pH Dependent Binding of Ligands to Serum Lipoproteins

Nicolas Simon,[1] Eric Dailly,[1] Pascale Jolliet,[1] Jean-Paul Tillement,[1] and Saïk Urien[1,2]

Received December 2, 1996; accepted January 30, 1997

Purpose. The binding interactions of binedaline, nicardipine and darodipine with lipoproteins (HDL, LDL, VLDL) were examined as a function of pH in order to evaluate the role of lipoprotein components and ligand protonation in the binding process.

Methods. Binding studies were performed by equilibrium dialysis with radiolabeled ligands and differential UV-visible spectroscopy.

Results. Deprotonated ligands had a markedly higher affinity for lipoproteins than the protonated forms, resulting in a concomitant decrease in the pK_a of bound ligands, i.e., a decrease in the basicity of the ligand in the bound state. The UV-visible difference spectra generated upon binding of auramine O to lipoproteins also showed that there was a contribution to the binding arising from the deprotonation of the ligand. Ligand binding was related to the phospholipid and cholesteryl ester content and to a lesser degree to the free cholesterol and protein content of lipoproteins, therefore to the surface monolayer components of lipoproteins. This relationship was even more accurate for the deprotonated, high-affinity, than for the protonated species.

Conclusions. It is suggested that among other possible interactions, ligand binding to lipoproteins involves proton exchange between the reactants and that the high affinity ligand species interact more specifically with the phospholipids of the lipoprotein surface monolayer.

KEY WORDS: plasma lipoproteins; protein binding; pH; ligand; drug transport.

INTRODUCTION

Lipoproteins (HDL, LDL, VLDL), the major carriers of lipids in blood, are also involved in the transport of various compounds, particularly basic and neutral drugs, including beta-blockers, antidepressants, neuroleptics and local anesthetics (1). The carrier function of lipoproteins has been found particularly interesting regarding some antioxidant compounds (alpha-tocopherol, probucol ...) that protect them from oxidation. Also, it has been suggested that the low density lipoprotein (LDL) receptor pathway is an important mechanism by which drug-lipoprotein complexes can concentrate in neoplastic cells. The delivery of anticancer drugs and photosensitizers by the LDL receptor pathway has been recently reviewed (2). Few studies, however, have investigated the molecular details of the lipoprotein-ligand interactions.

We now report a detailed analysis of the effect of pH on the binding of three basic ligands (binedaline pK_a 6.9, nicardipine pK_a 7.2, darodipine pK_a 11.4) to lipoproteins. The ligand-lipoprotein binding parameters were normalized according to specific lipoprotein components, cholesterol, phospholipids, apolipoprotein etc. to investigate the determinants of ligand-lipoprotein binding. The pH-induced binding variations were modelled by taking into account the protonated/deprotonated states of ligand or lipoprotein binding site. This approach allowed the determination of the ligand-lipoprotein association constants in protonated and deprotonated forms.

[1] Laboratoire de Pharmacologie, Faculté de Médecine, Université, Paris XII, France. INSERM, Paris, France.

[2] To whom correspondence should be addressed.

MATERIAL AND METHODS

Lipoproteins

They were prepared as previously described (3) then dissolved in Sörensen's phosphate buffer for use in the binding experiments. The molecular weights of HDL, LDL and VLDL were assumed to be 0.3×10^6, 3×10^6 and 10×10^6 daltons respectively. All values for the chemical composition of lipoproteins (protein, free and esterified cholesterol, phospholipids, triglycerides) were obtained from ref. 4.

Ligands

Radiolabeled or nonradiolabeled ligands were obtained from the following manufacturers: [^{14}C]-binedaline (Cassenne, 2.15 GBq/mmol, >96% pure), [^{14}C]-nicardipine (Sandoz, 0.935 GBq/mmol, 99% pure) and [^{14}C]-darodipine (Sandoz, 0.396 GBq/mmol, >98% pure).

Binding Experiments

Ligand binding was measured by equilibrium dialysis using a Dianorm apparatus. The cell chambers containing 200 µl each were separated by a Spectrapor 2 membrane. The ligand concentration ranges were 1 to 105 µM, 1 to 23 µM and 0.1 to 5 µM for binedaline, nicardipine and darodipine respectively. No isotopic dilution was used. Concentrated ligand solutions were made in ethanol and the final amount of ethanol in the dialysis system was always lower than 1%. Lipoprotein concentrations were 2 g/L. Measurement of radioactivity and differential UV-visible spectra were conducted as previously described (5,6). Sörensen's phosphate buffer was used for all experiments.

Data Analysis

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

$$B = \frac{n \cdot K \cdot F}{1 + F \cdot K} \cdot P_t \qquad (1)$$

where P_t is the total protein concentration, and n and K are the number of binding sites and association constant, respectively.

When the binding is apparently not saturable, the bound ligand as a function of the free ligand is a straight line and equation (1) reduces to

$$B = nK \cdot F \cdot Pt \qquad (2)$$

where nK is the binding constant (product of n by K).

When a ionizable ligand binds to a protein, and if the affinity depends on the ionization state of the ligand, K is an apparent association constant which relates protonated (LH^+)

0724-8741/97/0400-0527$12.50/0

and neutral (L) bound ligand concentrations to free ligand species concentrations:

$$K = \frac{[PLH^+] + [PL]}{[P] \cdot ([LH^+] + [L])} \quad (3)$$

The equilibria describing the binding of a basic ligand to a protein are shown in Fig. 1A and obey the following equations:

$$K_L = [PL]/[P][L] \quad (4)$$

$$K_{LH} = [PLH^+]/[P][LH^+] \quad (5)$$

Where K_L and K_{LH} are the association constant of deprotonated and protonated ligand, respectively.

$$K_F = [L][H^+]/[LH^+] \quad (6)$$

$$K_B = [PL][H^+]/[P \cdot LH^+] \quad (7)$$

with $pK_a(F) = -\log_{10}(K_F)$ and $pK_a(B) = -\log_{10}(K_B)$.

It follows that the observed association constant, K, can be expressed in terms of the binding constant of the protonated ligand, K_{LH}, the binding constant of the neutral ligand, K_L, and the ionization constant of the ligand in the free state, K_F:

$$K = \frac{K_{LH} \cdot ([H^+]/K_F) + K_L}{[H^+]/K_F + 1} \quad (8)$$

In addition, we have, for the ionization constant of the bound ligand:

$$K_B = \frac{K_L \cdot K_F}{K_{LH}} \quad (9)$$

If in contrast the affinity of the ligand-protein complex depends on the ionization state of the binding site but not of the ligand (Fig. 1B), then PH and P substitute to LH and L in equations (2) to (8), and K_F and K_B refer to the ionization constants of the binding site.

Practically, the pH values were chosen in order to involve significant changes in the apparent association constant, K, while remaining in values not too far from the physiological pH, 7.4. Also, the choice of pH was constrained by the solubility properties of the investigated ligands. By combining eq. 1 or 2 with eq. 8, the binding data obtained at different pH values can be analyzed together and described in terms of three unknowns, n, K_{LH} and K_L or two unknowns, nK_{LH} and nK_L in case of non-saturable binding. These were estimated by a nonlinear least-squares fit of at least 30 values of {F,B} at two or three different pH to the above equations with a commercially available software (MicroPharm^R, INSERM 1990).

RESULTS

At each pH value, the binding of binedaline to lipoproteins was slightly saturable, with high number of binding sites (Fig. 2). The n values were high relative to usual observations on other serum binding proteins, n = 12 ± 1 for HDL, n = 71 ± 15 for LDL and n = 632 ± 31 for VLDL, and the higher

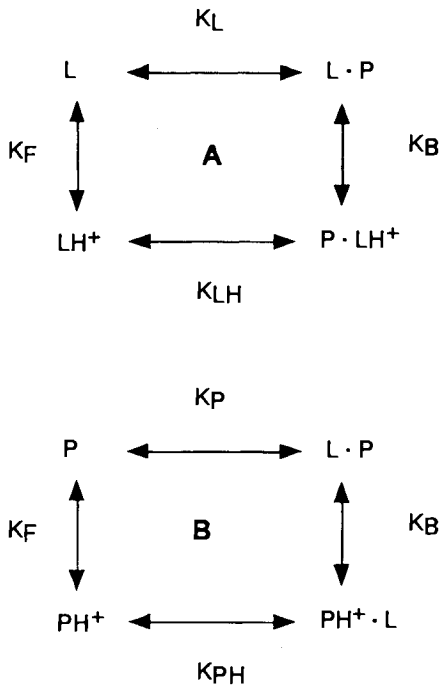

Fig. 1. A, equilibria describing the binding of deprotonated (L) and protonated (LH^+) ligand to lipoprotein (P). B, equilibria describing the binding of ligand (L) to deprotonated (P) and protonated (PH^+) lipoprotein sites.

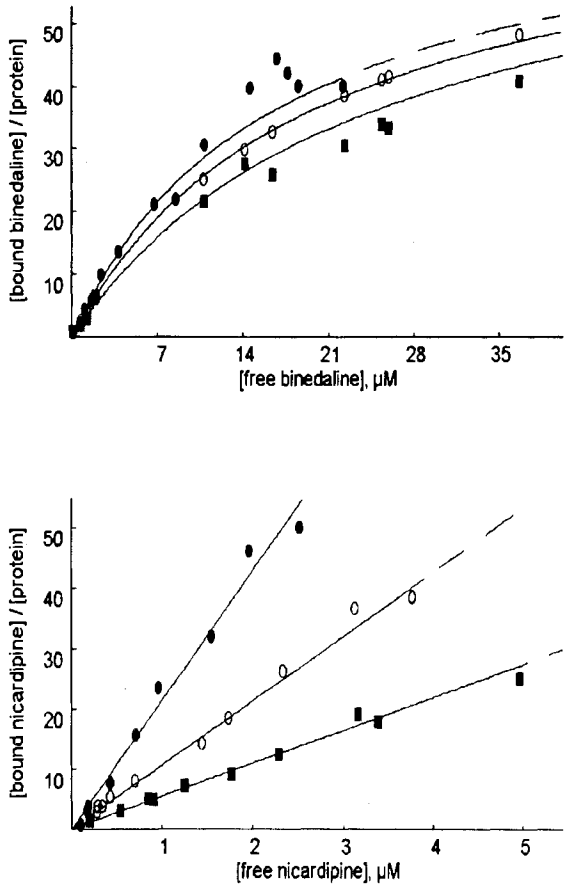

Fig. 2. Binding of binedaline at pH7.4 (●), pH7.0 (○), pH6.6 (■) and nicardipine at pH7.4 (●), pH6.7 (○) and pH6.3 (■) to low density lipoproteins. Curves are drawn according to equations 1 & 8 according to the values in table 2. Each point is the mean of two measurements.

Table 1. Binding Constants (Estimate ± Standard Deviation) of Ligands to Lipoproteins (HDL, LDL, VLDL) as a Function of pH

Ligand	pH	HDL nK (L/mmol)	LDL nK (L/mmol)	VLDL nK (L/mmol)
Binedaline	6.6	330 ± 60	2560 ± 62	6690 ± 1320
	7.0	409 ± 96	3140 ± 375	7460 ± 1420
	7.4	483 ± 165	36000 ± 590	10470 ± 2140
Nicardipine	6.3	452 ± 15	3960 ± 90	6010 ± 140
	6.7	941 ± 27	8080 ± 170	9650 ± 300
	7.4	1855 ± 186	15850 ± 445	23260 ± 300
Darodipine	6.3	700 ± 10	3370 ± 105	5510 ± 31
	7.4	705 ± 15	3580 ± 610	5790 ± 160
	7.8	740 ± 10	4040 ± 50	6060 ± 281

the nK value, the bigger the lipoprotein particle size (Table 1). The bindings of nicardipine and darodipine to lipoproteins were non-saturable in the studied range of ligand concentrations. Table 1 summarizes the effect of pH variations on the apparent binding constant of the studied ligand to lipoproteins (although n and K could be determined for binedaline, only the total binding constants were reported for comparison purpose). For binedaline and nicardipine there was a net increase in the apparent binding constant when the pH increased. By contrast, the effect of pH on darodipine-lipoprotein interactions was slight.

The binding data obtained at different pH were then analyzed together with the model that incorporates the effect of pH and assumes different values for the binding of deprotonated and protonated forms of the ligand (Fig. 1A). Binedaline and nicardipine data analyzed in this way are shown in Fig. 2. The results are shown in Table 2 and demonstrate that the binding constants of the deprotonated forms of these basic ligands were dramatically higher than those of the protonated forms ($nK_L >> nK_{LH}$). Moreover, there was a shift in the ionization constants of the bound ligands, the pK_a values of the bound ligands being about 0.8 (binedaline), 1 (nicardipine) or 2 (darodipine) unit lower than those of the free ligands. The apparent binding to lipoproteins of the practically unionizable ligand darodipine (pK_a of the free form = 11.4) was very slightly pH-dependent as shown in Table 1, providing additional evidence that the pH-induced change in the binding of the ligands was essentially due to different affinities of the two ionization states of binedaline and nicardipine.

An attempt to model darodipine binding as a function of ligand ionization (Fig. 1A) gave dramatically higher nK values for the deprotonated form (nK_L) than for the protonated form (nK_{LH}). When the ionization of the binding site was assumed to explain the lipoprotein-darodipine binding at different pH according to Fig. 1B, the curve-fitting gave binding constant estimates and the pK_a estimate of the unbound form of binding site(s) were 5.1–5.5 for the three lipoproteins (Table 3). There was a reverse shift in the pK_a of the binding site, the pK_a of the bound form was greater (2 units) than the pK_a of the unbound form.

To investigate the increase in nK values with the size of lipoprotein, the binding constants of ligand-lipoprotein complexes were also calculated assuming that only a specific component of lipoproteins could bind the ligand. For comparison purpose, the log(nK) values were calculated and for a given ligand, values were assumed to be similar when the difference between extreme values was less than 1 log unit. As shown in Table 4, there was no similarity between normalized nK values by triglycerides when either protonated or deprotonated ligands were considered. The nK values for the protonated ligands were reasonably similar when they were normalized by phospholipids, cholesterol ester and free cholesterol with maximum differences of 0.57, 0.65 and 0.70 respectively. For the deprotonated ligands, there was a fairly good similarity between the phospholipid and the cholesteryl ester nK values with maximum differences of 0.49 and 0.36 respectively. The degree of similarity between the free cholesterol and protein nK values was reasonable with maximum differences of 0.57 and 0.80 respectively. These results indicate that the surface components of lipoproteins, phospholipids and to a lesser degree free cholesterol, were involved in lipoprotein binding. The lipoprotein core should be excluded because there was no relationship between triglyceride lipoprotein content and nK values. However, the rather good correlation between cholesteryl ester lipoprotein content and nK values indicate that lipoprotein ligand binding should take place in the vicinity of this component, i.e., in the surface monolayer near the core.

For a compound that exhibits different absorption spectra in two ionization states, and whose pK_a changes upon binding to a protein, the difference spectrum generated upon binding

Table 2. Estimated Binding Constants (Estimate ± Standard Deviation, Expressed in mM^{-1}) of Lipoproteins (HDL, LDL, VLDL) for the Two States (Deprotonated,nK_L, and Protonated, nK_{LH}) of Binedaline, Nicardipine, and Darodipine

Ligand	Protein	pKa(F)	pKa(B)	nK(LH)	nK(L)
Binedaline	HDL	6.9	6.1	93 ± 22	362 ± 45
	LDL		6.0	675 ± 175	5740 ± 925
	VLDL		6.1	1770 ± 290	12960 ± 2080
Nicardipine	HDL	7.2	5.9	164 ± 61	3025 ± 200
	LDL		6.0	2145 ± 333	34326 ± 970
	VLDL		5.9	1840 ± 260	36370 ± 650
Darodipine	HDL	11.4	9.0	695 ± 10	181500 ± 60750
	LDL		8.7	3620 ± 80	1671520 ± 576300
	VLDL		8.8	5490 ± 170	2275810 ± 1058000

Note: pKa(F) and pKa(B) stand for the ionization constants of the unbound (F) and bound (B) ligand respectively.

Table 3. Estimated Binding Constants (Estimate ± Standard Deviation, Expressed in mM^{-1}) for the Interaction of Darodipine with the Deprotonated (nK_P) and Protonated (nK_{PH}) States of lipoprotein Binding Sites

Protein	pKa(F)	pKa(B)	nK(PH)	nK(P)
HDL	5.1	7.4	27 ± 7	742 ± 12
LDL	5.5	7.7	34 ± 16	4130 ± 1210
VLDL	5.2	8.1	74 ± 52	5900 ± 210

Note: pKa(F) and pKa(B) stand for the ionization constants of the unbound (F) and bound (B) lipoprotein site.

will contain a component arising from the change in the degree of protonation of the ligand. Figure 3 shows the difference spectra produced by auramine O binding to HDL, LDL and VLDL at pH7.4 (Fig. 3). This ligand was chosen for this dye presents a large-amplitude difference spectrum in the range 250–500 nm. On each plot the difference spectrum generated by the complexation was compared to the auramine O deprotonation spectrum (the difference spectrum between auramine O at pH4.4 and pH7.8, i.e., between deprotonated and protonated forms). Similarities are obvious between the difference spectra generated upon auramine O binding to lipoproteins and the deprotonation spectrum, particularly with VLDL. The band maxima of the binding difference spectra are red-shifted relative to the deprotonation spectrum. This indicates unambiguously that the deprotonation of auramine O contributes to the difference binding spectra. The difference spectra associated with auramine O binding to lipoproteins can be interpreted as being the sum of a deprotonation difference spectrum plus electronic changes in bound auramine O due to environmental changes.

DISCUSSION

The ligands examined here behaved similarly, their affinity to lipoproteins increased with increasing pH, i.e. with increasing proportion of deprotonated molecules in solution. This was also observed for the binding of basic ligands to $alpha_1$-acid glycoprotein (7). An increase in the total binding constant with the size and lipid content of lipoproteins was also observed.

As shown in Fig. 2, the binding data obtained at different pH values were efficiently fitted by the Fig. 1A model equations that ascribe different binding constants and acidity constants to the ligand in the bound and free state. The equilibrium model in Fig. 1A implies that K_B/K_F equals K_L/K_{LH} (eq. 9). In other words, the basicity of the bound ligand will be shifted to reflect the relative affinities of the deprotonated and protonated species. So, the higher affinity of the deprotonated species causes a proportional decrease in the basicity of the bound ligand, i.e. an increase in the proportion of high-affinity species. Moreover, this indicates that the variation of the association constants are not related to the structure or ionization properties of the ligands,

Table 4. The Deprotonated and Protonated Ligand Binding Constants to Lipoproteins Expressed as Log(nK_{LH}) and Log(nK_L) of Lipoprotein Components

		Binding constant of protonated ligand log[nK(LH)]				
	Lipoprotein fraction	Protein	Cholesterol ester	Free cholesterol	Phospholipid	Triglyceride
binedaline	HDL	1.97	0.29	0.76	0.01	0.84
	LDL	2.83	−0.22	0.45	0.01	0.31
	VLDL	3.25	0.18	0.44	−0.07	−0.49
nicardipine	HDL	2.21	0.53	1.01	0.26	1.08
	LDL	3.33	0.29	0.95	0.51	0.81
	VLDL	3.26	0.20	0.45	−0.06	−0.47
darodipine	HDL	2.84	1.16	1.63	0.89	1.71
	LDL	3.56	0.51	1.18	0.74	1.04
	VLDL	3.74	0.68	0.93	0.42	0.01
		Binding constant of deprotonated ligand log[nK(L)]				
	Lipoprotein fraction	Protein	Cholesterol ester	Free cholesterol	Phospholipid	Triglyceride
binedaline	HDL	0.47	0.88	1.35	0.60	1.43
	LDL	0.94	0.69	1.36	0.92	1.22
	VLDL	1.14	1.05	1.30	0.79	0.37
nicardipine	HDL	1.39	1.80	2.27	1.53	2.35
	LDL	1.74	1.49	2.16	1.72	2.02
	VLDL	1.59	1.50	1.75	1.24	0.82
darodipine	HDL	3.17	3.58	4.05	3.30	4.13
	LDL	3.42	3.18	3.84	3.40	3.70
	VLDL	3.38	3.29	3.54	3.04	2.62

Note: These constants were obtained as follows: (molar constant)/(molecular weight)/(lipid fraction). The log value of nK_{LH} or nK_L was used to facilitate comparisons. The chemical composition of lipoproteins was obtained from Shen *et al.* (1977).

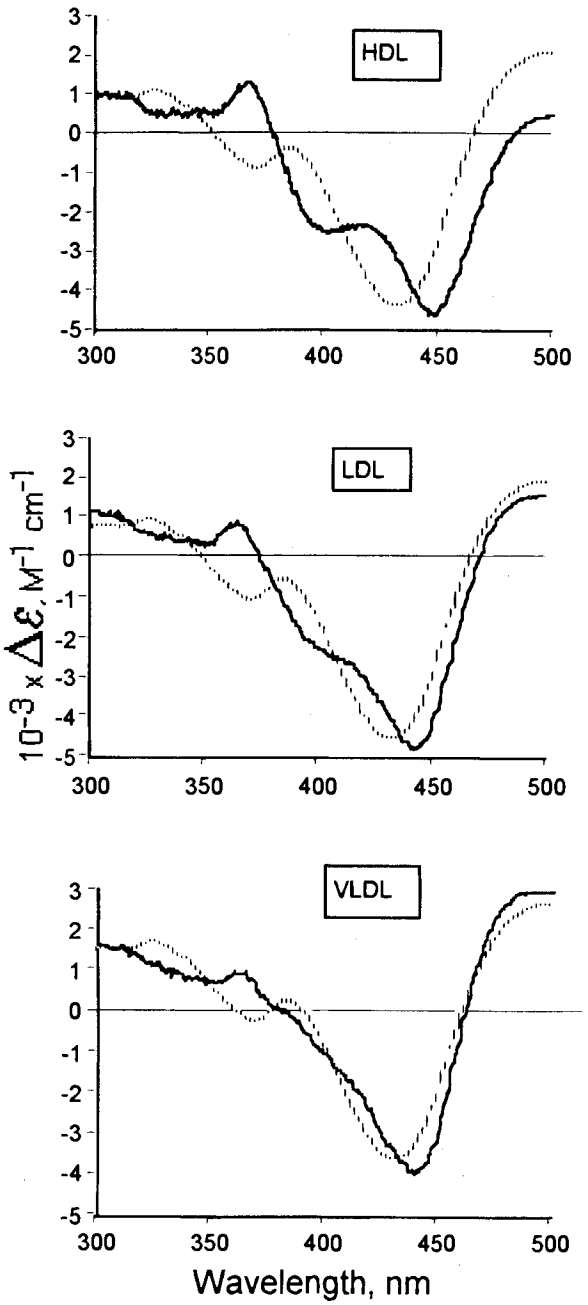

Fig. 3. Difference spectra generated upon auramine O binding to lipoproteins (black lines, HDL, LDL, VLDL) and deprotonation spectrum of free auramine O (dashed line). The difference spectra are obtained by using matched quartz split-compartment cells with each compartment of path length 0.4375 cm. Background, unmixed compartments, is run first, then sample, mixed compartments, is run. The deprotonation spectrum of auramine O is the difference between spectra at pH4.0 and pH7.8

but they depend on the pH domain investigated and on the difference $\{\log(K_L) - \log(K_{LH})\}$, i.e., the difference between the affinities of the deprotonated and protonated ligands.

Conversely, scheme B (Fig. 1) was applied to the reaction between darodipine and lipoproteins, an inverse pK_a shift between the free binding site and the occupied site was observed, indicating that the basicity of the binding site was increased upon binding. Interestingly, the shift was of the same magnitude (2 units or slightly more) when both schemes (Fig. 1A and 1B) were applied to darodipine, showing that the decrease in the basicity of the ligand was compensated by an increase in the basicity of the binding site. In other words, a proton was "exchanged" between the ligand and the binding site demonstrating that a mutually induced "pK fit" took place in the ligand-lipoprotein complexation. The general resemblance between the difference spectrum generated by deprotonation of auramine O and that observed when auramine O binds to lipoproteins is an additional support to this conclusion.

If the binding depends on a particular component, the normalisation of the nK parameters by the lipoprotein component should produce similar nK values for a ligand complexation with each lipoprotein fraction. This was observed with phospholipids, cholesteryl ester, free cholesterol and to a lesser degree with the protein fraction, indicating that the binding is expected to take place in the amphiphilic surface monolayer of lipoproteins (4). Thus, the ligand-lipoprotein complexation involves more complex mechanisms than a simple liposolubilisation. The degree of similarity was better between the normalized binding constants for the deprotonated ligands, high affinity species than for the protonated, low affinity, species. In other words, the binding of the high affinity species is more accurately related to specific lipoprotein components. This result reinforces the concept of a different binding, in which the deprotonated, high affinity, ligand can interact more specifically with lipoprotein components. Other studies advocate the location of various ligands in the lipoprotein surface monolayer, particularly in the vicinity of phospholipids, namely the dyes N,N-dipentadecylaminostyrylpyridinium (8), 5-(N-hexadecanoylamino)fluorescein (HAF) (9) and nile red (10).

Various models of phospholipids (dispersion, micelles, membrane models) have been shown to bind positively or negatively charged ligands, such as 1-anilino-8-naphthalene sulfonate (ANS) (11), nile red (10), mefloquine (12) and even peptides such as prothrombin (13), and one mechanism invoked was a close electrostatic interaction between the complementary electric charges of the phospholipids and the ligand. Our hypothesis that ligand binding involved the surface monolayer of lipoproteins, in which phospholipids and titratable residues of proteins are ionic at neutral pH, agrees well with the concept of a hydrogen bond formation between the ligand and some surface component of the lipoprotein, probably the phospholipids. Accordingly, the ligand must be able to exchange a proton with the surface component of the lipoprotein, i.e., its basicity should be reduced upon lipoprotein binding.

ACKNOWLEDGMENTS

We acknowledge financial support from the Ministère de l'Education Nationale (EA N°427) and the Réseau de Pharmacologie Clinique (Agence Française du Médicament.

REFERENCES

1. J. P. Tillement, G. Houin, R. Zini, S. Urien, E. Albengres, J. Barré, M. Lecomte, P. D'Athis, and B. Sébille. *Adv Drug Res* **13**:59–94 (1984).
2. J. C. Mazière, P. Morlière, and R. Santus. *J Photochem Photobiol Biol* **8**:351–360 (1991).

3. S. Glasson, R. Zini, and J. P. Tillement. *Biochem Pharmacol* **31**:831–835 (1982).
4. B. W. Shen, A. M. Scanu, and F. J. Kézdy. *Proc Natl Acad Sci USA* **74**:837–841 (1977).
5. S. Urien, E. Albengres, R. Zini, and J. P. Tillement. *Biochem Pharmacol* **31**:3687–89 (1982).
6. S. Urien, P. D'Athis, and J. P. Tillement. *Biochem Pharmacol.* **33**:2283–89 (1984).
7. S. Urien, F. Brèe, B. Testa, and J. P. Tillement. *Biochem J* **280**:277–280 (1991).
8. J. P. Corsetti, C. H. Weidner, J. Cianci, and C. E. Sparks. *Anal Biochem* **195**:122–128 (1991).
9. L. A. Sklar, M. C. Doody, A. M. Gotto Jr, and H. J. Pownall. *Biochemistry* **19**:1294–1301 (1980).
10. P. Greenspan and S. D. Fowler. *J Lipid Res* **26**:781–789 (1985).
11. J. Y. C. Ma, J. K. H. Ma, and K. C. Weber. *J Lipid Res* **26**:735–744 (1985).
12. R. Chelvi and C. D. Fitch. *Antimicrob Agents Chemother* **21**:581–586 (1982).
13. G. A. Cutsforth, R. N. Whitaker, J. Hermans, and B. R. Lentz. *Biochemistry* **28**:7453–7461 (1989).