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INTERACTION OF TETRAPYRROLIC RINGS WITH RHODAMINE 110 AND 123 AND WITH RHODAMINE 110 DERIVATIVES BEARING A PEPTIDIC SIDE CHAIN

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SUMMARY: Rhodamines 110 and 123, and rhodamine 110 linked via peptide bonds to Arg, Cbz-Arg and Cbz-Ile-Pr-Arg interact with free base porphyrins or cytochrome C. Rhodamines 110 and 123 essentially form 1:1 complexes while the other derivatives form 2:1 complexes. The possible biological implications of these results are discussed.

Rhodamines are fluorescent dyes that can penetrate living cells. Thus the cationic rhodamine 123 (Rh123) can be used as a fluorescent marker of mitochondria (1). Since it has been shown to preferably accumulate in transformed cells, it has been suggested as a potential anticancer drug (2). The anticancer activity is attributed, at least partly, to its effect on phosphorylation (3). Rhodamine 110 (Rh110) is the fluorogenic chromophore of a variety of serine proteinase substrates (4)(5). These substrates, bearing a basic Arg residue, can enter living cells as demonstrated by recent microspectrofluorometric studies (6). In these studies, we reported that one of these substrates (Cbz-Ile-Pro-Arg-NH)$_2$-Rh110 (BZiPAR) was strongly interacting with exogenous porphyrins

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Abbreviations : Rh123, rhodamine 123; Rh110, rhodamine 110; Cyt C, cytochrome C; UP, uroporphyrin I; HP, hematoporphyrin; BZiPAR, (Cbz-Ile-Pro-Arg-NH)$_2$-Rh110; BZAR, (Cbz-Arg-NH)$_2$-Rh110; BAR, (Arg-NH)$_2$-Rh110.

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used as photosensitizers (6). We present here results unambigously demonstrating that Rh110, Rh123 and peptides linked to Rh110 moderately or strongly bind to porphyrins either as free bases or chelated to iron ions such as in cytochrome C.

MATERIALS and METHODS

Rhodamines 123 and 110 were purchased from Eastman Kodak Company. The peptides BZiPAR, BZAR ($(Cbz\text{-}Arg\text{-}NH)_2$-Rh110), BAR ($(Arg\text{-}NH)_2$-Rh110) were synthesized and purified as described (4)(5). Stock solutions (10 mM) were prepared in dimethylformamide (DMF) and diluted as desired in 10 mM HEPES buffer (pH7). Water-soluble porphyrins such as uroporphyrin I (UP) and hematoporphyrin (HP) were obtained from Sigma and used as received. Cytochrome C (Cyt C) from horse heart was supplied by Koch-Light. Stock solutions (0.1 mM) of Cyt C were also prepared in the HEPES buffer.

Optical absorption spectroscopy was carried out with a Perkin-Elmer Lambda 5 spectrophotometer while fluorescence spectra were recorded with either a Perkin Elmer LS5 or a SPEX spectrofluorometer with emission and excitation spectrum correction. All experiments were performed at 20°C.

RESULTS AND DISCUSSION

Complex formation between rhodamine derivatives and free-base porphyrins

As shown in Fig. 1, addition of increasing BZiPAR concentrations to micromolar solutions of UP (or HP) leads to strong hypochromic effects on the Soret and to the appearance of isosbestic points in the visible absorption bands of the porphyrins (Insert Fig. 1). Addition of a large excess (i.e. one hundred fold) of BZiPAR further induces a 8 nm red shift of the Soret band accompanied by a hyperchromic effect. Comparable shifts are also observed in the visible range of the absorption spectra. These shifts cannot be due to the contribution of free BZiPAR to the absorption spectrum of the complexed porphyrins. Thus, although BZiPAR has the same absorption maximum (492 nm) as the strongly absorbing Rh110 (ϵ = 66,800 $M^{-1}cm^{-1}$), it is characterized by a very low molar extinction coefficient (ϵ = 23 $M^{-1}cm^{-1}$) (5).

The same results are obtained with fluorescence spectroscopy (Fig. 2a). The well-known UP fluorescence characterized by two emissions bands (max. wavel. : 615 and 678 nm) is readily quenched upon BZiPAR addition. Trivial considerations about energy levels and concentration conditions

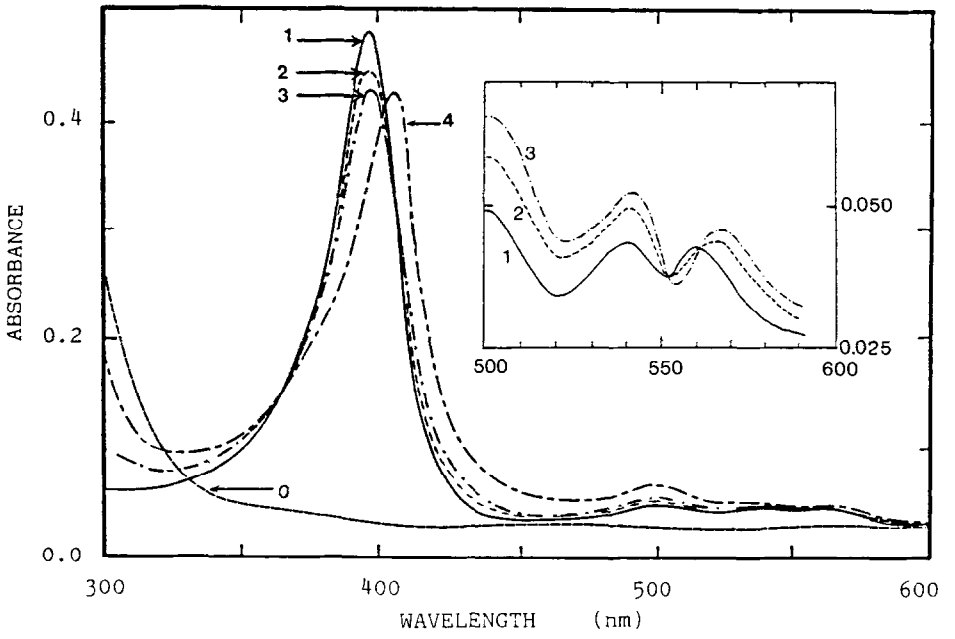

Fig. 1. Absorbance (1 cm light path) of 1.35 uM UP in the presence of 0 uM (1), 2 uM (2), 4 uM (3) and 100 uM (4) BZiPAR. Insert : Close-up of the 500-600 nm absorbances of 1, 2 and 3. Solvent : Hepes buffer (pH 7).

allow to rule out energy transfer from the first excited singlet state of porphyrins to the rhodamines. At high BZiPAR concentration, the fluorescence increases and is red shifted suggesting the formation of a second

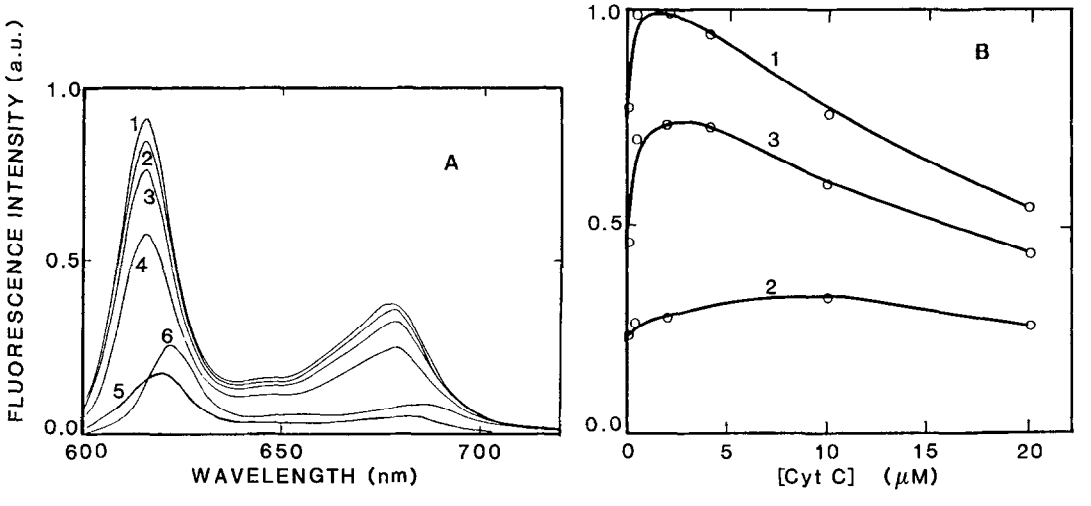

Fig. 2. (A) : Fluorescence spectra upon excitation at 560 nm (isosbestic point) of 1.5 uM UP in the presence of 0 uM (1), 1 uM (2), 2 uM (3), 3.2 uM (4), 7.2 uM (5) and 193 uM (6) BZiPAR. (B) : Relative fluorescence intensities (at 530 nm) of 0.2 uM BAR (1), BZiPAR (2) and BZAR (3) solutions (pH 7, Hepes buffer) in the presence of increasing |Cyt C| upon excitation at 454 nm. The |Cyt C| was such that, at the excitation and emission wavelengths, absorbance was always lower than 0.22 (1 cm light path) leading to negligible inner filter effects.

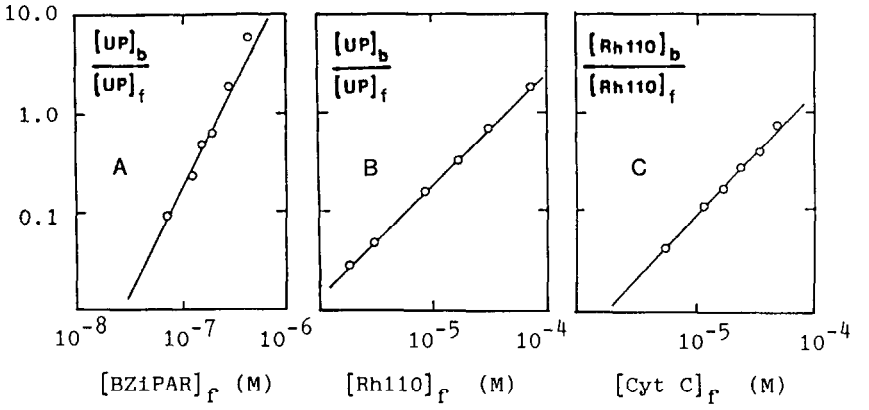

Fig. 3. Log-log plots (according to equation 1) of the variation of the concentration of free and bound UP ($|UP|_0$ = 3.4 uM) (A,B) or Rh110 ($|Rh110|_0$ = 125 nM) (C) as a function of free BZiPAR (A,B) or free Cyt C (C). Concentrations were calculated as described in the text using fluorescence intensities assuming either 1:1 or 2:1 complexes. Excitation and emission wavelengths for UP were 560 nm and 615 nm respectively (A,B). Excitation and emission wavelengths for Rh110 were 490 nm and 530 nm respectively (C). Absorbance at excitation and emission wavelengths was lower than 0.2 (1 cm light path).

complex , Identical observations can be made when HP replaces UP. It must be emphasized that, at low BZiPAR concentration, the formation of the first complex is not accompanied by the modification of the fluorescence decay of the porphyrin[$]. It can thus be assumed that the first complex is unfluorescent.

The thermodynamical analysis of the complex formation can be readily carried out at low BZiPAR concentration where only one complex is present. For low optical densities at the fluorescence excitation wavelength, the fluorescence intensity is proportional to the free UP concentration ($|UP|_f$). It can be readily shown that :

$$\log (|UP|_b / |UP|_f) = \log K + n \log |BZiPAR|_f \qquad \text{(eq. 1)}$$

where $|BZiPAR|_f$ represents the free BZiPAR , $|UP|_b$ the complexed UP, K, the equilibrium constant and n the number of ligands.

Data in Fig. 3 demonstrate that results are best explained by a 2:1 complex. It is obvious that complex stabilization occurs upon modifying chain length and/or composition. No attempt was made to analyze the

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Table I. Binding parameters (according to equation 1) of rhodamine derivatives to UP or Cyt C. Fluorescence data used in the calculations were obtained under the same excitation and emission conditions as in Fig. 3

Name of complex	K (n)
BZiPAR (UP)	1.4×10^{13} M^{-2} (2.04)
BZAR (UP)	2.04×10^{10} M^{-2} (2.02)
BAR (UP)	2.7×10^{10} M^{-2} (1.9)
Rh110 (UP)	1.7×10^{4} M^{-1} (1.06)
Rh123 (UP)	3.0×10^{5} M^{-1} (1.1)
Rh110 (Cyt C)	1.05×10^{4} M^{-1} (1.05)
Rh123 (Cyt C)	1.05×10^{4} M^{-1} (1.12)

stoichiometry of complex formation at high BZiPAR concentration because of large overlap of the fluorescence spectra of free and bound UP.

As shown in table I the same results can be obtained with the other rhodamines. It can be observed that the complex stoichiometry is changed from 2:1 to 1:1 in the absence of a peptidic side chain linked to Rh110. Also, upon addition of Rh110 or Rh123 to UP, only weak (if any) modification of the absorption spectrum of the porphyrins are observed (data not shown). In the 1:1 complexes, while the equilibrium constant ($\neq 10^{4}$ M^{-1}) is moderately high for Rh 110, it is one order of magnitude larger for Rh 123, suggesting that the latter can form strong complexes with free base porphyrins. In the case of the 2:1 complexes, their strength is illustrated by the total inhibition by BZiPAR of the photosensitization of L cell fibroblasts by HP under UV light excitation (6).

Interaction of rhodamines with cytochrome C

Since cytochrome C is not fluorescent, the complex formation between Cyt C and the rhodamines was investigated by looking at the quenching of the fluorescence of rhodamines (max. wavel. ≠ 520-530 nm) by increasing

amounts of Cyt C. Rhodamine 110 and 123 led to 1:1 complexes (Fig. 3C) as observed with free base porphyrins (Fig. 3B). However, in this case, the equilibrium constants are very similar for both rhodamines. While the Rh 110 and Rh 123 fluorescence was uniformly quenched by Cyt C, the peptidic rhodamines behave quite differently. At low |Cyt C| / |peptide| ratio (Fig. 2b), addition of Cyt C induces an increase in the rhodamine fluorescence. On the other hand, large excess of Cyt C leads to fluorescence quenching. This may be due to the presence of binding sites of lower affinity on Cyt C through interaction(s) of the apoprotein with the peptidic chain of BAR, BZAR and BZiPAR. Similar complex formation was observed with either oxidized or reduced Cyt C. Thus these results demonstrate that BAR, BZAR and BZiPAR bind to Cyt C.

CONCLUSION

These results have two implications. First the cationic rhodamines under study associate in vitro with a hemoprotein of the mitochondrial electron transport chain. It can be envisioned that they may also interact with other extramitochondrial hemoproteins. Second, it may be suggested that they can form complexes with extramitochondrial intermediates of heme biosynthesis (7). These results may not only help explain the alteration of vital cellular functions by rhodamines (8), but they may also lead to interesting developments in the management (6) of photosensitization encountered in porphyria diseases (7)(9).

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