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BINDING OF 5-METHOXYPSORALEN TO HUMAN SERUM LOW DENSITY LIPOPROTEINS

Teresa de Sa E Melo[÷§], Patrice Morlière[∿], Sonia Goldstein[×], René Santus[÷ω], Louis Dubertret[∿] and Dominique Lagrange[∞].

÷ Laboratoire de Physico-Chimie de l'Adaptation Biologique
Muséum National d'Histoire Naturelle
43 Rue Cuvier - 75231 PARIS CEDEX 05 (France)

∿ Hôpital Henri Mondor
Laboratoire de Recherche Dermatologique
94010 CRETEIL (France)

× Laboratoire de Biochimie B
Faculté de Médecine St Antoine
27 Rue Chaligny - 75012 PARIS (France)

∞ INSERM U.177 - Institut Biomédical des Cordeliers
15-21 Rue de l'Ecole de Médecine - 75006 PARIS (France).

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SUMMARY. 5-methoxypsoralen (5-MOP) binds to human serum low density lipoproteins (LDL) according to a two-step process. Scatchard analysis of the first step yields $K = 1.4 \times 10^5\ M^{-1}$ and 4 binding sites. It involves the LDL apoprotein. The second step corresponds to a solubilization, in the lipidic core, of $\simeq$ 45 molecules of 5MOP per LDL molecule. It is accompanied by a large blue shift of the 5MOP fluorescence. The ability of LDL to bind 5MOP and to carry it into various cells may explain some biological effects sometimes encountered during PUVA therapy.

The use of 5-methoxypsoralen as a photosensitizing agent for the oral photochemotherapy of psoriasis and in sun-tan preparations requires the full knowledge of its pharmacokinetics and metabolization processes. It has been established that serum albumin plays a major role in its transport through the whole body (1)(2). Thus, the extent of 5-MOP binding to serum albumin has been correlated with the hydrophobicity of the molecule (1). However, it has

§ Present address : Centro de Quimica Estrutural, Complexo I. Instituto Superior Technico, 1096 LISBOA CODEX - PORTUGAL.

ω:to whom correspondence should be addressed.

ABBREVIATIONS : 5-MOP : 5-methoxypsoralen, 8-MOP : 8-methoxypsoralen, L.D.L. : Low density lipoproteins.

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been also shown that 5-MOP and 8-MOP can be competitively displaced from albumin molecules by other drugs such as benzodiazepines (1). Moreover, the plasma levels of 8-MOP has been shown (3) to vary notably on food or drug ingestion and intrinsic hepatic clearance. The displacement of 5-MOP and 8-MOP from albumin suggests the possibility of the involvement of other blood proteins in the transport of psoralens The hydrophobicity of 5-MOP also suggests that it could be solubilized in the micelle-like lipoproteins present in the serum. The L.D.L. are of special interest because these cholesterol transporting proteins can be internalized into the cells via a receptor-mediated endocytosis (4). The eventual incorporation of 5-MOP into L.D.L. may be followed by their transport to the lysosomes of various cells such as endothelial and adreno-cortical cells, hepatocytes and lymphocytes. The incorporated 5-MOP may then be metabolized. It may also induce some photobiological effects in cells exposed to light during PUVA therapy (5). We show here that each L.D.L. molecule can incorporate about fifty 5-MOP molecules by a two-step process involving a conformational change of the L.D.L. apoprotein.

MATERIALS AND METHODS

Chemicals of analytical grade were used throughout this study. Bergapten and radioactive 5-MOP (^3H - labelled - Specific activity : 400 Ci $mole^{-1}$) were kindly supplied by "Laboratoire GOUPIL S.A.". The L.D.L. were isolated from human normolipidaemic serum by a sequential ultracentifugal flotation procedure previously described (6) and extensively dialysed at 4°C in a Spectrapor membrane tubing (molecular weight cut-off ≃ 3500) against a solution containing 0.02 % w/v NaN_3, 0.04 % EDTA, 5 mM Tris pH = 7.4 buffer and 50 mM NaCl. Before use, the L.D.L. solution was extensively dialyzed against 5 mM pH = 7.5 Tris buffer containing 50 mM NaCl. The protein concentration was determined according to Lowry's method (7). The L.D.L. concentration in the reacting solution was calculated knowing that L.D.L., whose molecular weight is ≃ 2,500 000 contains ≃ 25 % of protein, mainly apoprotein B. Absorption spectra were recorded with a Perkin Elmer Lambda 5 spectrophotometer. Fluorescence spectra were obtained with a Perkin Elmer LS 5 spectrofluorometer equipped with excitation spectrum correction in a 5 x 5 mm cell. Equilibrium dialyses were performed at 15°C with a Diachema Dianorm^R equipment. The solutions contained 0.2 µM L.D.L. and varying radioactive 5-MOP concentrations ($[5\text{-MOP}]_0$ = 0.15 to 19 µM) and were dialyzed during 48 hours against 5mM pH 7.5 Tris buffer and 50 mM NaCl. The concentration of bound ($[5\text{-MOP}]_b$) and free ($[5\text{-MOP}]_f$) 5-MOP were calculated from radioactivity counting data according to :

$$[5\text{-MOP}]_f = \frac{R_{5\text{-MOP}} \times [5\text{-MOP}]_0}{R_0} \quad \text{and} \quad [5\text{-MOP}]_b = \frac{(R_{L.D.L.} - R_{5\text{-MOP}})[5\text{-MOP}]_0}{R_0}$$

where $R_{L.D.L.}$ and $R_{5\text{-MOP}}$ were the radioactivity measured after dialysis in the compartments containing the macromolecule (at concentration $[L.D.L.]_0$) and the

small molecule respectively ; $[5\text{-MOP}]_0$ was the concentration of a reference solution of 5-MOP in ethanol giving a radioactivity counting R_0. Experimental data showed a slight loss (≃ 7%) of material due to 5-MOP binding to the dialysis membrane. Radioactivity counting was required because the very weak solubility of 5-MOP in aqueous media precludes difference absorption spectroscopy. This solubility limit was ≃ 2 x 10^{-5} M in Tris buffer containing 2.5 % ethanol as determined by both the radioactivity counting and the fluorescence technique. Microcrystals were eliminated by centrifugation (10,000 rpm during 5 min.) 12 hours after preparation of the solution.

RESULTS AND DISCUSSION

a) Fluorescence study of the binding of 5-MOP to L.D.L.

The fluorescence emission of 5-MOP in buffered aqueous solutions is a weak (fluorescence quantum yield ≃ 10^{-3}), broad and structureless band with a maximum at 500 nm. The fluorescence intensity is proportional to the 5-MOP concentration up to 20 µM which corresponds to the 5-MOP solubility limit in this medium (see experimental section).

The excitation of L.D.L. solutions with 290 nm radiations leads to a fluorescence emission centered at ≃ 334 nm and attributed to the fluorescence of Trp residues (8)(9). The L.D.L. contains 13 ± 2 Trp residues. Almost all the fluorescent residues are located in the same environment near the lipid phase (9)(10), 15 Å below the lipid-water interface (8). Addition of 5-MOP to the L.D.L. solution induces a quenching of the L.D.L. fluorescence (Fig. 1a). The fluo-

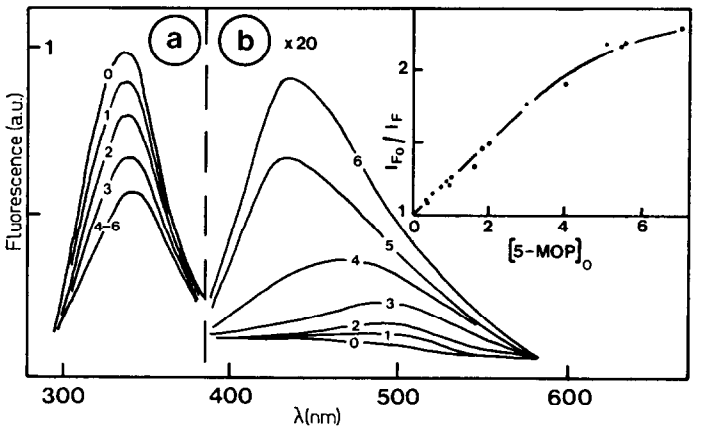

Fig. 1. Fluorescence of 0.2 µM L.D.L. solutions containing various concentrations of 5-MOP : 0(0), 4.5 µM (1), 9 µM (2), 18 µM (3), 36 µM (4), 54 µM (5) and 72 µM (6). a) fluorescence of Trp residues, λ_{exc} = 290 nm. b) fluorescence of 5-MOP, λ_{exc} = 330 nm.
Insert : Stern-Volmer plot of the L.D.L. fluorescence as a function of the total 5-MOP concentration, λ_{exc} = 290 nm, λ_{em} = 335 nm.

rescence quenching does not depend on the excitation wavelength in the 265-295 nm range which, at least, suggests that Tyr residues are negligibly involved in the overall energy transfer process from L.D.L. to 5-MOP. In addition, there is no indication in the literature of extensive Forster-type singlet-singlet energy transfer from Tyr to Trp residues in L.D.L. (8). Such a transfer occurs sometimes in proteins containing both Trp and Tyr residues (11).

The fluorescence quenching follows a STERN-VOLMER law (insert of Fig. 1) at 5-MOP concentrations smaller than the one corresponding to the solubility limit. At higher 5-MOP concentrations, one reaches a plateau while the fluorescence maximum of the Trp residues undergoes a 3.5 nm red-shift. On the basis of published data (9) the presence of two classes of Trp residues leading to distinct fluorescence emission properties and different accessibility to bound 5-MOP molecules seems likely. At the same time, one observes a corresponding blue shift (50 nm) (see Fig. 1B) of the 5-MOP fluorescence emission upon excitation with 330 nm radiations which are only absorbed by the psoralen derivative. The blue shifted emission is not reminiscent of the 5-MOP fluorescence in hydroxylic solvents such as ethanol (fluorescence maximum at $\simeq$ 470 nm). It may be due to interactions of 5-MOP molecules with the hydrocarbon-like chain of the fatty acids in the lipidic core.

The good overlap between the L.D.L. fluorescence spectrum (λ_{max} = 335 nm) and the absorption spectrum of 5-MOP (λ_{max} = 313 nm) should allow efficient Forster-type energy transfer from L.D.L. to 5-MOP. However the decay of the fluorescence of the Trp residues of L.D.L. is pratically unaffected (12) even at the highest 5-MOP concentration used (26 µM). Since, at the same time 50 % of the fluorescence is quenched, one must admit that the fluorescence quenching is not dynamic in nature (13). It is tempting to propose that the strong and sudden blue shift in the fluorescence of bound 5-MOP molecules is due to a conformational change of the L.D.L. triggered by excess 5-MOP. This would explain the 3.5 nm red-shift in the fluorescence maximum of the Trp residues observed upon addition of 5-MOP at concentrations

above the solubility limit and why bound 5-MOP molecules could, at that time, enter the lipidic core of the L.D.L. (see below).

b) Equilibrium dialysis analysis

The fluorescence analysis demonstrates that the binding of 5-MOP to L.D.L. can be explained as a two step process. In keeping with the fluorescence results, at very low 5-MOP concentrations (lower than the solubility limit in pure buffer), the binding followed by equilibrium dialysis can be represented by a good Scatchard plot (14). Thus, $r/[5\text{-MOP}]_f$ versus r ($r = [5\text{-MOP}]_b / [L.D.L.]_0$) is a straight line yielding $K = 1.4 \times 10^5\ M^{-1}$ and 4 binding sites (Fig. 2A). This small number of binding sites explains why the fluorescence observed at low 5-MOP concentration in 0.2 µM L.D.L. solutions is identical to that of free 5-MOP (see Fig. 1a).

However, above the solubility limit, the presence of 0.2 µM L.D.L. strongly increases solubility of 5-MOP in the solution (Fig. 2B). From the new solubility limit, it can be deduced that a single L.D.L. molecule can

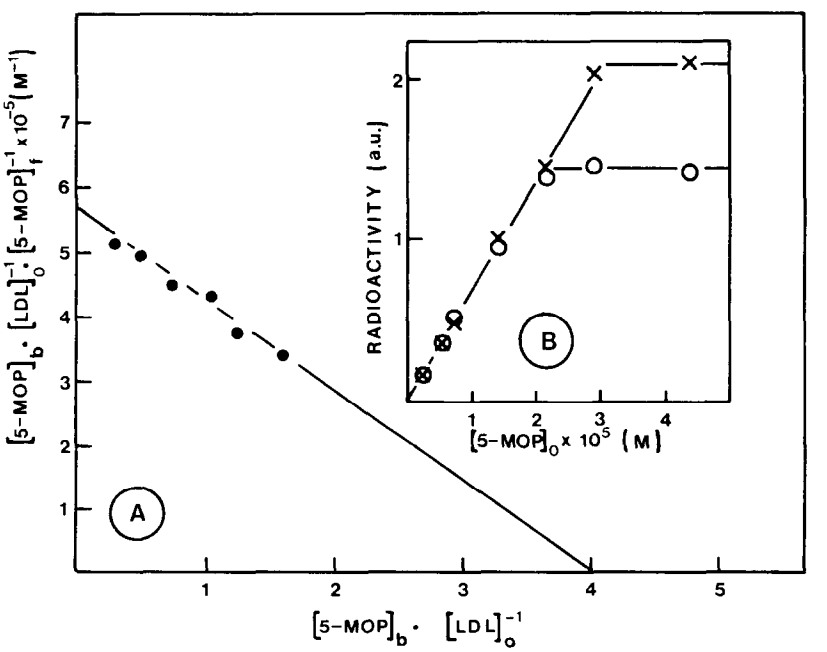

Fig. 2. A) Scatchard plot of the 5-MOP binding to L.D.L., $[L.D.L.]_0 = 0.2$ µM. B) Radioactivity of 5-MOP solutions containing no L.D.L. (o) and containing 0.2 µM L.D.L. (x). $[5\text{-MOP}]_0$ is the initial concentration. Solutions were centrifuged 12 hours after preparation to eliminate microcrystals before radioactivity counting.

incorporate about 45 psoralen molecules. This second step may reflect a process similar to solubilization into the micelle-like L.D.L. lipidic core.

The presence of 4 equivalent primary sites on the L.D.L. apoprotein, clearly evidenced by the above results, does not rule out the occurrence of non-specific secondary binding sites. These secondary sites may be operative at the solubility limit of the psoralen because the protein has a greater affinity for 5-MOP than the solvent molecules. They may induce the triggering of the conformational change of L.D.L. revealed by the fluorescence technique. Both the quenching of the Trp residue fluorescence and the conformational change suggest that the L.D.L. apoprotein plays a key role in channelling the 5-MOP molecules from the buffer to the lipid core.

CONCLUSION

The serum lipoproteins easily incorporate 5-MOP, as demonstrated in this study dealing with L.D.L. The lipoprotein concentration in human serum is about 1 % that of albumin. However, under conditions where 5-MOP binding to albumin is impaired as a consequence of drug ingestion or of diets with high lipid content, lipoproteins may play a role in its clearance via L.D.L. endocytosis in specific cells. Furthermore, the rapid L.D.L. turn-over (≅ 15 minutes at 37° C) may give rise to a continuous shift of the binding equilibrium of 5-MOP from albumin to lipoproteins. The 5-MOP delivery via L.D.L. endocytosis may also be involved in lymphocyte activation sometimes observed during PUVA therapy (5). However it remains to demonstrate that the conformational change induced by the 5-MOP binding to L.D.L. does not hinder their internalization. This is presently investigated in cultured cells.

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