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Optical Studies into the Nature of the High Affinity Binding Site of Human Serum Albumin for Phenylbutazone

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SUMMARY

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The binding of phenylbutazone to human serum albumin was investigated by UV difference-spectroscopy. From those measurements at two different wavelengths an association constant of 6.5×10^5 M^{-1} at 27.5° was calculated. The intrinsic tryptophan fluorescence of human serum albumin was quenched by binding of phenylbutazone. This quenching was not due to absorption or nonradiative energy transfer, but to a direct perturbation of the molecular environment of the fluorophore. Fluorescence measurements allowed us to calculate the binding parameters of the phenylbutazone-albumin interaction and to evaluate the thermodynamics of the binding. The association constant varied from 6.2 to 9.5×10^5 M^{-1} at temperatures from 18° to 45° corresponding with free energy changes from −7.7 to −8.7 kcal/mole. The reaction is endothermic (ΔH = +3.0 kcal/mole) and strongly entropy-driven (ΔS = +36.8 e.u.). This is characteristic for a hydrophobic interaction with solvent perturbation. Spectrophotometric and fluorescence measurements both suggested that upon binding, phenylbutazone forms a π-π complex with the tryptophan residue of the protein. This points toward the presence of that residue in the high affinity binding site. Yet the alternative hypothesis of a conformational change in the protein causing the optical changes could not be definitely excluded.

INTRODUCTION

Drug-albumin binding is a very important factor in the pharmacodynamics and the toxicology of a drug. It is of great interest to examine the physico-chemical aspects of the drug-albumin interaction in order to get information on the nature of the binding. The anti-inflammatory agent phenylbutazone is an acidic drug with high affinity for albumin. The binding of this compound has been investigated mainly by equilibrium dialysis (1-5). In these publications the authors agree on one binding site with high affinity for phenylbutazone. Chignell (3, 4) found two secondary sites while Wagner (5) reported eight weaker binding sites. In his optical studies of the phenylbutazone-albumin complex, Chignell (3) advanced several indications that the interaction had a hydrophobic character.

In our study, optical methods were used to gain better insight into the physico-chemical nature of the binding and the amino-acids involved. UV difference-spectroscopic measurements allowed us to cal-

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culate an approximate value of the association constant of phenylbutazone and pointed out the role of the tryptophan residue in the binding. It was further observed that the intrinsic fluorescence of the protein was quenched by binding of phenylbutazone. Fluorescence measurements were therefore used to investigate the binding characteristics, affording a more rapid and less tedious method than dialysis for the thermodynamic equilibrium studies of the drug-albumin interaction. The results allowed us to compute the values for the free energy, the enthalpy and the entropy changes of the reaction. These results confirm that phenylbutazone is bound to the protein in a hydrophobic environment. The observed optical changes strongly suggest that the tryptophan residue forms a π-π complex with phenylbutazone and is therefore an essential part of the binding site. However, we could not refute the alternative explanation that our observations are indirect effects due to a drug-induced conformational change.

MATERIALS AND METHODS

Materials. Human serum albumin (essentially fatty acid free, less than 0.005%; prepared from fraction V human albumin) was obtained from Sigma Chemical Company (n° A-1887). The concentration of HSA[2] solutions was checked spectrophotometrically at 280 nm using an extinction coefficient of $E^{1\%}_{1cm} = 5.3$. All solutions were made in sodium phosphate buffer (0.01 M, pH 7.4, containing 0.9% NaCl). The albumin solutions (20 ml) were dialysed for 12 hours against buffer solution (1L) and filtered through Millipore filter (pore size .22 μ) before use. Phenylbutazone was kindly provided by Drs. Keberle and Scheibli of Geigy Pharmaceuticals.

Titrations with difference spectrum measurements. Spectrophotometric measurements were performed with a Unicam SP 1750 ultraviolet recording spectrophotometer. A solution of phenylbutazone 500 μM was prepared in buffer containing HSA 15 μM. Aliquots (10, 20 and 100 μl) of this solution were added to 2 ml of a HSA solution of the same concentration. After each addition the absorption of the HSA-phenylbutazone mixture was measured at 285 nm and 265 nm. Blanks were made up in the same conditions without HSA. All measurements were made at 27.5° in a thermally regulated cell. Each titration was performed twice independently.

Titrations with fluorescence measurements. Fluorescence was measured with a JY-3 (Jobin-Yvon) spectrofluorometer equipped with a thermally-regulated cell. A solution of phenylbutazone 100 μM was prepared in buffer containing HSA, 1.5 μM. Aliquots (5, 10, 20 and 100 μl) of this solution were added to 2 ml of a HSA solution of the same concentration to obtain a molar drug to albumin ratio ranging from 0 to about 13. After each addition of phenylbutazone the protein fluorescence spectrum was recorded using an activation wavelength of 290 nm. The maximum fluorescence-values at 335 nm were noted. Bandwidths of excitation and emission were both 10 nm. The titrations were performed four times independently at different temperatures (18°, 28°, 37° and 45°).

RESULTS

Spectrophotometric measurements. The binding of phenylbutazone to HSA generates a difference spectrum with a maximum at 285 nm. The ΔOD was calculated as the difference between the measured OD and the sum of the OD of the free components in the same conditions. The addition of phenylbutazone to HSA leads to a progressive increase in ΔOD, reaching a maximum at saturation of the albumin (Fig. 1). All calculations were made assuming that only binding at the high affinity site, mentioned by several workers, was responsible for the observed optical changes. In order to calculate the fractions of bound and free ligand, absorption measurements were performed at the maxima of free phenylbutazone (265 nm) and the HSA-phenylbutazone difference spectrum (285 nm). In this way two equations with two unknowns, the molar concentrations of bound and free ligand, could be formed. The observed optical density (OD_{obs}) can be considered as the

[2] The abbreviation used is HSA, human serum albumin.

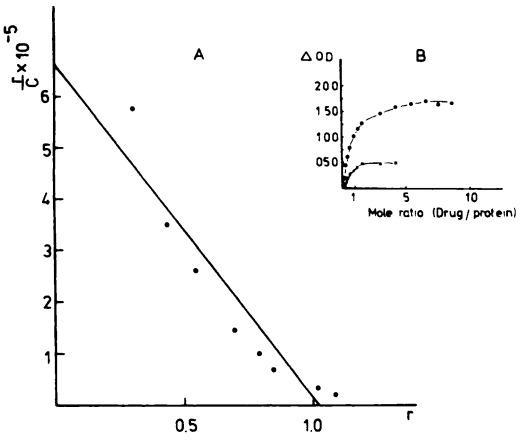

FIG. 1. *Scatchard plot for the binding of phenylbutazone to HSA measured by UV difference spectroscopy*

(A) r = number of moles phenylbutazone bound per mole albumin, C = molar concentration of free phenylbutazone. Calculation of the best fit yielded a correlation coefficient of 0.94. Binding measurements and calculations are described under MATERIALS AND METHODS and RESULTS, respectively. (B) Optical density differences at 285 and 265 nm generated when phenylbutazone is bound to HSA. The concentration of HSA was 15 μM. Phenylbutazone solution of 500 μM was added to obtain a molar drug to protein ratio of 0–9. Measurements were made at 27.5°. ● = 285 nm; × = 265 nm.

sum of the optical densities of the free albumin, the free ligand and the complex. This can be written in terms of molar extinction coefficients

$$OD_{obs} = ([HSA]_{tot} - [B]) \cdot \epsilon_{HSA} + [C] \cdot \epsilon_{Phe} + [B] \cdot \epsilon_{complex} \quad (1)$$

$$OD_{obs} - [HSA]_{tot} \cdot \epsilon_{HSA} = [C] \cdot \epsilon_{Phe} + [B](\epsilon_{complex} - \epsilon_{HSA}) \quad (2)$$

where [C] and [B] are the molar concentrations of free and bound ligand respectively, and $[HSA]_{tot}$ is the total molar concentration of HSA. To solve the equations, the molar extinction coefficients at 265 and 285 nm of all compounds must be known. The values for free HSA and ligand were directly obtained from their absorption spectra. The $\epsilon_{complex}$ could be calculated from the experimental data at complete binding of the ligand (first titration point) or at saturation of the HSA (last titration point; Fig. 1). At complete binding of the drug

$[Phe]_{tot}$ = [complex] and

$$OD_{obs} = ([HSA]_{tot} - [Phe]_{tot}) \cdot \epsilon_{HSA} + [Phe]_{tot} \cdot \epsilon_{com.} \quad (3)$$

When the albumin is saturated

$[HSA]_{tot}$ = [complex] and

$$OD_{obs} = ([Phe]_{tot} - [HSA]_{tot}) \cdot \epsilon_{Phe} + [HSA]_{tot} \cdot \epsilon_{com.} \quad (4)$$

where $[Phe]_{tot}$ is the total molar concentration of phenylbutazone. The molar extinction coefficients are assembled in Table 1. By substitution of these values in equation (2) for both wavelengths two equations were obtained from which [B] and [C] could be derived. Table 2 shows the calculated values of [B] and [C] together with their sum. Conformity of the calculated sum of free and bound ligand and the total quantity phenylbutazone added in the titration proves the validity of the calculations. The binding results were plotted according

TABLE 1

Molar extinction coefficients of HSA, phenylbutazone and the HSA-phenylbutazone complex at 265 and 285 nm

	ϵ_{265}	ϵ_{285}
HSA	26,900	29,800
Phe_{H_2O}	20,300	10,300
$Phe_{ethanol}$	23,100	15,800
Complex	49,600	49,300

TABLE 2

Bound and free phenylbutazone concentrations as calculated from the UV difference spectra

$[Phe]_{tot}$[a]	[B][b]	[C][c]	[B] + [C]
	(M × 10^6)		
2.49	2.64	−0.15	2.49
4.95	4.59	0.53	5.12
7.39	6.48	1.23	7.71
9.80	8.18	2.08	10.26
14.56	10.40	4.77	15.17
19.23	11.88	8.10	19.98
23.81	12.72	12.03	24.75
45.45	15.33	30.60	45.93
65.22	16.33	49.17	65.50

[a] Total molar concentration of phenylbutazone added.

[b] Molar concentration of bound phenylbutazone calculated from Eq. (2)

[c] Molar concentration of free phenylbutazone calculated from Eq. (2).

to the method of Scatchard (6) using the equation

$$\frac{r}{C} = nK - rK \qquad (5)$$

where r = the number of moles of ligand bound per mole protein, n = the number of binding sites, K = the association constant and C = the concentration of free ligand. From the results of these spectrophotometric measurements an association constant $K = 6.5 \pm 0.9 \times 10^5\ M^{-1}$ was calculated (Fig. 1).

Fluorescence measurements. With increasing phenylbutazone concentrations the intrinsic protein fluorescence progressively decreased. The successive spectra show a shift in emission maximum from 335 nm, the tryptophan maximum (7), to shorter wavelengths. This indicates that the quenching occurs at the higher wavelengths, thus involving the tryptophan residue.

Corrections for the quenching due to the inner filter effect were calculated according to Parker (8), using absorption values at 290 nm of 0.03 for 1.5 μM HSA and 0.6 for 10^{-4} M phenylbutazone. After this correction for self-absorption, the actual quenching of the fluorescence by the bound drug was obtained (Fig. 2). Quenching was defined as $Q = 1 - F/F_o$ where F_o and F are the corrected fluorescence values of the HSA-solutions and the HSA-phenylbutazone mixtures respectively. In further calculations the mean values of the different titrations at one temperature were used. At low molar phenylbutazone to albumin ratios, the fluorescence steadily dropped to a limit value indicating a saturation of the drug binding. The relative quenching increased with the temperature. Maximum quenching values were determined by extrapolating the mean corrected values according to Wallach (9). The plots of the reciprocal of the quenching versus the reciprocal of the drug concentration were linear (Fig. 3). The maximum quenching values at the four temperatures were not significantly different; therefore, the mean value of 62 ± 2% was used further. The fraction of bound phenylbutazone at each titration point was calculated using the formula

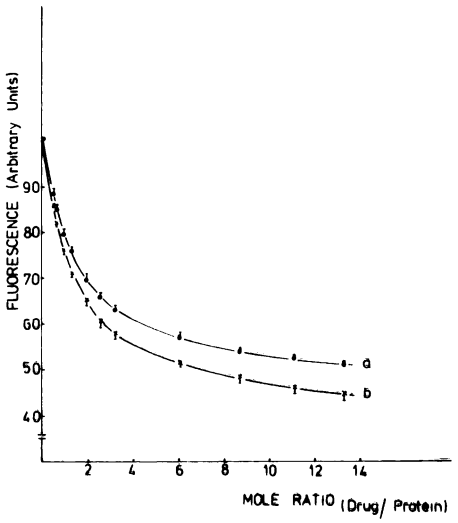

FIG. 2. *The quenching of the fluorescence of HSA by the binding of phenylbutazone at two different temperatures*

The concentration of HSA was 1.5 μM. Curves a and b represent mean corrected values at 18° and 37°, respectively. The activation and emission wavelengths were 290 and 335 nm. Bandwidths were 10 nm.

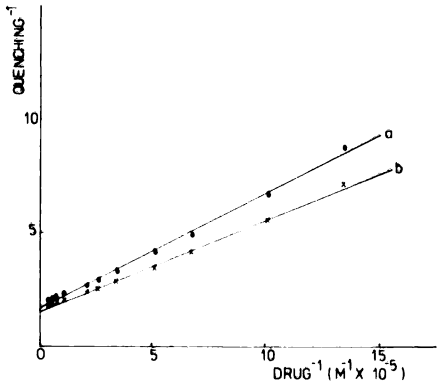

FIG. 3. *Plot of reciprocal of fluorescence quenching at 335 nm versus reciprocal of phenylbutazone concentration at 18° (a) and 37° (b)*

The values were corrected for self-absorption. HSA concentration was 1.5 μM. Activation and emission wavelengths were 290 nm and 335 nm. Bandwidths were 10 nm. The intercept at 1/drug concentration: 0 gives the reciprocal of the maximum quenching value for infinite ligand concentration. Correlation coefficient of the best fit = .996 at 18° and .997 at 37°.

$$[B] = \frac{Q}{Q_m} \times 1.5\ \mu M \qquad (6)$$

where [B] = bound phenylbutazone, Q = the quenching of the HSA-phenylbutazone mixture, Q_m = maximum quenching for the

1.5 μM HSA solution. These binding data were plotted according to Scatchard (6). For each temperature the Scatchard plot was linear with an intercept of about 0.9 at the r-axis which indicated the presence of one binding site (Fig. 4). Table 3 shows the binding parameters calculated from the Scatchard plots. The Hill coefficient at the different temperatures was calculated assuming the Hill plot was linear (Table 3). The plots (Fig. 4) denoted a Hill coefficient tending to 1.00 at low ligand concentrations. The top of the plot was slightly curved. This indicates that at low drug concentration, binding occurs to one independent site; at higher ligand concentration, secondary binding sites take part in the drug binding (10).

The association constant increased with the temperature. An enthalpy change ΔH of +3.0 ± 0.6 kcal/mole was determined from a Van 't Hoff plot with correlation coefficient 0.961. The binding of phenylbutazone to HSA is an endothermic reaction. The free energy change ΔG, calculated from

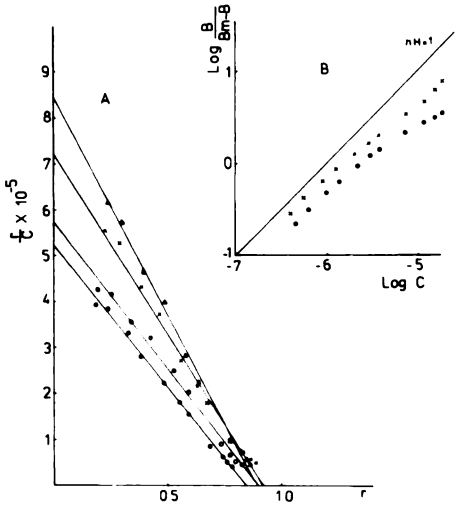

FIG. 4. *Scatchard plots (A) and Hill plots (B) for the binding of phenylbutazone to HSA*

Binding was measured by fluorescence titration at 18° (●), 28° (○), 37° (×) and 45° (▲). Correlation coefficients of 1.00, 0.99, 0.99 and 1.00, respectively, were calculated for the Scatchard plots. At the top the Hill plots clearly show a deviation from the theoretical plot with coefficient nH = 1. r = number of moles phenylbutazone bound per mole albumin. C = molar concentration of free phenylbutazone. B = molar concentration of bound phenylbutazone. B_m = maximum molar concentration of bound phenylbutazone.

TABLE 3

Binding parameters and free energy change for the binding of phenylbutazone to HSA at different temperatures

Temperature	Association constant K	Number of sites	Hill coefficient	Free energy change ΔG
	(M^{-1})			(*kcal/mole*)
18	$6.2 \pm 0.1\ 10^5$	.84	.728	−7.7
28	$6.5 \pm 0.2\ 10^5$	.89	.774	−8.0
37	$8.0 \pm 0.2\ 10^5$	.92	.826	−8.4
45	$9.5 \pm 0.2\ 10^5$	.89	.742	−8.7

the thermodynamic relationship ΔG = − RTlnK, is given in Table 3. The negative ΔG values indicate that the complex formation is favored notwithstanding the endothermy of the reaction. It means that the high positive entropy charge, ΔS = +36.8 e.u. calculated from the formula ΔS = ΔH − TΔS, is the driving force for the binding of phenylbutazone to HSA.

DISCUSSION

Upon binding to HSA the absorption of phenylbutazone shifts to a longer wavelength with an increase in intensity, leading to a difference spectrum with a maximum at 285 nm. Our findings are in agreement with those of Chignell (3). He suggested that the spectrum was generated by the movement of the phenylbutazone phenyl rings from an aqueous to a more hydrophobic environment as the spectrum is similar to the difference spectra of the drug dissolved in 95% ethanol or 0.05% cetrimide (3, 11). However, after quantitative comparison of the ΔOD at 285 nm of the phenylbutazone-HSA complex and phenylbutazone dissolved in ethanol (made alkaline with a trace amount of NaOH to convert all molecules into their enolic form) we found a greater molar ΔOD for the complex (Table 1). This suggests that the environment of phenylbutazone in the protein molecule is more hydrophobic than in ethanol or that possibly changes in the protein molecule, more specifically in the environment of the tryptophan residue, contribute to the difference spectrum.

From the experimental data the molar extinction coefficient of the ligand-albumin complex could be obtained. By measuring at two wavelengths, characteristic for the bound and free forms of phenylbutazone,

we were able to calculate the fractions of bound and free drug in the experiments and combine them in a Scatchard plot (Fig. 1).

The rather high standard deviation of the association constant calculated from the UV difference-spectroscopy is due to the low sensitivity of this method.

Since spectrophotometric measurements suggest a change in the tryptophan characteristics of the protein molecule, it was decided to investigate whether a binding study could be performed by fluorescence measurements. Human serum albumin contains only one tryptophan residue (12–14) that emits fluorescence with a maximum at 335 nm by excitation at 290 nm (7). This fluorescence is quenched by the binding of phenylbutazone. The fluorescence titrations were used to derive the binding parameters according to Scatchard. The results correspond with the values reported by other workers. Using equilibrium dialysis with radioactive ligand at 37°, Wagner (5) found one binding site of high affinity ($K_1 = 4.61 \times 10^5$ M^{-1}) and eight weaker binding sites ($K_2 = 1.61 \times 10^3$ M^{-1}). Chignell (4) reports one high affinity site ($K_1 = 1.0 \times 10^6$ M^{-1}) and two secondary sites ($K_2 = 3 \times 10^4$ M^{-1}). The binding site with strong affinity for phenylbutazone mentioned by both authors corresponds with the site responsible for the fluorescence quenching. The fact that defatted albumin was used may explain why the association constant is higher than the value reported by Wagner, as it is known that fatty acids compete with phenylbutazone for binding on human albumin (2). The presence of secondary sites is suggested by the aspect of the Hill plot, showing a slight curvature in the concentration range where binding at these sites may start.

The association constant obtained from the spectrophotometric measurements (at 27.5°) is in good agreement with the value calculated from fluorescence quenching at 28°.

The thermodynamic analysis indicates that the binding of phenylbutazone to the high affinity site of human albumin is endothermic and strongly entropy-driven. This is characteristic for a solvation disturbance at a hydrophobic site (15).

Warfarin, an anticoagulant of the coumarin type, is known to compete with phenylbutazone for the same binding site on human albumin (2, 16–18). Thermodynamic analysis of the warfarin-albumin interaction (19) revealed that the binding was based on hydrogen bonds (negative ΔH) and hydrophobic interactions (positive ΔS). So although both drugs bind at the same site, the physico-chemical interactions at the molecular level appear to be of a different nature.

Both optical measurements strongly suggest that upon binding to HSA, phenylbutazone affects the electron structure of the albumin tryptophan residue.

As the drug exhibits no absorbance at wavelengths above 310 nm, there is no appreciable overlap of the emission spectrum of the protein and the absorption band of phenylbutazone. Therefore it is not likely that the quenching phenomenon is due to nonradiative energy transfer (20), as was claimed for the fluorescence quenching of HSA by binding of the nitro-derivative of phenylbutazone (21). The increase in absorption at 290 nm (the activation wavelength) due to the drug-albumin complex formation is negligible for the concentration used in the experiments and cannot be the cause of the observed fluorescence decrement. The excitation spectrum of a HSA-phenylbutazone mixture shows no shift in wavelength compared to that of the free protein. This excludes the possibility that the bound drug prevents excitation of tryptophan by incident light. We finally concluded that the quenching had its origin at the source of the fluorescence emission namely in a direct perturbation of the fluorophore.

Together with the spectroscopic observations this suggests that upon binding to the protein, the phenylbutazone molecule comes in the direct proximity of the tryptophan residue. The ligand possibly influences the π-electrons of the indole ring: a π-π complex is formed between tryptophan and phenylbutazone (causing the observed optical changes) (22). This implies that the tryptophan is part of the binding site of phenylbutazone and that only binding at this site results in the observed optical changes.

Various authors already have suggested

that the single tryptophan residue in human albumin is part of the major binding site. In their reports Swaney and Klotz (23) and Lindup (24) cite several workers supporting this conclusion. In his review dealing with fatty acid binding to plasma albumin, Spector (25) points out the importance of hydrophobic interactions in the binding of large organic anions and the presence of tryptophan in the binding area.

An alternative explanation for the spectroscopic and fluorescence changes cannot be definitely excluded. Upon binding, phenylbutazone could induce a conformational change in the protein, causing quenching groups to move into the adjacent area of the tryptophan residue. The thermodynamic analysis strongly suggests the hydrophobic nature of the phenylbutazone-HSA complex which is in agreement with the presence of the hydrophobic tryptophan residue in the binding site.

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