STUDY OF LIPID–PROTEIN INTERACTIONS IN MEMBRANE MODELS: INTRINSIC FLUORESCENCE OF CYTOCHROME b_5 –PHOSPHOLIPID COMPLEXES

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1. Introduction

Until now, few integral membrane proteins have been isolated and studied; cytochrome b_5 is one of them. Strittmatter et al. [1] and Ememoto and Sato [2] first demonstrated that pure detergent extracted cytochrome b_5 can bind to microsomes. Furthermore, Sullivan and Holloway [3] showed that this protein interacts with egg phosphatidylcholine. By reconstitution experiments with single shell vesicles, Dufourcq et al. [4] and Robinson and Tanford [5] obtained a more detailed knowledge of the lipid–protein complexes.

Cytochrome b_5 can be described as a protein composed of two different moieties: one, bearing the heme, is hydrophilic and can be obtained by trypsin action on microsomes (cytochrome t-b_5). The second one is composed of about fifty four residues for bovine liver extracted protein, and would be responsible for the binding of the whole protein to the membrane [6,7]. Furthermore, this hydrophobic peptide contains several aromatic residues; four of them are tryptophan residues [8].

Fluorescence is a classical method to study the environment of aromatic residues in soluble proteins [9,10]. This technique has also been used to look at interactions between apolipoproteins and lipids [11,12,13].

In this paper, we report variations of the fluorescence parameters during interactions of purified bovine liver cytochrome b_5 with selected phospholipids, in order to obtain information on the structure of the lipid–protein complexes as an approach to the structure of membranes.

2. Materials and methods

Cytochrome b_5 is extracted from bovine liver according to the method of Spatz et al. [7] and Ozols [8] with slight modifications, as previously described [4]. On polyacrylamide sodium dodecyl sulfate gel electrophoresis, the purified protein shows a singel band. The ultraviolet spectra of the oxidized or reduced states of the cytochrome b_5 are identical to those already published elsewhere [5,7,8].

Natural phosphatidylcholine is extracted from egg yolk according to the method of Singleton et al. [14]. Phosphatidylserine is extracted from bovine brain [15] and phosphatidylinositol is obtained from baker's yeast [16]. Lysolecithin and dipalmitoylphosphatidylcholine were obtained from Nutritional Biochemicals Corporation.

Selected phospholipids suspended in 20 mM Tris–acetate buffer pH = 7.7 are sonicated for 15 min under nitrogen at 4°C; this mainly generates single shell vesicles which are directly used to interact with the protein in the case of natural phospholipids. In experiments with synthetic phosphatidylcholine, the crude sonicated phospholipid suspension, obtained at 45°C, is filtered on a Sepharose 4B column to remove large unfractionated particles [17]. Liposomes are then incubated with cytochrome b_5 at 44°C for 30 min and centrifugated at 375 000 g for 3 hr. The lipids to protein ratio is measured by determination of cytochrome content from the 413 nm Soret band (ϵ = 117 000 M^{-1} cm^{-1}) [18], and phospholipid content by ^{14}C radioactivity measurements, the initial material being labelled with 1% natural lecithin ^{14}C (specific activity 1.5 Ci/mmole).

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Uncorrected fluorescence spectra are recorded on a Perkin–Elmer MPF 3 spectrofluorometer, with an excitation wavelength of 275 nm. Absorption spectra are obtained on a Pye Unicam 1800 B spectrophotometer.

3. Results

3.1. *Effect of lipid binding on the fluorescence spectrum of cytochrome b_5*

Cytochrome b_5 is a class B protein [9], so its luminescence spectrum is dominated by emission of tryptophan residues. The observed spectrum, shown in fig. 1a, has its maximum at 333 nm, which is characteristic of tryptophan residues in an hydrophobic environment [10].

Addition of egg phosphatidylcholine liposomes to the protein solution leads to a large change in the spectrum, as shown in fig.1b. The main effect of the for-

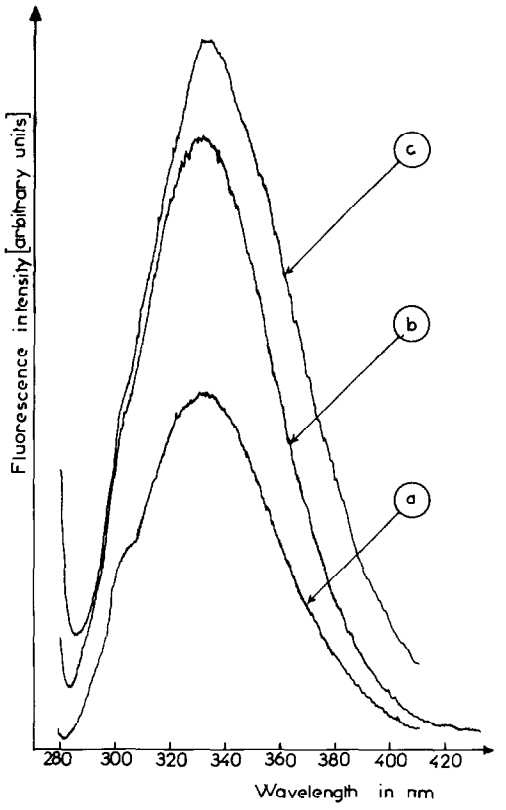

Fig.1. Fluorescence spectra of cytochrome b_5 (3 μM; pH = 7.7): (a) pure protein; (b) protein with a 50-fold molar excess of egg phosphatidylcholine; (c) same experiment as (b) after limited tryptic digestion and removal of cytochrome t-b_5.

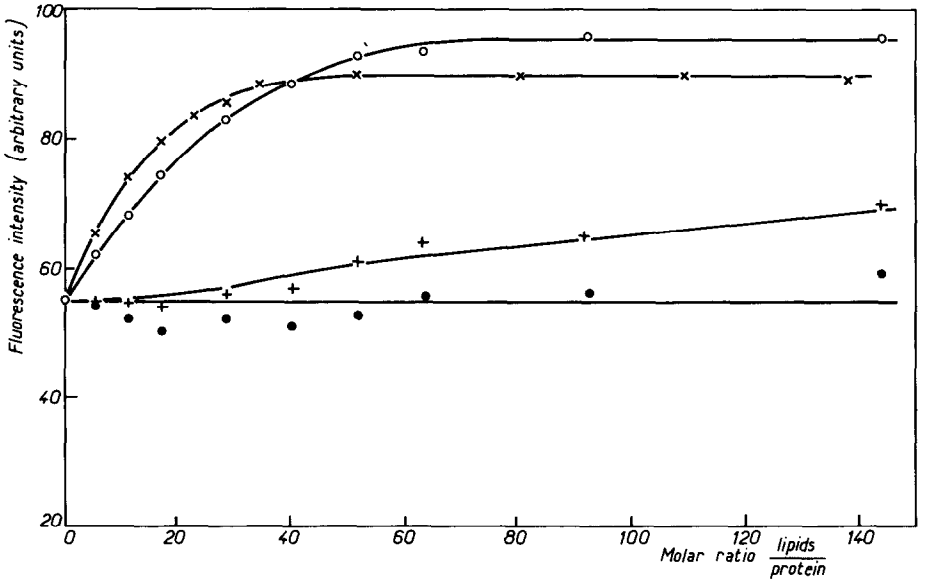

Fig.2. Fluorescence intensity of cytochrome b_5 (3 μM) mixed with increasing amounts of egg phosphatidylcholine (○), lysophosphatidylcholine (x), phosphatidylinositol (+) and phosphatidylserine (•).

mation of the lipid–protein complex is a strong increase of the fluorescence intensity. Moreover, a slight hypsochrome shift is observed (1–2 nm).

By partial trypsin digestion followed by gel filtration on a Sepharose 4B column, it is possible to quantitatively eliminate the hydrophilic moiety of cytochrome b_5 from the reconstituted species [4]. The fluorescence spectrum obtained with these vesicles, containing only the hydrophobic peptide, remains mainly the same as that of the initial complex. This is consistent with the previous results of Huntley and Strittmatter [19], who showed that cytochrome *t*-b_5 contains one tryptophan residue, but has no fluorescence because of the quencing by the heme. So, the tryptophan spectrum of the whole cytochrome b_5 is only related to the hydrophobic peptide which is concerned in the interaction with phospholipids.

Fig.2 shows the fluorescence intensities when increasing amounts of phospholipids are added to the protein solution. With egg phosphatidylcholine and lysophosphatidylcholine, there is a strong increase, up to 60%, when the lipid to protein ratio increases from zero to 50 and 35 respectively. Further addition of lipids has no effect. With phosphatidylinositol, only a small monotonous increase in fluorescence intensity is observed (about 25%), but in the case of phosphatidylserine, there is no effect at all.

3.2. *Protein denaturation*

Denaturation of cytochrome b_5 is followed by a large shift of the maximum of emission, from 333 to 346 nm, when urea concentration increases up to 8 M (fig.3a). This effect is similar to those generally observed on proteins [10], and it can be interpreted as an exposure of the tryptophan residues to the solvent.

Addition of urea to a solution of cytochrome b_5 bound to lysophosphatidylcholine or dipalmitoylphosphatidylcholine leads to different results. At maximal urea concentration (9 M), the peak is shifted only to 340 nm. Moreover, as it can be seen in fig.3a, the protein becomes sensitive to urea only at concentrations higher than in the case of the protein alone. So, a protective effect on the tryptophan residues is a consequence of the binding of cytochrome b_5 to liposomes. During the same experiments, the absorption changes at 413 nm, which are related to the unfolding of the heme region, are the same for cytochrome b_5 alone in solution and bound to phospholipids (fig.3b and 3c).

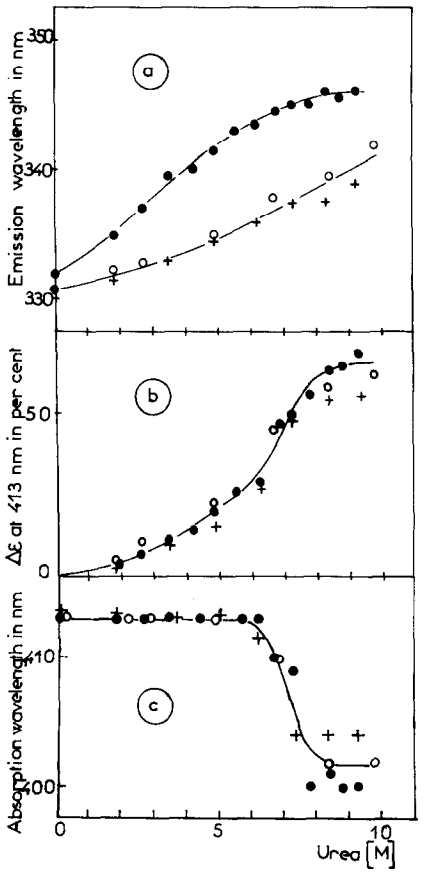

Fig.3. Urea denaturation of cytochrome b_5 (4 μM, pH = 7.7): (•) pure protein; (○) protein bound to dipalmitoylphosphatidylcholine (1:200); (+) protein bound to lysophosphatidylcholine (1:200). (a) Shifts of the fluorescence emission maxima. (b) Relative decreases of the molar coefficient of extinction at 413 nm. (c) Shifts of the maxima of the Soret band.

3.3. *Effect of temperature on saturated phosphatidylcholine-cytochrome b_5 complexes*

In fig.4 are plotted versus temperature, the fluorescence intensities of cytochrome b_5 alone or bound to dipalmitoylphosphatidylcholine, and that of ovalbumin in the presence of the same amount of lipids, used as a reference. When temperature increases from 20°C up to 46°C, the fluorescence intensities regularly decrease in the case of cytochrome b_5 alone and ovalbumin. This shows that ovalbumin fluorescence is totally insensitive to the phase transition of dipalmitoylphosphatidylcholine which occurs at 41°C.

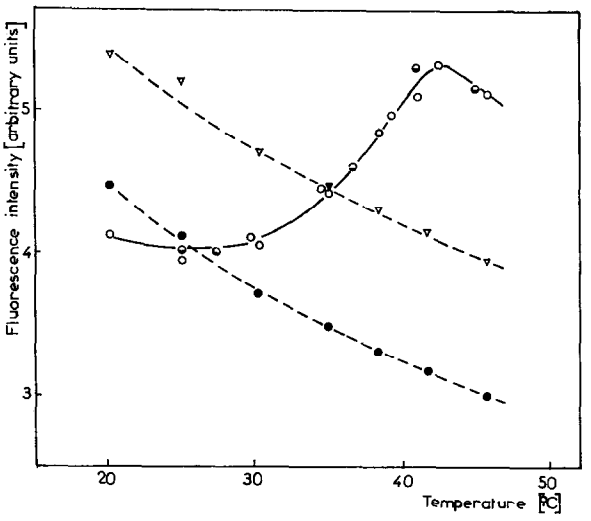

Fig.4. Effect of temperature on the fluorescence intensity of cytochrome b_5: (•) pure protein; (○) dipalmitoylphosphatidylcholine–cytochrome b_5 complex (1 protein for 44 lipid molecules); (○) increasing temperature; (⊖) decreasing temperature; (▽) ovalbumin in presence of dipalmitoylphosphatidylcholine.

On the contrary, for bound cytochrome b_5 at almost saturation level (one protein for 44 lipid molecules), the fluorescence intensity varies very little from 20°C to 30°C, and then increases by about 30% between 30°C and 42°C. Above this temperature, intensity decreases sharply in the same way as cytochrome b_5 alone or ovalbumin.

Experiments carried out with cytochrome b_5 bound to dimirystoylphosphatidylcholine give similar results, the increase of intensity being centered around 23°C, which is the phase transition temperature of the pure lipid.

4. Discussion

The first point to be mentioned is that intrinsic fluorescence intensity is very sensitive for detecting the formation of cytochrome b_5–phospholipids complexes. Furthermore, most, if not all, of the observed fluorescence is due to tryptophan residues located in the hydrophobic peptide remaining bound to the phospholipids after trypsin action.

From the plots of variation of intensity versus the lipid to protein ratio it is possible to evaluate the minimum lipid content for which all proteins are bound. This corresponds to 50 egg phosphatidylcholine and 35 lysophosphatidylcholine molecules for one protein molecule. This difference can be related to the fact that in the case of sonicated dispersions a few multibilayer particles are still present for which the binding ratio of cytochrome b_5 is lower. These values agree fairly well with that obtained by Strittmatter et al. [1] on microsomes (1:40), and that we determined, 1 protein for 35 egg phosphatidylcholine, by isolating the complexes by gel filtration [20]. Therefore, lysophosphatidylcholine micelles and egg phosphatidylcholine bilayers would interact with cytochrome b_5 in a similar way although they have very different structures [21].

These results can be compared to those obtained by the same fluorescence method for binding of apolipoprotein to lysophosphatidylcholine by Verdery III et al. [12]. They essentially observed the same phenomena, i.e. no change on the emission wavelength of the hydrophobic residues (λ_{max} = 331 nm) but an increase in intensity which corresponds to the complex formation.

Very different results are obtained with negatively charged phospholipids. They lead to the conclusion that there is no interaction between the protein and lipids. This can be related to the fact that cytochrome b_5 bears a net negative charge at the experimental pH (7.7); electrostatic forces probably prevent the formation of a complex.

From the change of the fluorescence intensity of the cytochrome b_5–dipalmitoylphosphatidylcholine complex, versus temperature, two important conclusions can be inferred. First of all, the interpretation of the fluorescence intensity increase as a consequence of the melting of the phospholipids aliphatic chains, leads to the conclusion that the transition is broad, but still does occur at temperatures closely related to those of transition for pure phospholipids. A similar conclusion has been proposed by Barratt et al. [22] from the study of dansylated apolipoprotein bound to dimyristoylphosphatidylcholine. This can also be compared to the results obtained by differential scanning calorimetry on natural membranes and lipids extracts [23]. On the other hand, since the tryptophan residues are sensitive to the phase transition, one can conclude that they are closely in contact with aliphatic

chains. So, part of the protein is embedded in the hydrophobic core of the bilayer.

The protective effect observed on denaturation by urea can be interpreted in the same way. It has also been observed in the case of the binding of apolipoprotein to lysophosphatidylcholine [12]. Furthermore, this protective effect is localized within the hydrophobic region of the protein since unfolding of the heme moiety is the same for both protein alone or bound to liposomes. This is a new potent argument for the two domain structure of cytochrome b_5, proposed by several authors [1,2] and, more recently by Visser et al. [24].

Although the structure of the hydrophilic region is very well known [25], only the amino acid composition of the hydrophobic peptide has been determined [7,8]. Intrinsic fluorescence spectroscopy should allow us to evaluate the respective roles of the modification of the medium and (or) of the protein structure in the observed phenomena. So developments of this work seem to be a possible means to investigate the structure of the hydrophobic peptide interacting with lipids.

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