ligand	T (°C)	First binding class K_A Observed	First binding class K_A Calculated	Second binding class nK'_A Observed	Second binding class nK'_A Calculated
Nimesulide	20	487	412	38.8	16.9
	31	246	267	8.00	15.2
	42	136	171	6.67	13.6
BBO	20	171	250	23.2	27.9
	31	94.8	147	16.6	23.5
	42	81.6	90	24.7	19.7
Riluzole	20	11.5	8.95		
	31	5.47	5.79		
	37	3.82	4.63		
	42	4.31	3.86		
Isradipine	20	94.0	73.0		
	31	33.2	61.6		
	42	30.3	52.7		

riluzole (basic ligand, pK_a 3.6) and isradipine (neutral ligand) was to one class of site only (Figure 2).

When the binding data obtained at various temperatures were fitted to the integrated model [eqn. (6)], the number of binding sites and thermodynamic parameters were satisfactorily determined for each drug (Table 2). The n values were in the range 1.07–6.31, indicating that the stoichiometry of ligand binding to HSA was variable, depending on the ligand. For all of the ionizable ligands, a net enthalpy decrease with a small entropy decrease was observed on binding to the first class of site. For the neutral compound, isradipine, a rather small enthalpy decrease was associated with a net entropy increase. Similarly, the binding of acidic ligands to the low-affinity class of sites was also characterized by a small enthalpy decrease with a net entropy increase. To illustrate the validity of the integrated model, Figures 3 and 4 depict the curve fitting of binding data for BBO and isradipine respectively obtained at three temperatures using the thermodynamic parameters estimated in Table 2. Moreover, the K_A values calculated on the basis of the integrated model [using eqn. (3)] were compared with those estimated at each temperature (values given in Table 1). As shown in Table 3, the concordance is good and an analysis of variance for repeated

measures indicated that the differences between the two values were not significant.

DISCUSSION

The results of the present study demonstrate that standard thermodynamic relationships can serve to simultaneously analyse ligand–protein binding data obtained at various temperatures. In the case of the two acidic ligands examined, the usual analysis of data at each temperature could only identify a second non-saturable class of sites. The experimental points that relate to the second low-affinity class of sites correspond to very high concentrations of ligand, above the maximal concentration of protein-binding sites, and these experimental values are often difficult to obtain because of physicochemical constraints such as poor solubility of the ligand. Moreover, there is generally a certain degree of data dispersion at high ligand concentration and the points can only be fitted to a linear relationship and not a hyperbolic one. Thermodynamically oriented analysis of binding can discriminate between binding-site classes that react quite differently on a thermodynamic point. Both nimesulide and BBO bound to two distinct classes of binding site and, on HSA, because the thermodynamic parameters of binding at each site were quite different ($\Delta H \ll 0$ with $\Delta S \leqslant 0$ for the first class and $\Delta H \leqslant 0$ with $\Delta S \gg 0$ for the second class), analysis of their temperature-dependent binding to HSA could easily resolve the overall binding into two distinct saturable contributions (Figure 3). Binding of the basic and neutral ligands could not be resolved into more than one type, indicating, at least for riluzole, that all of the binding sites were equivalent from an energetic point of view.

Interestingly, we observed that the ionizable ligands, namely nimesulide, BBO and riluzole, bound to the main binding site with comparable enthalpy (-32 kJ/mol) and entropy (-2.5 to -24.9 J/mol per K) changes. This indicates that the binding is mainly enthalpy-driven and the forces involved are probably the same. The large decrease in enthalpy suggests that these forces are essentially electrostatic (hydrogen bonds, ion–ion, dipole–dipole). The decrease in entropy suggests an increased degree of orderliness on complexation, resulting from the bimolecular $2 \rightarrow 1$ reaction (Testa et al., 1987). The thermodynamics of hydrogen-bonding involve a ΔH of -36 to -38.5 kJ/mol and a ΔS of -92 to -100 J/mol per K (Blatz and Tompkins, 1992). On binding to the high-affinity sites of HSA, the ΔH variation is similar but the ΔS decrease is smaller, probably because solvent molecules are released into the bulk on complexation. The entropy change was not significant for the acidic ligands, nimesulide and BBO, suggesting a compensation between the increased order and the concomitant increase in disorder.

The binding of isradipine and the binding of the acidic ligands to their secondary sites resulted in similar thermodynamic events, a small or insignificant decrease in enthalpy and a net entropy increase, indicating that mainly hydrophobic interactions were involved with no or negligible electrostatic interactions (Testa et al., 1987). The net entropy increase ($\Delta S \gg 0$) mainly results from the disruption of ordered solvent cages around the hydrophobic surfaces of either the protein or the ligand on complexation (Smithrud and Diederich, 1990). Moreover, the energetics of these interactions, $\Delta H \leqslant 0$ and $\Delta S \gg 0$, are similar to those reported for the binding of a series of carbamate derivatives (non-ionized and lipophilic compounds): ΔH between -2.5 and -10.9 kJ/mol and ΔS between $+16.3$ and $+59.4$ J/mol per K (Brown et al., 1982).

In conclusion, the integrated analysis of binding data obtained at different temperatures allowed the discrimination of discrete classes of binding site on the protein molecule along with the estimation of the corresponding number of sites and thermodynamic contents.

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