Rhodamine 101 as a fluorescent probe for sensing haemoglobin conformational changes at the lipid-water interface

Olga K. Zakharenko, Valeriya M. Trusova* and Galyna P. Gorbenko

Department of Biological and Medical Physics, V. N. Karazin Kharkov National University, 4 Svobody Sq., 61077 Kharkov, Ukraine

The spectral behaviour of the xanthene dye rhodamine 101 (R101) in model systems containing haemoglobin (Hb) and liposomes from phosphatidylcholine (PC) and cardiolipin (CL) was examined. Fluorescence parameters of R101 remained virtually unchanged in the presence of liposomes, while in Hb–lipid systems the emission maximum of R101 exhibited a hypsochromic shift coupled with fluorescence intensity changes. These effects were interpreted in terms of R101's ability to form complexes with free and lipid-bound Hb, differing in their spectral characteristics. It is proposed that R101 senses lipid-induced destabilization of Hb structure, implicating protein unfolding and splitting of haem-globin bonds.

Keywords: haemoglobin, liposomes, protein conformational changes, rhodamine 101

1. INTRODUCTION

Fluorescence spectroscopy currently represents one of the most powerful tools for elucidating structural features of biological macromolecules and their assemblies [1–4]. In particular, this technique is highly informative regarding the structural characterization of the complexes between two major membrane constituents—proteins and lipids [5, 6]. One problem encountered in such studies lies in the correct differentiation of spectral signals pertaining to lipid-bound and protein-bound states of the reporter molecule. Most fluorescent probes exhibit preferential binding to a lipid bilayer, and thus are inappropriate for detecting conformational transitions of interfacially adsorbed proteins. For this reason, there still exists a strong need to look for new probes selectively sensitive to lipid-induced changes in protein structure.

The present study has been undertaken to evaluate the possibility of employing one representative of a large group of xanthene dyes, rhodamine 101 (R101), as a specific probe for reporting conformational changes of membrane-bound proteins. In approaching this problem, the spectral behaviour of R101 in the model systems whose protein and lipid components were represented by haemoglobin (Hb) and phospholipid vesicles (liposomes) has been examined. Although Hb is not a membrane protein, it avidly associates with lipids via electrostatic and hydrophobic interactions [7–10]. To ensure varying contributions of electrostatic and hydrophobic binding components to the formation of Hb-lipid complexes, liposomes were prepared from a mixture of zwitterionic lipid phosphatidylcholine (PC) with anionic lipid cardiolipin (CL) in the molar ratio 4:1.

*Corresponding author. Tel.: +38 057 364 29 34; +38 057 343 82 44. Fax: +38 057 705 00 96. E-mail: valtrusova@yahoo.com

2. MATERIALS AND METHODS

2.1. Materials

Egg yolk phosphatidylcholine and beef heart cardiolipin were purchased from Biolek (Kharkov, Ukraine). Both phospholipids gave single spots by thin layer chromatography in the solvent system chloroform:methanol:acetic acid:water, 25:15:4:2 v/v. Horse haemoglobin (in the ferric form) was from Reanal (Hungary), butylated hydroxytoluene (BHT) was from Merck (Germany), thiourea (TU) was from Sigma (USA) and rhodamine 101 was from Fluka (Switzerland). Toyopearl gel HW-60F was from Toyo Soda (Japan). All other chemicals employed were of analytical grade.

2.2. Preparation of lipid vesicles

Unilamellar lipid vesicles composed of PC mixed with 20 mol% CL were prepared by the extrusion method [11]. Appropriate amounts of lipid stock solutions were mixed in ethanol, evaporated under vacuum and then left under reduced pressure for 1.5 h to remove any residual solvent. The obtained thin lipid films were hydrated with 1.2 mL of 5 mM sodium phosphate buffer (pH 7.4) at room temperature to yield a final lipid concentration of 10 mM. Thereafter lipid suspensions were extruded through a 100 nm pore size polycarbonate filter (Nucleopore, Pleasanton, Cal.). The phospholipid concentration was determined according to the procedure of Bartlett [12].

2.3. Spectroscopic measurements

Fluorescence measurements were performed with a CM 2203 spectrofluorimeter equipped with a magnetically stirred, thermostated cuvette holder (SOLAR, Belarus). Fluorescence spectra of rhodamine 101 were recorded at an excitation wavelength of 520 nm with excitation and emission bandwidths set at 2 nm. The fluorescence

Journal of Biological Physics and Chemistry **8** (2008) 43–48
Received 14 January 2008; *accepted* 12 May 2008

intensity of the probe measured in the presence of Hb was corrected for reabsorption and inner filter effects using the following coefficient [13]:

$$k = \frac{\left(1-10^{-A_o^{ex}}\right)\left(A_o^{ex}+A_{Hb}^{ex}\right)}{\left(1-10^{-\left(A_o^{ex}+A_{Hb}^{ex}\right)}\right)A_o^{ex}} \frac{\left(1-10^{-A_o^{em}}\right)\left(A_o^{em}+A_{Hb}^{em}\right)}{\left(1-10^{-\left(A_o^{em}+A_{Hb}^{em}\right)}\right)A_o^{em}} \quad (1)$$

where A_o^{ex} and A_o^{em} are the probe optical densities at the excitation and emission wavelengths in the absence of Hb, A_{Hb}^{ex} and A_{Hb}^{em} are the Hb optical densities at the excitation and emission wavelengths. Protein concentration was determined using the extinction coefficient $\varepsilon_{407} = 1.4\times10^5$ $M^{-1}cm^{-1}$ per haem. The concentration of R101 was calculated taking the molar absorptivity of the dye as $\varepsilon_{576} = 9.5\times10^4$ $M^{-1}cm^{-1}$. Stock solutions of R101 were prepared in ethanol.

2.4. Deconvolution of fluorescence spectra

Individual emission spectra of R101 were analysed using a nonlinear least-squares fitting routine based on the Levenberg-Marquardt algorithm implemented in "Origin" (version 7). After subtraction of the linear baseline the spectra were deconvoluted into Gaussian components:

$$I(\lambda) = \sum_{i=1}^{m} A_i \frac{1}{w\sqrt{\pi/2}} \exp\left[\frac{-2\left(\lambda-\lambda_{max}^{i}\right)^2}{w^2}\right] \quad (2)$$

where A_i, w_i and λ_{max}^{i} stand for area, width and maximum position of the ith component, respectively, and m is the number of components. All fits give the minimum number of components required to achieve a correlation coefficient (R^2) not less than 0.99. The reproducibility of the curve fitting was verified by varying the starting parameters.

2.5. Size-exclusion chromatography

Hb-liposome complexes and free Hb were separated by size-exclusion chromatography on Toyopearl HW-60F gel. Hb (26 μM) was incubated with liposomes (4 mM) at 25 °C for 1 h and then 0.6 mL of this mixture was applied to the column (20 × 1 cm) equilibrated with 5 mM sodium phosphate buffer. Elution was carried out at 25 °C at a rate of 0.2 mL/min. The collected 1 mL samples were analysed for Hb content by measuring the protein absorbance at 406 nm.

2.6. Dye-protein binding studies

Quantitative characteristics of R101 binding to Hb were determined by analysing Hb-induced changes of the probe fluorescence intensity at 596 nm. It was assumed that the measured fluorescence intensity decrease (ΔI) is proportional to the concentration of bound probe (B_z):

$$\Delta I = a_z B_z \quad (3)$$

where a_z is a coefficient of proportionality. If one protein molecule contains n binding sites for R101 the association constant (K_a) is given by:

$$K_a = \frac{B_z}{F_z\left(nP-B_z\right)} = \frac{B_z}{\left(Z_0-B_z\right)\left(nP-B_z\right)} \quad (4)$$

where P is the total protein concentration and F_z is the concentration of free dye. Eqns (2) and (3) can be rearranged to give:

$$\Delta I = a_z B_z = \quad (5)$$

$$\left(a_z/2\right)\left[Z_0+nP+1/K_a - \sqrt{\left(Z_0+nP+\frac{1}{K_a}\right)^2 - 4Z_0nP}\right]$$

Approximation of the experimental dependencies $\Delta I(P)$ by eqn (4) allowed us to determine the binding parameters (K_a and n) through minimizing the function:

$$f = \frac{1}{N_e}\sum_{i=1}^{N_e}\left(\Delta I_i^{theor} - \Delta I_i^{exp}\right)^2 \quad (6)$$

where ΔI_i^{exp} and ΔI_i^{theor} are the ΔI values measured experimentally and calculated from eqn (5), respectively, and N_e is the number of experimental points.

3. RESULTS AND DISCUSSION

At the first step of the study we made an attempt to reveal the lipid-associating properties of R101. As illustrated in Fig. 1, the emission spectrum of this probe remained virtually invariable upon addition of liposomes, suggesting that either R101 does not associate with lipid vesicles or its fluorescence parameters are insensitive to probe transfer into the lipid phase. The latter assumption seems to be rather realistic because fluorescence lifetime and quantum yield of R101 are reported to be nearly independent of changes in solvent characteristics and temperature [14, 15]. Such a property can be rationalized in terms of the early "loose bolt" concept of Lewis and Calvin [16], suggesting that flexible groups attached to chromophores are capable of promoting nonradiative deactivation of the excited state. The flexibility of the amino moieties has been proposed to play an essential rôle in nonradiative deactivation of rhodamine dyes [15, 17]. In contrast to other xanthene chromophores, the amino groups of R101 are held rigidly by bridging carbon structures, thereby rendering the dye only slightly sensitive to external influences. Nevertheless, our further experiments conducted with Hb-lipid systems proved to be suggestive of R101 sensitivity to changes in Hb conformational state.

As shown in Fig. 2, titration of the pre-incubated R101–Hb mixture with liposomes resulted in marked changes in the shape of the emission spectrum, suggesting the existence of several spectral bands and enhancement

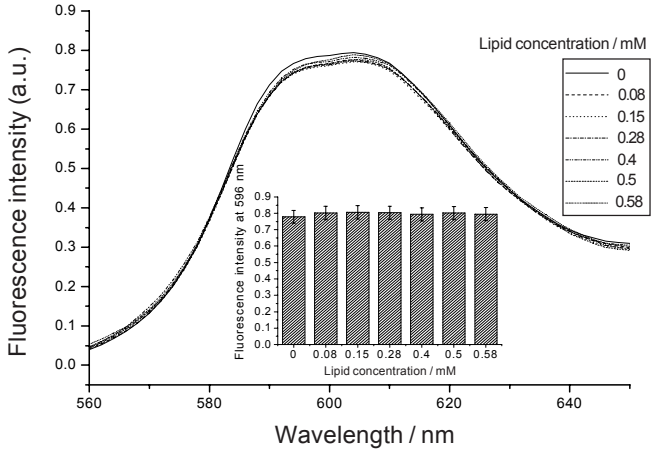

Figure 1. Fluorescence spectra of rhodamine 101 in suspension of PC/CL (20 mol% CL) liposomes. Shown in the inset is the dependency of rhodamine 101 fluorescence intensity at 596 nm on lipid concentration. Probe concentration was 10 nM.

of the shorter-wavelength components with increasing amounts of membrane-bound Hb. To explain this effect, two hypotheses have been tested. The former relates to the ability of Hb to induce lipid peroxidation [18, 19] followed by the formation of highly reactive species capable of influencing the spectral properties of R101, while the latter implicates direct interactions between R101 and Hb. To test the first hypothesis, fluorescence measurements have been performed in the presence of the antioxidants BHT and TU. The changes in R101 fluorescence in Hb-lipid systems were not suppressed by antioxidants (Fig. 3). Hence, the first of the above proposals can apparently be ruled out. Next, we evaluated the direct interactions between R101 and Hb. As shown in Fig. 4, titration of R101 with Hb was indeed accompanied by progressive decrease of the probe fluorescence. Approximation of the experimental $\Delta I_{596}(P)$ dependencies (Fig. 5) by eqn (5) yielded the following parameters of R101 complexation with Hb: $K_a = (2.6 \pm 0.3) \times 10^5$ M^{-1}, $n = 1.5 \pm 0.4$. Taking into account the fact that R101 is a zwitterion with rather high hydrophobicity, and the fact that under the experimental conditions employed (pH 7.4, ionic strength 5 mM) Hb bears a slight negative charge (the protein isoelectric point is about 6.8 [19]), it can be assumed that both ionic and hydrophobic forces are involved in the formation of dye-protein complexes.

In the following, to allow more unambiguous conclusions to be reached, Hb-liposome complexes and free Hb were separated using gel filtration technique. Shown in Fig. 6 is a typical elution profile consisting of two peaks corresponding to lipid-bound and free Hb. Subsequent addition of isolated Hb-liposome complexes to R101 in buffer was accompanied by changes in the shape of the emission spectrum similar to those recovered from the titration of the R101–Hb mixture with liposomes (cf. Figs 2 and 7).

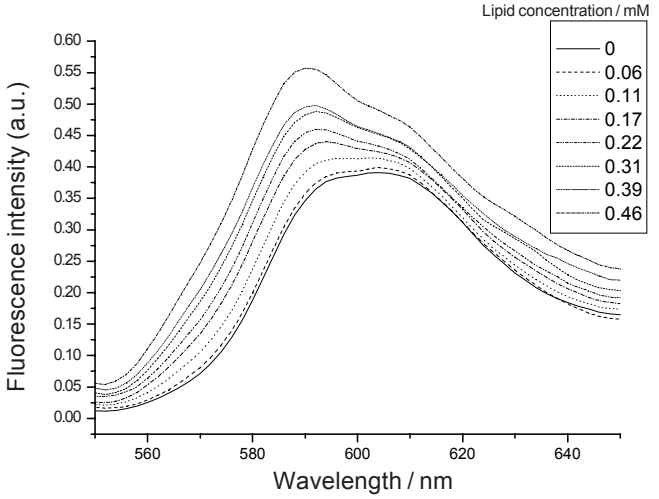

Figure 2. Fluorescence spectra of rhodamine 101 in haemoglobin–liposome mixtures. Haemoglobin concentration was 1.2 μM. Probe concentration was 5 nM.

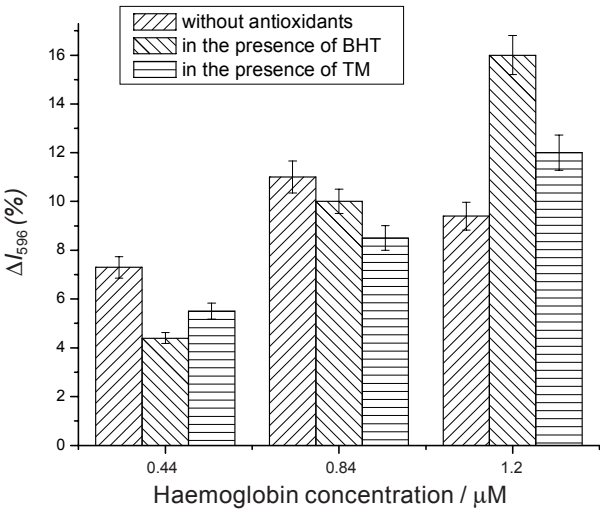

Figure 3. Effect of antioxidants on the changes of rhodamine 101 fluorescence intensity at 596 nm. Concentrations of butylated hydroxytoluene and thiourea were 0.04 mM and 67 mM, respectively.

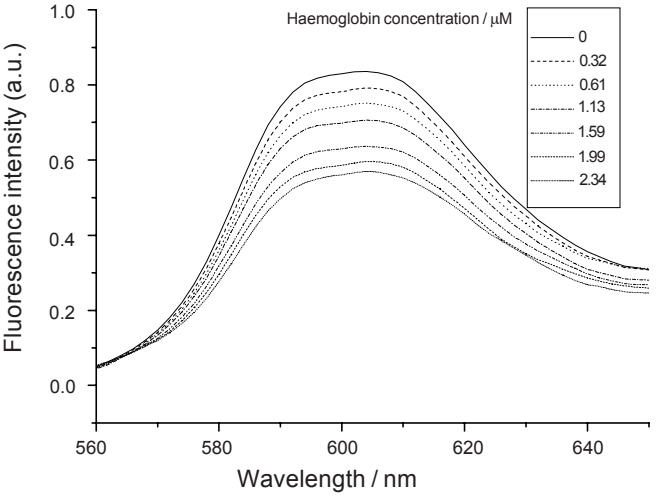

Figure 4. Fluorescence spectra of rhodamine 101 at varying haemoglobin concentrations. Probe concentration was 10.6 nM.

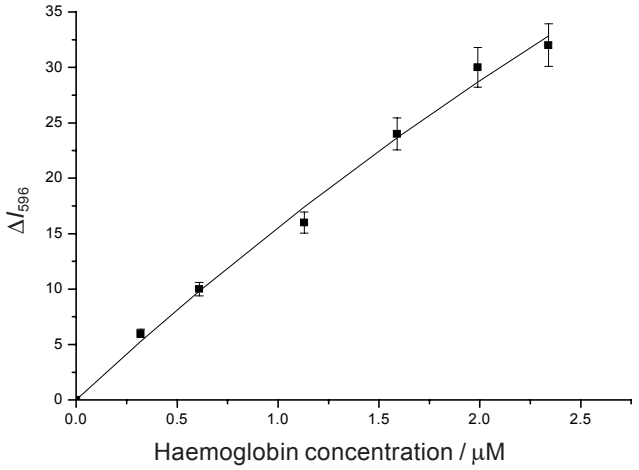

Figure 5. Decrease of rhodamine 101 fluorescence intensity at 596 nm as a function of haemoglobin concentration. Dye concentration was 10 nM. The solid line represents a theoretical curve calculated from eqn (5) with the binding parameters $K_a = 2.6 \times 10^5\ M^{-1}$, $n = 1.5$.

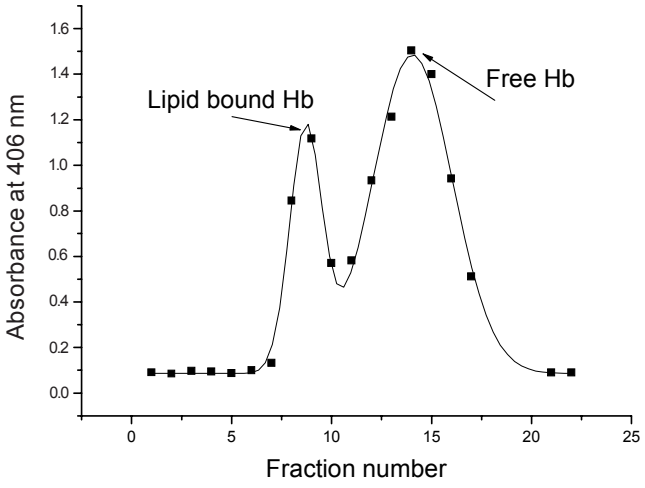

Figure 6. Elution profile of Hb-liposome mixture obtained by size-exclusion chromatography on Toyopearl HW-60F gel.

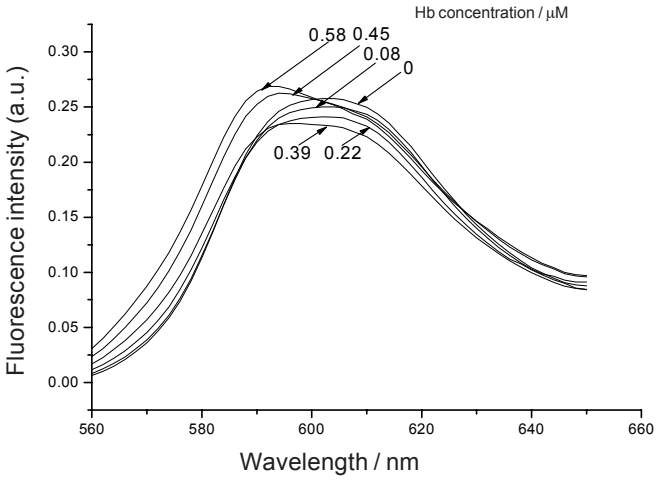

Figure 7. Fluorimetric titration of rhodamine 101 with isolated Hb-liposome complexes. Dye concentration was 3.2 nM. Lipid-to-Hb molar ratio was 440.

In the last step of the study it seemed of importance to analyse the spectral behaviour of R101 in more detail. To this end, we made an attempt to decompose the emission spectra of the probe in buffer, Hb solution or a suspension of isolated Hb-liposome complexes into several components. After baseline subtraction a sufficient accuracy of deconvolution (correlation coefficient *ca* 0.99) was achieved with two Gaussians (Fig. 8), characterized by their position (λ_{max}), width (w) and area (A). Analysis of these parameters shows that peculiarities of the R101 emission spectra in Hb-liposome mixtures could be attributed rather to the altered relative contributions of the short- and long-wavelength components in the overall spectrum than to changes in their positions, because the differences in $\lambda_{max}^{1,2}$ between R101 in buffer (or Hb solution) and the Hb-lipid systems do not exceed 2 nm (Table 1). More specifically, Hb complexation with liposomes resulted in an increase of the relative area (ratio of the peak area to total area under the spectrum) of the short-wavelength component to 0.37, compared to 0.11 in Hb solution or 0.18 in buffer. This may account for the observed changes in spectral countour and the shift of the spectral maximum to *ca* 590 nm in Hb-liposome mixtures.

The demonstrated ability of R101 to interact with Hb lends support to the hypothesis that spectral effects revealed for Hb-lipid systems reflect the behaviour of the probe associated with lipid-bound protein. To date, lipid-associating properties of different forms of haemoglobin are rather well characterized. A good deal of evidence suggests that Hb-bilayer interactions implicate a number of closely interrelated processes, viz.: (i) formation of electrostatic and hydrogen-bonding protein-lipid contacts [10, 19]; (ii) conformational changes of the Hb molecule, which may involve protein unfolding [19, 20], haem displacement and reorientation [19], dissociation of the haem–globin complex [8, 20], and iron release from the porphyrin ring [21]; (iii) modification of the lipid bilayer structure [7, 9]; and (iv) protein penetration into the membrane interior [19, 22]. The nature and relative significance of these processes largely depend on bilayer composition and experimental conditions.

Within the range of relatively low protein concentrations employed in our experiments (0.3–1.2 μM), tetrameric Hb molecule tends to dissociate into αβ dimers [7]. Such a change in Hb quaternary structure, accompanied by exposure of the nonpolar face, is thought to be responsible for the substantially greater Hb propensity for hydrophobic interactions with lipids as compared to other water soluble proteins [23]. Another factor that may enhance the hydrophobic component of Hb-lipid binding involves lowering the activation energy barrier for protein

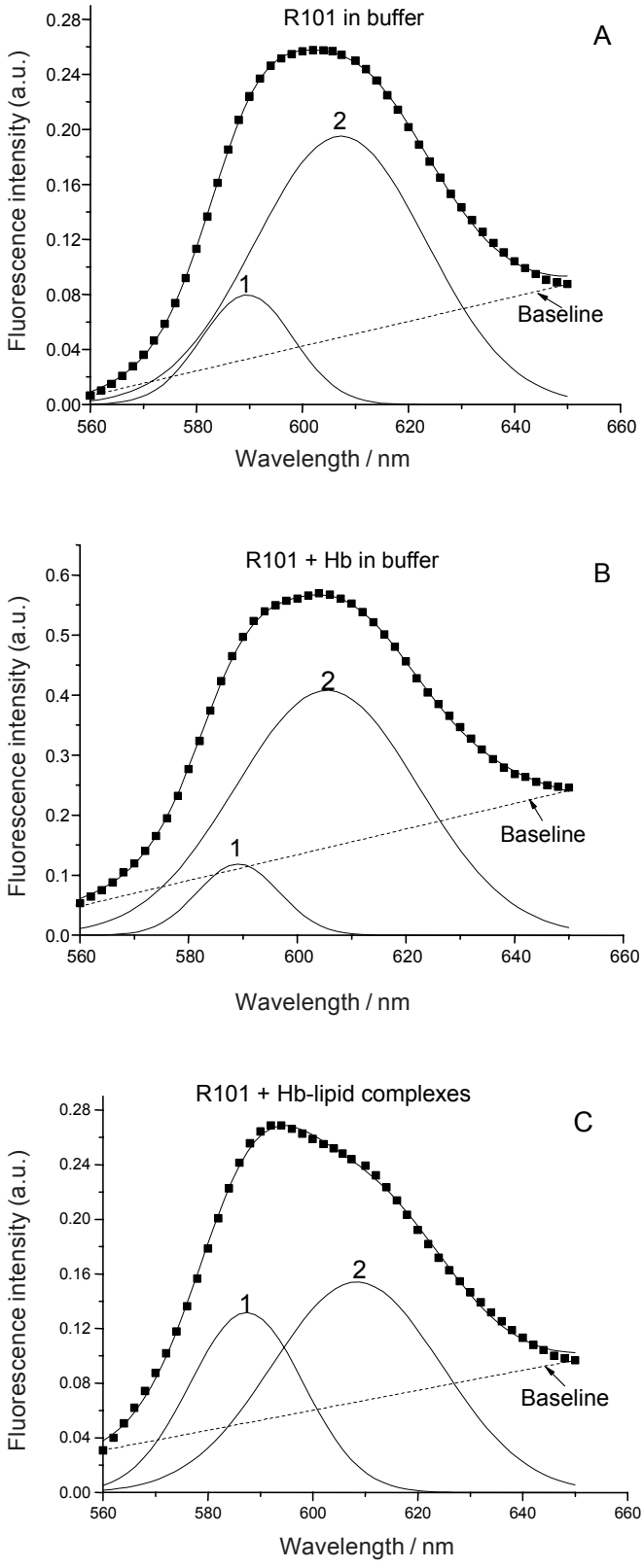

Figure 8. Deconvolution of rhodamine 101 emission spectra in buffer (A), Hb solution (B) and suspension of isolated Hb-liposome complexes (C) into two Gaussian components. Probe concentration was 3.2 nM (A, C), 10.6 nM (B). Hb concentration was 2.34 μM (B), 0.58 μM (C).

Table 1. Parameters of deconvolution of rhodamine 101 emission.

Parameter	R101 in buffer	R101 + Hb	R101 + Hb-liposome complexes
λ^1_{max} / nm	589.6 ± 0.2	589.2 ± 0.2	587.4 ± 0.6
w_1 / nm	17.3 ± 0.7	15.1 ± 0.5	21.6 ± 0.9
A_1	1.7 ± 0.2	2.2 ± 0.2	3.6 ± 0.6
λ^2_{max} / nm	607.3 ± 0.5	605.5 ± 0.2	608.4 ± 1.7
w_2 / nm	32.3 ± 0.4	33.7 ± 0.2	32.2 ± 1.6
A_2	7.9 ± 0.2	17.2 ± 0.2	6.2 ± 0.6
f_1 ($A_1/(A_1+A_2)$)	0.18	0.11	0.37
f_2 ($A_2/(A_1+A_2)$)	0.82	0.89	0.63

unfolding at the lipid-water interface. It is supposed that unfolding of proteins adsorbing onto negatively charged membranes is promoted by the low interfacial pH created by proton accumulation in the vicinity of anionic lipid headgroups. The more highly charged state of the protein at low pH may enhance side chain charge repulsion on the protein surface, thereby giving rise to a more open structure with exposed hydrophobic patches. However, pH lowering is not the only way by which a lipid bilayer may act as a structure-forming environment. Factors such as formation of ionic protein-lipid contacts and hydrophobic mismatch may also considerably modify protein conformation. Specifically, it has been hypothesized that interfacial destabilization of Hb structure is largely determined by electrostatic attraction between positively charged iron in the haem and the negative charges of the lipid headgroups [19, 20]. Possible consequences of ionic haem-membrane interactions involve displacement and reorientation of the haem group, detachment of haem from the globin and deconjugation of the porphyrin ring.

All these considerations provide a basis for the assumption that lipid-induced modification of Hb structure is coupled with the appearance of new protein-binding sites for R101. There exist at least two processes that may account for the altered reactivity of membrane-bound Hb: (i) protein unfolding at the lipid-water interface; and (ii) loosening and splitting of the bonds between haem and globin. These processes are closely interrelated and strongly depend on the membrane surface charge. On the other hand, it seems highly probable that membrane-mediated detachment of the prosthetic group from the globin favours specific association of R101 with the haem cavity. The validity of this idea comes from the facts that aromatic dyes, particularly Rose Bengal and 1-anilino-8-naphthalene sulfonate, can specifically bind to apohaemoglobin and compete with haem for globin binding sites [24, 25].

To summarize, the present study demonstrates that xanthene dye R101, which is commonly regarded as environmentally insensitive, exhibits spectral features pointing to the possibility of its use for detecting conformational transitions of Hb. Significant changes in the shape of R101 fluorescence spectra and the appearance of an emission maximum around 590 seem to be the major hallmarks of Hb structural alterations at the lipid-water interface. Although the ability of R101 to sense conformational changes of other proteins remains to be clarified, this probe may prove useful in elucidating both general and specific mechanisms by which lipids tend to modify Hb structure. Such studies are of interest in view of lipid involvement in the low affinity Hb binding to the inner surface of red blood cell membranes [26]. Finally, better understanding of conformational behaviour of interfacially adsorbed Hb is of great significance in practical aspects related to a more rational design of haemosome-based blood substitutes [27].

REFERENCES

1. Lakowicz, J.R. *Principles of Fluorescent Spectroscopy*, 3rd edn. New York: Plenum Press (2006).
2. Valeur, B. *Molecular Fluorescence*. Weinheim: Wiley VCH (2002).
3. Klymchenko, A.S., Duportail, G., Demchenko, A.P. and Mely, Y. Bimodal distribution and fluorescence response of environment-sensitive probes in lipid bilayers. *Biophys. J.* **86** (2004) 2929–2941.
4. Cohen, B.E., Pralle, A., Yao, X., Swaminath, G., Gandhi, C.S., Jan, Y. Kobilka, B.K. Isakoff, E.Y. and Jan, L.Y. A fluorescent probe designed for studying protein conformational changes. *Proc. Natl Acad. Sci. USA* **102** (2005) 965–970.
5. Haidekker, M.A., Ling, T., Anglo, M., Stevens, H.Y., Frangos, J.A. and Theodorakis, E.A. New fluorescent probes for the measurement of cell membrane viscosity. *Chem. Biol.* **8** (2001) 123–131.
6. Chattopadhyay, A. and Raghuraman, H. Application of fluorescence spectroscopy to membrane protein structure and dynamics. *Current Science* **87** (2004) 175–180.
7. Bossi, L., Alema, S., Calissano, P. and Marra, E. Interaction of different forms of haemoglobin with artificial lipid membranes. *Biochim. Biophys. Acta* **375** (1975) 477–482.
8. Shviro, Y., Zilber, I. and Shaklai, N. The interaction of haemoglobin with phosphatidylserine vesicles. *Biochim. Biophys. Acta* **687** (1982) 63–70.
9. Chupin, V., Ushakova, I., Bondarenko, S., Vasilenko, I., Serebrennikova, G., Evstigneeva, R., Rosenberg, G. and Koltsova, G. ^{31}P-NMR study of methemoglobin interaction with model membranes. *Bioorg. Chem.* **9** (1982) 1275–1280.
10. Pitcher III, W., Keller, S. and Huestis, W. Interaction of nominally soluble proteins with phospholipid monolayers at the air-water interface. *Biochim. Biophys. Acta* **1564** (2002) 107–113.
11. Mui, B., Chow, L. and Hope, M.J. Extrusion technique to generate liposomes of defined size. *Meth. Enzymol.* **367** (2003) 3–14.
12. Bartlett, G. Phosphorus assay in column chromatography. *J. Biol. Chem.* **234** (1959) 466–468.
13. Bulychev, A.A., Verchoturov, V.N. and Gulaev, B.A. *Current Methods of Biophysical Studies*. Moscow: Vyschaya Shkola (1988).
14. Karstens, T. and Kobs, K. Rhodamine B and rhodamine 101 as reference substances for fluorescence quantum yield measurements. *J. Phys. Chem.* **84** (1980)1871–1872.
15. Drexhage, K.H. Structure and properties of laser dyes. In: *Dye Lasers*. New York: Springer (1977).
16. Lewis, G.N. and Calvin, M. The color of organic substances. *Chem. Rev.* **25** (1939) 320–328.
17. Drexhage, K.H. What's ahead in laser dyes? *Laser Focus* **9** (1973) 35–39.
18. Gutteridge, J. Age pigments and free radicals: fluorescent lipid complexes formed by iron and copper-containing proteins. *Biochim. Biophys. Acta* **834** (1985) 144–148.
19. Szebeni, J., Hauser, H., Eskelson, C., Watson, R. and Winterhalter, K. Interaction of haemoglobin derivatives with liposomes. Membrane cholesterol protects against the changes of haemoglobin. *Biochemistry* **27** (1988) 6425–6434.
20. Marva, E. and Hubbel, R. Denaturing interaction between sickle haemoglobin and phosphatidylserine liposomes. *Blood* **83** (1994) 242–249.
21. Szebeni, J., Di Lorio, E., Hauser, H. and Winterhalter, K. Encapsulation of haemoglobin in phospholipid liposomes: characterization and stability. *Biochemistry* **24** (1985) 2827–2832.
22. Gorbenko, G.P. Resonance energy transfer study of haemoglobin complexes with model phospholipid membranes. *Biophys. Chem.* **81** (1999) 93–105.
23. Kimelberg, H. Protein-liposome interactions and their relevance to the structure and function of cell membranes. *Mol. Cell. Biochem.* **10** (1976) 171–190.
24. Coulson, A.P. and Yonetani, T. Interaction of Rose Bengal with apo-haemoproteins: an essential histidine residue in cytochrome c peroxidase. *Eur. J. Biochem.* **26** (1972) 125–131.
25. Stryer, L. The interaction of a naphthalene dye with apomyoglobin and apohemoglobin. A fluorescent probe of non-polar binding sites. *J. Mol. Biol.* **13** (1965) 482–495
26. Shaklai, N., Yguerabide, J., Ranney, H. Classification and localization of haemoglobin binding sites on the red blood cell membrane. *Biochemistry* **16** (1977) 5593–5597.
27. Stowell, C.P. Haemoglobin-based oxygen carriers. *Current Opinion Haematol.* **9** (2002) 537–543.