Protoporphyrin IX-induced structural and functional changes in human red blood cells, haemoglobin and myoglobin

SUSMITA SIL, TANIA BOSE, DIBYENDU ROY and ABHAY SANKAR CHAKRABORTI*

Department of Biophysics, Molecular Biology and Genetics, University College of Science, 92, Acharyya Prafulla Chandra Road, Kolkata 700 009, India

**Corresponding author (Email, aschak@cubmb.ernet.in)*

Protoporphyrin IX and its derivatives are used as photosensitizers in the photodynamic therapy of cancer. Protoporphyrin IX penetrates into human red blood cells and releases oxygen from them. This leads to a change in the morphology of the cells. Spectrophotometric studies reveal that protoporphyrin IX interacts with haemoglobin and myoglobin forming ground state complexes. For both proteins, the binding affinity constant decreases, while the possible number of binding sites increases, as the aggregation state of the porphyrin is increased. The interactions lead to conformational changes of both haemoglobin and myoglobin as observed in circular dichroism studies. Upon binding with the proteins, protoporphyrin IX releases the heme-bound oxygen from the oxyproteins, which is dependent on the stoichiometric ratios of the porphyrin:protein. The peroxidase activities of haemoglobin and myoglobin are potentiated by the protein-porphyrin complexation. Possible mechanisms underlying the relation between the porphyrin-induced structural modifications of the heme proteins and alterations in their functional properties have been discussed. The findings may have a role in establishing efficacy of therapeutic uses of porphyrins as well as in elucidating their mechanisms of action as therapeutic agents.

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1. Introduction

Porphyrins are a class of tetrapyrroles that have attracted the attention of researchers worldwide owing to their extensive application as photosensitizing drugs in medicine (Joussen *et al* 1997; Nauta *et al* 1997; Peng *et al* 1997; Lilge and Wilson 1998; Nseyo *et al* 1998). Porphyrin IX species such as deuteroporphyrin IX, haematoporphyrin IX, haematoporphyrin derivative, mesoporphyrin IX and protoporphyrin IX are widely used photosensitizers in photodynamic therapy (PDT), a new modality of cancer treatment (Stables and Ash 1995; Dougherty *et al* 1998). This treatment involves administration of photosensitizer and then an incubation period of 48–72 h is allowed, followed by treatment with light. During this interval, porphyrins get localized in tissues, preferentially in tumours. When the tumour region is exposed to visible light or near infrared light, the photosensitizers are activated, yielding highly reactive oxygen species, which causes oxidative damage to variety of cellular targets and a subsequent cell death resulting in tumour ablation (Fisher *et al* 1995; Reynolds 1997). PDT-induced apoptosis has been reported under both *in vitro* and *in vivo* situations (He *et al* 1994; Ahmed *et al* 1998). However, the mechanism of the PDT-mediated cytotoxic effect is not yet fully understood. Serum albumin, low density and high density lipoproteins act as the endogenous carriers of porphyrins in the circulation (Kessel 1986; Jain 1992). Migration of porphyrins within the tissue involves initial binding to very hydrophobic loci, representing lipid-rich membrane domains and then slow movement to more hydrophilic intracellular regions (Ricchelli *et al* 1995).

Keywords. Drug-protein binding; haemoglobin; myoglobin; protoporphyrin IX; red blood cells

Abbreviations used: cd, Circular dichroism; EPP, erythropoietic protoporphyria; Hb, haemoglobin; HRP, horseradish peroxidase; Mb, oxymyoglobin; PDT, photodynamic therapy; PP, protoporphyrin; RBC, red blood cells; ZPP, zinc protoporphyrin.

Cellular specificity as well as extra cellular tumour characteristics are suggested to be responsible for the specific uptake of porphyrins by malignant tissues (Jain 1992). However, in the process of porphyrin transport, it may also be concentrated in normal cells. It, therefore, seems logical to conceive that porphyrins can also penetrate the membranes of the red blood cells (RBC) and in turn, may affect their biological activities by interacting with haemoglobin (Hb). Cross-linking of cytoskeleton proteins in the erythrocytes has been reported due to membrane photodamage of protoporphyrin IX (PP) (Dadosh and Shaklai 1988) and hematoporphyrin derivative (Beaton *et al* 1995). Several reports have been published on Hb-porphyrin interactions (Sil and Chakraborti 1996; Sil *et al* 1997, 2000; Chakraborti 2003; Hirsch *et al* 1993). Interactions between Hb and PP are particularly important because in erythropoietic protoporphyria (EPP) the amount of PP in the erythrocytes is significantly increased (Lamon *et al* 1990). PP remains bound to Hb in the erythrocytes and it is transferred from the erythocytes to skin endothelial cells for the expression of photosensitivity in these patients. Hb, an allosteric protein, plays an important role in carrying oxygen from lungs to different tissues. The oxygen affinity of Hb has been reported to increase in erythropoietic protoporphyric red cells and their hemolysates (Hirsch *et al* 1993). In lead intoxication also porphyrin is accumulated in the erythrocytes, but it is present as zinc protoporphyrin (ZPP) (Lamola *et al* 1975). The interaction of ZPP with Hb causes the opposite effect (Hirsch *et al* 1989). The oxygen affinity of Hb decreases with increasing mole ratio of ZPP. Different protoporphyrins (PP, ZPP) may, therefore, alter Hb function in different ways.

Oxymyoglobin (Mb) is another important heme-containing oxygen storage protein and present in muscle cells. Under conditions of oxygen deprivation e.g. severe exercise, the protein readily releases its bound oxygen for the oxidative synthesis of ATP by muscle mitochondria. Mb is very similar to Hb in its a-helical conformation and three-dimensional structural organization. However, unlike tetrameric Hb with its four interacting subunits (a_2b_2), Mb is a single chain monomeric protein and has no subunit interaction (Stryer 1995). Porphyrins are also believed to penetrate the membranes of the muscle cells and come in contact with Mb.

To verify the membrane permeability of porphyrins, here we have studied the effect of PP on the morphology of the RBC in the absence of light exposure and if it affects their function with respect to oxygen storage and transport. Interaction of Hb as well as Mb with exogenously added PP was reported earlier from this laboratory by spectrofluorometric study (Sil and Chakraborti 1996). In the present study, absorption difference spectroscopy has been used to find ground state complex formation between PP and heme proteins – Hb and Mb. The binding parameters namely, binding affinity constant and the possible number of binding sites have been estimated from the spectral changes of the proteins due to gradual addition of the porphyrin. Since there is no report on the exogenously added PP on structural and functional properties of Hb or Mb, we have studied PP-induced changes of their functions with respect to oxygen storage. Besides, being the transporter of oxygen, both Hb and Mb possess other functional activities such as peroxidase activity (Giardina *et al* 1995; Patel *et al* 1996), which has been reported to be enhanced by hematoporphyrin (HP) (Sil *et al* 2000; Sil and Chakraborti 2002). We have investigated here the PP-induced change in peroxidase activities of Hb and Mb. The findings may be important in suggesting the altered Hb function in erythropoietic protoporphyria. The functional modifications of Hb and Mb may be associated with their structural modifications as demonstrated by circular dichroic study. The effects of PP on RBC, Hb and Mb as reported in this communication should be given due consideration in elucidating the details of the mechanism of porphyrin actions in therapy.

2. Materials and methods

2.1 *Materials*

Disodium protoporphyrin IX (PP), horse heart myoglobin, o-dianisidine, superoxide dismutase (SOD), NADH, sephadex G-100 and G-25 were purchased from Sigma Chemical Company, USA. Other chemicals used were of analytical grade and purchased locally.

2.2 *Preparation of PP solution*

PP crystals taken in 0·15 M NaCl was stirred for an hour and centrifuged. A known amount of aliquot from the supernatant (stock solution) was diluted in final concentration of 2·7 N HCl. The concentration of PP in this acidic solution was determined using extinction coefficient $e_{408nm} = 262\ mM^{-1}\ cm^{-1}$ in 2·7 N HCl (Falk 1964). The stock solution was properly diluted with 0·15 M NaCl for the experiments. Solutions of PP were always freshly prepared and protected from light.

2.3 *Preparation of RBC and Hb*

Fresh RBC were purified from human blood donated by healthy non-smoking volunteers, aged 22–25 years. Hb was isolated and purified by hemolysing the intact RBC and gel filtration (sephadex G-100) according to the method of Bhattacharyya *et al* (1990). The isolated Hb in 0·15 M

NaCl showed the characteristic absorption spectrum possessing three peaks at 415 nm (Soret band), 540 nm and 577 nm. The concentration of the stock solution was determined from its soret absorbances using an extinction coefficient value of 125 mM^{-1} cm^{-1} (heme basis). The concentration of Hb was expressed in heme basis, unless otherwise stated. The percent of oxygenation (Y) for Hb was estimated from its absorption spectrum according to relation of Huang and Redfield (1976):

$$Y = [12{\cdot}23 - 9{\cdot}92\ (A_{560}/A_{540})]/[3{\cdot}77 + 3{\cdot}88\ (A_{560}/A_{540})],$$

where A_{560} and A_{540} are the Hb absorbances at 560 nm and 540 nm, respectively. The protein was found to be nearly 99% oxygenated.

2.4 *Preparation of Mb solution*

The myoglobin purchased was mostly in the form of metmyoglobin. It was converted to the oxy form by sodium dithionite and purified by sephadex G-25 column chromatography as described before (Sil and Chakraborti 2002). The absorption spectra of the untreated and treated myoglobin samples in normal saline were distinctly different which confirmed the conversion of metmyoglobin to Mb (Smith and Gibson 1959; Dixon and McIntosh 1967). The absorption spectrum of metmyoglobin in the wavelength range 380–700 nm showed absorption peaks at 408 nm (Soret peak), 501 nm and 630 nm. The peaks at 501 nm and 630 nm were quite broad. Mb in the same wavelength region also showed three absorption peaks but at different positions – at 418 nm (Soret peak), 540 nm and 577 nm. The concentration of Mb solution so prepared was determined from its molar extinction coefficient value $e_{418nm} = 128\ mM^{-1}\ cm^{-1}$ (Witenberg and Witenberg 1981). Its oxygen content was measured (Huang and Redfield 1976) and was found to be approximately 98% oxygenated.

2.5 *Light microscopic experiments on porphyrin-treated and untreated RBC*

The effect of PP on RBC was studied using a compound light microscope (Carl Zeiss Axioscope). The cells were suspended in normal saline and incubated in dark with or without PP at 25°C for 5 min. Thirty µl of the control or PP-treated (8·0 µM and 12·0 µM) RBC (approximately 10^7 cells) were taken on a clean glass slide, covered with a cover slip and observed under microscope. The cells were photographed within 15 min of treatment. Alteration in the morphology of the cells was determined from the increase in diameter and crenated surface.

2.6 *Oxygen release experiments*

Oxygen release from RBC, Hb and Mb in the presence of PP was measured in a Gilson 5/6-oxygraph machine. The change in partial pressure due to released oxygen in the Hb or Mb solution (8 µM) or RBC suspension ($1{\cdot}8 \times 10^7$ cells) in 1·8 ml 0·15 M NaCl in a stoppered cell was detected by the membrane-covered oxygen electrode fitted with the cell. The output signal was recorded in the oxygraph chart as a function of time. Normal saline alone showed no change in the output signal even after 30 min stirring. The amount of free dissolved oxygen in normal saline was taken to be 250 nmol (West 1985). Calibration of the oxygraph chart in terms of the nmol oxygen release was made from the change in the output signal due to the total depletion of free oxygen from 2 ml normal saline when 0·1 g sodium metabisulfite was added. No oxygen was found to be released from RBC suspension or Hb and Mb solution in the absence of the porphyrin. The temperature during the experiment was maintained at 25°C.

2.7 *Hb-PP and Mb-PP binding experiments by absorption spectroscopy*

The interactions of PP with Hb and Mb were studied spectrophotometrically (difference absorption spectroscopy). The experiments were done in a Hitachi U2000 spectrophotometer using 3 ml quartz cuvette of pathlength 1 cm. Three ml of fixed concentration of Hb or Mb in normal saline was taken in the sample cuvette and the same volume of normal saline was taken in the reference cuvette. Absorption spectrum from 380–650 nm was recorded. The protein absorbances were titrated by gradual addition of PP from a concentrated stock solution to both the sample and reference cuvettes. Binding studies were also done by reverse titration. In this case, the absorption change of a fixed concentration of porphyrin was observed with increasing concentration of Hb or Mb. The volume increment due to addition of protein to both the sample and reference cuvettes was negligible. The binding affinity constants and the number of binding sites were estimated following the methods as described in §3.

2.8 *Circular dichroic experiments*

Circular dichroism (cd) measurements (210–250 nm) of 1 µM Hb and Mb were done in a Jasco-600 spectropolarimeter using a quartz cuvette of pathlength 1 cm. Molar ellipticity, $[q]$ values were obtained using the relation (Geraci and Parkhurst 1981):

$$[q] = [M_r W]\ q/10{\cdot}l{\cdot}c,$$

where c (g ml^{-1}) is the concentration of protein, q (mdeg), obtained directly from the chart, is the observed rotation, l (cm) is the pathlength and M_rW is the mean residual molecular weight of the protein ($[M_rW] = 110$). The a-helical contents of the protein in the presence and absence of the porphyrin were estimated from the spectra according to the relation (Chen *et al* 1972):

$$\text{Fraction of } a\text{-helix} = ([q]_{222} + 2340)/-30300,$$

where $[q]_{222}$ is the ellipticity at 222 nm.

2.9 *Assay of peroxidase activities of Hb and Mb*

The peroxidase activity of Hb or Mb with respect to o-dianisidine oxidation was assayed spectrophotometrically (Everse *et al* 1994). The reaction mixture (2 ml) contained 1·8 ml of sodium phosphate-citrate buffer (50 mM), pH 5·4, Hb (1·5 μM) or Mb (0·43 μM) and various concentrations of PP as indicated. The reaction was initiated by addition of a mixture 0·002% o-dianisidine and 17·6 mM H_2O_2. The assay was done at ambient temperature with Hb or Mb and buffer in the reference cuvette. The increase in absorbance at 450 nm was monitored continuously for 2 min.

The peroxidase activity was also assayed with respect to NADH oxidation. The reaction mixture (1 ml) contained 0·5 ml of sodium phosphate-citrate buffer, 2·3 μM Hb or 1·9 μM Mb, 0·1 M NADH and various concentrations of PP. The reaction was initiated by addition of 17·6 mM H_2O_2. The change in absorbance at 340 nm was followed for 2 min with Hb or Mb and buffer in the reference cuvette. The effect of SOD on Hb or Mb-catalysed peroxidation was studied by adding SOD instead of PP.

3. Results

3.1 *Effect of PP on the morphology of human RBC*

Normal human RBC was observed under the light microscope in the absence and presence of PP. Untreated cells exhibited their normal discoid shapes with smooth membranous boundary (figure 1a). Upon treatment with PP (8·0 μM and 12·0 μM), the regular smooth surface of these cells gradually turned wrinkled and the cells became enlarged, which have been termed as being deformed (figure 1b,c). As shown in the figure, the extent of the induced enlargement of the cell diameter, crenation of the cell surface as well as the proportion of the number of affected cells in a particular field were found to be dependent on the concentration of the porphyrin. The diameters of 40 randomly selected untreated and treated cells from each plate were measured cross-diagonally and averaged and shown in table 1. The average normal cell diameter of 7·10 μm (figure 1a) increased to an average of 7·94 μm (figure 1b) in the presence of 8·0 μM PP, with an approximately 50% cells being affected. With further increase in PP concentration (12·0 μM), the average cell diameter was found to enhance to 8·45 μm (figure 1c) and approximately

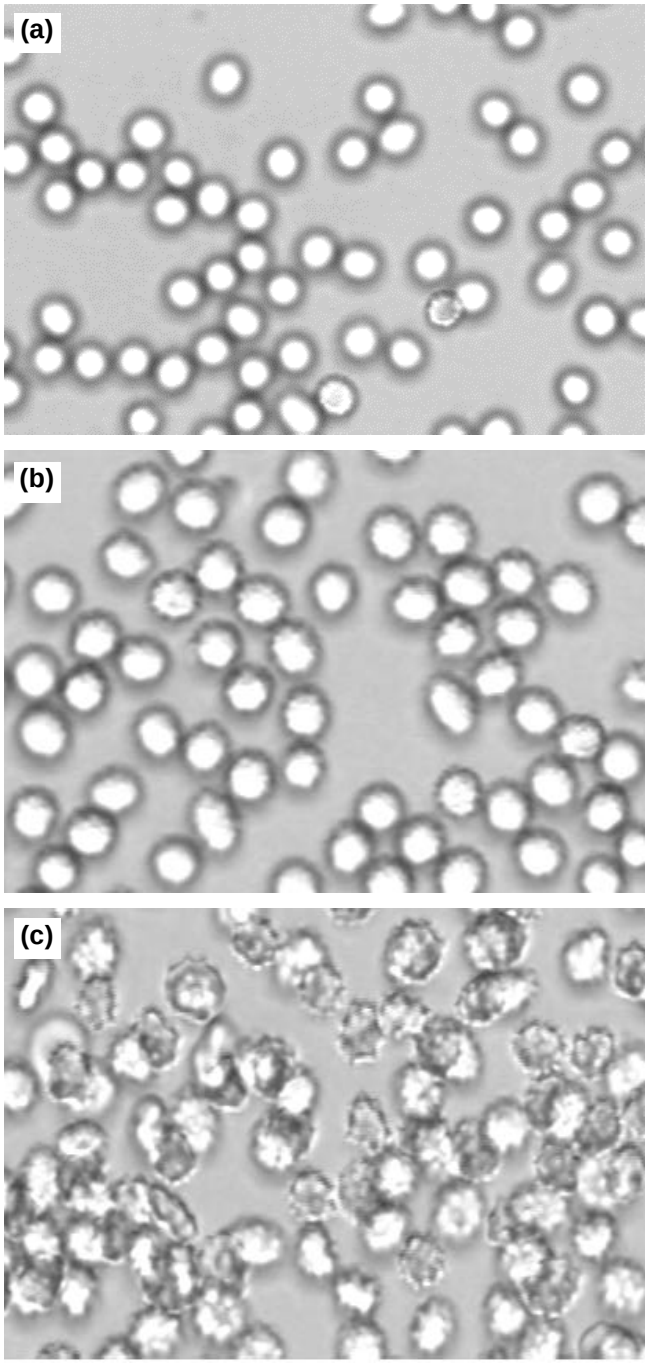

Figure 1. Photomicrographs of human red blood cells untreated **(a)** and treated with 8·0 μM PP **(b)** and 12·0 μM PP **(c)** for 5 min at 25°C. (Magnification = 1000 ×).

76% cells appeared to be deformed. Longer incubation with PP or treatment with higher concentration of the drug initiated considerable lysis of the cells. For example, incubation of RBC with 12·0 μM PP for 30 min caused considerable cell lysis (data not shown).

3.2 *Effect of PP on oxygen release from RBC*

Oxygraph experiment done with freshly isolated RBC (approximately $1{\cdot}8 \times 10^7$ cells) showed that PP liberated oxygen from these cells. Figure 2 is the oxygraph chart record exhibiting the PP-induced oxygen release. Although the extent of released oxygen was low, there was an increase in the proportion of the released oxygen with increased porphyrin concentration. Oxygen was not released from RBC suspension in the absence of PP. This result suggests that PP penetrated into RBC and interacted with Hb, from which oxygen was released.

3.3 *Spectrophotometric study on interaction of PP with Hb and Mb*

Absorption spectrophotometric technique (difference spectroscopy) was used to investigate the ground state complex formation between PP and Hb as well as Mb.

Table 1. Alteration in the morphology of human RBCs induced by PP.

	Treatment with PP (in dark)		
Measurement	0 μM	8·0 μM	12·0 μM
RBC diameter (μm)	7·10 ± 0·61	7·94 ± 0·80	8·45 ± 0·86
Deformed cells (%)	0	50	76

Number of cells examined in each case = 40.

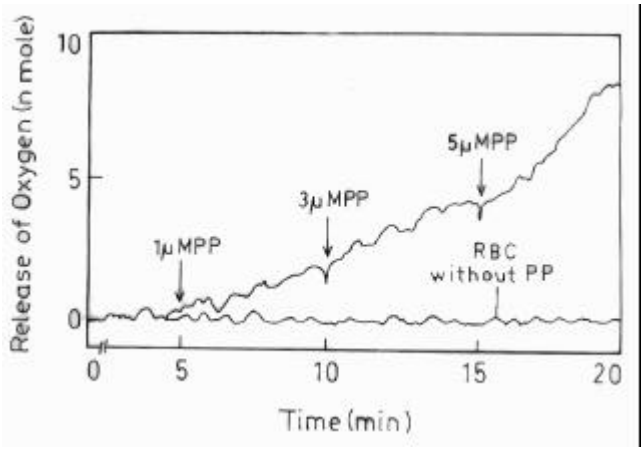

Figure 2. Gilson 5/6 oxygraph chart of the release of oxygen from red blood cells (10^7 cells/ml) in 0·15 M NaCl due to gradual addition of PP. Arrows indicate the addition of porphyrins (final concentration at regular time interval of 5 min).

Figure 3 shows the absorption spectral change of Hb in the presence of different concentration of PP in the wavelength region 380–650 nm. In this representative experiment, PP concentration range used was 2·0 μM to 6·7 μM to titrate 5·0 μM Hb. The spectral change in the wavelength region 500–600 nm is shown with an enlarged scale (inset). Three isosbestic points were noticed at 431 nm, 523 nm and 584 nm. Figure 4 represents absorption spectral change of Mb (8·2 μM) due to gradual addition of PP (0·8 μM to 5·4 μM). The single isosbestic point appeared at 430 nm. Thus PP formed stable ground state complexes with both Hb and Mb.

The binding affinity constant, *K* for the interactions was determined by analysing the reduced absorbances at Soret band (415 nm for Hb and 418 nm for Mb) due to

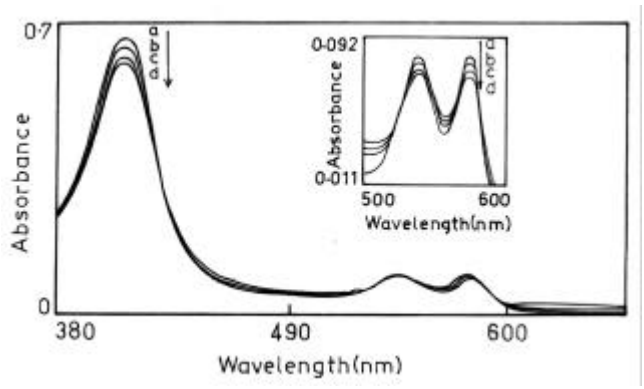

Figure 3. Representative difference absorption spectra of 5·0 μM Hb titrated with varying concentrations of PP. Porphyrin concentrations used during titration of the protein are 0 μM PP (a), 2·1 μM PP (b), 3·9 μM PP (c) and 6·7 μM PP (d). *Inset*: The same absorption spectra of Hb in the absence and presence of PP from 500–600 nm with enlarged scale.

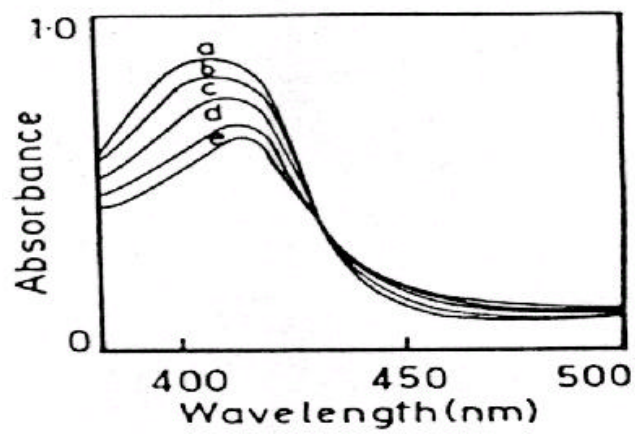

Figure 4. Representative difference absorption spectra of 8·2 μM Mb in the absence (a) and presence of 0·8 μM PP (b), 1·9 μM PP (c), 3·7 μM PP (d) and 5·4 μM PP (e).

gradual addition of PP to the protein solutions following the relation of Kapp *et al* (1990):

$$A_0/\Delta A = A_0/\Delta A_{max} + A_0/\Delta A_{max} \cdot 1/K \cdot 1/L_t,$$

where $\Delta A = A_0 - A$; A_0 and A are the protein absorbances in the absence and presence of the ligand (L_t), respectively. ΔA_{max} represents the maximally reduced absorbance. In the linear plots $A_0/\Delta A$ vs $1/L_t$, $A_0/\Delta A_{max}$ is obtained from the intercept on the $A_0/\Delta A$ axis, corresponding to $1/L_t = 0$. In figure 5 representative plots have been shown for Hb-PP interactions using two different concentration ranges of PP (0·4 μM to 1·2 μM and 1·6 μM to 2·9 μM). Similarly, plots were obtained with different concentration ranges of PP for interactions with Mb (not shown). The binding affinity constants for the interaction of PP with Hb and Mb were evaluated from the slope of the plots and the results have been summarised in table 2. K value was found to decrease with increasing concentration ranges of PP.

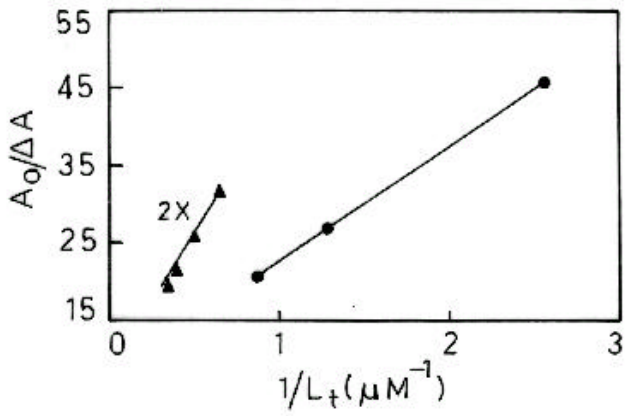

Figure 5. Plot of $A_0/\Delta A$ versus $1/L_t$ for estimation of the binding affinity constant in the interaction of Hb with PP. $\Delta A = A_0 - A$, where A_0 and A were Hb absorbances in the absence and presence of PP (L_t), respectively. Five μM Hb was titrated with 0·4–1·2 μM PP (•) and 1·6–2·9 μM PP (▲). $A_0/\Delta A$ values obtained in the higher concentration range of PP were multiplied by 2 to have a suitable scale for graphical representation.

The possible number of binding sites, p was determined from the linear plot of $1/(1–q)$ vs L_t/q as shown in figure 6 for Hb-PP interactions following the relation (Kapp *et al* 1990):

$$1/(1 - q) = K \cdot L_t/q - K \cdot p \cdot A_t,$$

where q (= $\Delta A/\Delta A_{max}$) is the fractional saturation of PP binding sites and A_t is the fixed concentration of the protein. As shown in table 2, increased concentration ranges of porphyrin led to an increase in the values of the possible number of binding sites for both Hb-PP and Mb-PP interactions.

In aqueous medium, porphyrins tend to aggregate among themselves; the smaller aggregate being a dimer and the higher order species being micelle-like polymers (Margalit *et al* 1983). Therefore, one possible objection to the above results is that we have gradually increased the concentration of PP in binding experiments and each addition may shift the monomer/dimer/higher aggregation distribution, which may affect the accurate determination of the binding parameters. The answer to this objection is

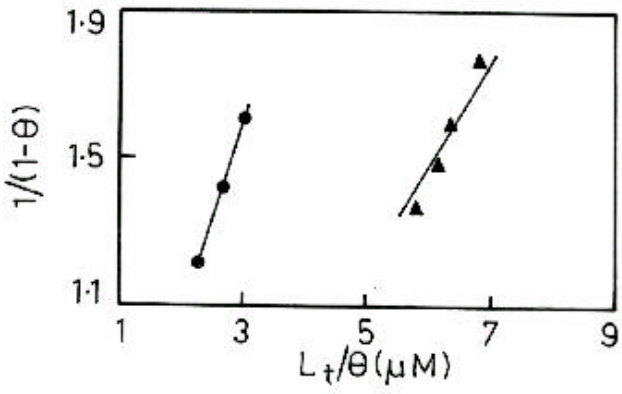

Figure 6. Plot of $1/(1–q)$ versus L_t/q for spectrophotometric estimation of the possible number of binding sites (p) involved in the interaction of Hb with PP. q = extent of binding ($\Delta A/\Delta A_{max}$) and L_t = PP concentration. Five μM Hb was titrated with 0·4–1·2 μM PP (•) and 1·6–2·9 μM PP (▲).

Table 2. Estimated values of binding affinity constant (K) and possible number of binding sites (p) for binding of Hb and Mb with PP.

PP concentration range (μM)	Affinity constant, K ($\times 10^5$ M^{-1})		Number of binding sites, p	
	Hb	Mb	Hb	Mb
0·4–1·2	5·25 ± 0·64	9·04 ± 1·06	0·12 ± 0·01	0·04 ± 0·006
1·6–2·9	2·42 ± 0·18	4·00 ± 0·35	0·54 ± 0·04	0·08 ± 0·006
3·5–6·0	1·06 ± 0·15	0·63 ± 0·10	1·06 ± 0·12	0·31 ± 0·03

5 μM Hb (heme basis) and 8 μM Mb were used. Number of binding sites for Hb was expressed on tetramer basis. Results are mean of 4 experiments ± SD.

that the linearity of the titration experiments for PP added to Hb or Mb in different concentration ranges (0·4–1·2 μM, 1·6–2·9 μM and 3·5–6·0 μM) is consistent with binding between the proteins and porphyrin. However, to avoid the possibility of interference due to the shift in PP species distribution during titration, the binding experiment was also done by reverse titration i.e. the absorbance of a fixed concentration of PP was titrated by gradual increase of Hb or Mb concentration. As in the case of Hb titration with PP (figure 3), titration of PP (4·3 μM) with Hb also exhibited three isosbestic points around 400 nm, 500 nm, and 550 nm (figure 7). The binding affinity constant, K and the possible number of binding sites, p calculated from the reverse titration method were found to be $3{\cdot}49 \times 10^5\ M^{-1}$ and 0·14, respectively (the plots not shown). The results are again in conformity with those obtained by titration of the protein with gradual addition of the porphyrin. Similar reverse titration experiments with Mb and PP were also consistent with that of the corresponding previous ones (data not shown).

3.4 *Effect of PP on conformations of Hb and Mb*

To understand the conformational change of Hb and Mb, if any, upon binding with PP the far UV cd spectra was studied. Figure 8 shows the far UV (210–250 nm) cd spectra of Mb in the absence and presence of various concentration of PP. In this region of wavelength, a cd measurement of a protein gives information about its conformation in relation to the secondary structure. The untreated Mb showed its characteristic cd spectrum asserting its high α-helical content (approximately 70%). The negative ellipticity of the protein at 222 nm gradually reduced with increased addition of PP. This suggests that the complexation of Mb with PP decreased the α-helical content of the protein. Similar result was obtained with Hb (spectra not shown). However, the extent of decrease in α-helicity of Hb induced by PP was less than that of Mb. For example, in one representative experiment shown in table 3, 4·5 μM PP reduced the α-helicity of 1·0 μM Hb by about 5·2%, while that of 1·0 μM Mb by about 16%.

3.5 *Effect of PP on oxygen release from Hb and Mb*

The reversible binding of oxygen with heme iron in Hb and Mb is brought about by the folding of the polypeptide chains around the heme groups to enclose them in hydrophobic pockets. Therefore, any alteration in the three-dimensional structure of the proteins caused by ligand binding, can significantly affect the oxygen affinity of these oxyproteins. To test this functional modification, effect of PP on oxygen affinity of Hb and Mb was recorded in a Gilson 5/6 oxygraph machine. Oxygen (in nmol) released from a fixed concentration (8 μM) of Hb and Mb was estimated after addition of different concentration of PP (oxygraph charts not shown). The percentage of oxy-

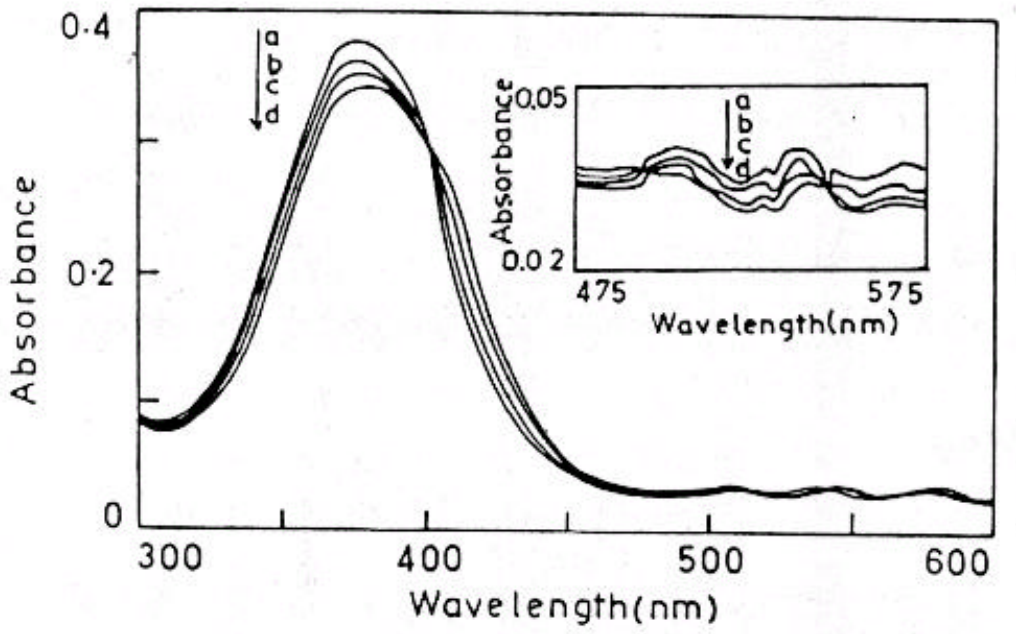

Figure 7. Representative difference absorption spectra of 4·3 μM PP in the absence (a) and presence of 0·6 μM Hb (b), 1·1 μM Hb (c) and 1·9 μM Hb (d). *Inset*: The same absorption spectra of porphyrins in the absence and presence of Hb from 475–575 nm with enlarged scale.

gen release was directly proportional to the stoichiometric ratio of porphyrin : protein as shown in table 4. The conformational change in the globin part of the protein molecules due to interaction with PP might thus be related with their oxygen affinities.

3.6 *Effect of PP on peroxidase activities of Hb and Mb*

The effect of PP on the peroxidase activities of Hb and Mb with respect to the oxidation of o-dianisidine and NADH were studied. The results have been presented in table 5. In the absence of Hb or Mb, PP had very little peroxidase activities with respect to the oxidation of o-dianisidine or NADH. However, both Hb and Mb-catalysed H_2O_2-mediated oxidation of o-dianisidine or NADH were increased by PP. PP-induced structural modification of proteins might thus be related to the enhanced peroxidase activities. SOD, which removes superoxide radicals, was also found to potentiate the peroxidase activities of Hb and Mb (table 5).

4. Discussion

PP treatment caused alteration in the morphology of RBC (figure 1a–c). In this study, the cells were not exposed to light, except during taking the photomicrographs. Light-induced effects of porphyrins on erythrocytes are known (Beaton *et al* 1995; Bolodon *et al* 1996). The present study suggests that PP also causes alteration in red cell morphology in the dark. The interaction of cell membranes is probably favoured with hydrophobic substituents of porphyrin molecules, causing alteration in the morphology of the cells. In the presence of light, the morphological change is likely to be more pronounced due to photodamage. The membrane structure alteration induced by porphyrins may facilitate their entry within the cells and may alter the physiological functions of the cells, which is evident from PP-induced oxygen release from RBC (figure 2). Interaction of PP with Hb may be responsible for the release of oxygen from these cells. Like RBC, PP may also accumulate within other cells e.g. muscle cells and may come in contact with Mb, leading to its functional modification.

We reported earlier in a spectrofluorometric study the formation of an excited state complex during interaction of PP with Hb and Mb (Sil and Chakraborti 1996). The mode of interaction of PP with Hb was found to be electrostatic, and that with Mb was hydrophobic. The thermodynamic analysis also corroborated this finding. The isosbestic points obtained in titration of Hb and Mb with PP in the present spectrophotometric study (figures 3 and 4) indicate the ground state complex formation between the porphyrin and proteins. This has also been confirmed by reverse titration i.e. titration of a fixed concentration

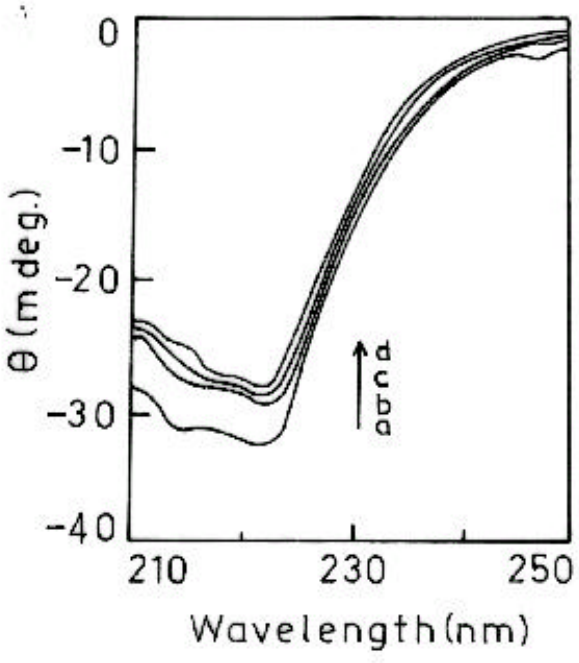

Figure 8. Representative circular dichroic spectra of Mb (1·0 μM) in the absence and presence of PP. PP concentrations used were 0 μM (a), 0·5 μM (b), 1·0 μM (c) and 3·0 μM (d).

Table 3. Effect of PP on *a*-helix contents of Hb and Mb.

	Percentage decrease in *a*-helicity	
PP concentration (μM)	Hb	Mb
0·5	1·3	10·2
1·0	3·7	11·2
1·5	4·0	13·5
3·0	4·8	14·0
4·5	5·2	16·1

Protein concentration used was 1 μM.

Table 4. Release of oxygen from Hb and Mb as a function of molar ratio of PP : protein.

Molar ratio of PP : Hb	Release of oxygen (%)	Molar ratio of PP : Mb	Release of oxygen (%)
0·25	18	0·25	11
0·50	32	0·50	29
0·75	35	0·75	40
1·00	40	1·00	52

Percent release of oxygen was estimated as nmol oxygen released from 1·8 ml 8 μM Hb or 8 μM Mb (oxygen content in each case = 14·5 nmol) in 0·15 M NaCl due to addition of different concentration of PP. The results are mean of four experiments (SD < 15%).

of the porphyrin with different concentrations of proteins (figure 7). The binding parameters (binding affinity constants and number of binding sites) of PP with both Hb and Mb change with the concentration ranges of the porphyrin. The binding affinity constant decreases, but the number of binding sites increases with increasing concentration ranges of PP (figures 5, 6 and table 2). Similar results were obtained by spectrofluorometric study (Sil and Chakraborti 1996). The findings may be explained by assuming the existence of independent binding sites on the proteins for different aggregated states of the porphyrin. Smaller aggregates present in low concentration range of PP probably bind with higher affinity than larger aggregates present in higher concentration range. Rotenberg *et al* (1987) have also reported the existence of two different sites, one for monomers and one for dimers, for porphyrin (deuteroporphyrin and HP) binding to human and bovine serum albumin. The interaction of proteins with aggregated states of porphyrins may be significant under certain physiological conditions. For example, high level of PP present in EPP (90–270 μM PP within the erythrocytes) may be in different aggregation states (Hirsch *et al* 1993). High concentrations of porphyrins (0·5–2 mg/kg body weight) administered in PDT get diluted after administration, but complete monomerization is not possible and may exert their effects as aggregated states (Margalit *et al* 1983; Smith and Ghiggino 1993).

Interaction of PP with Hb and Mb causes alterations in the secondary structures of the proteins reflected in the reduction of their *α*-helix contents (figure 8 and table 3). Compared to Hb, the effect is more pronounced in Mb, the reason of which may the subunit interaction property present and absent in the tetrameric Hb and monomeric Mb, respectively. The present finding is in agreement with the reports that HP induces alteration of secondary structures of Hb (Sil *et al* 2000), Mb (Sil and Chakraborti 2002) and bovine serum albumin (Timmins and Davies 1994). PP-induced conformational changes in the enzymes *d*-aminolevulinic acid dehydratase and porphobilinogen deaminase have been reported by Afonso *et al* (1997).

PP-induced conformational change of Hb and Mb might be associated with their functional modifications, because release of oxygen is closely coupled with the amount of porphyrin-bound heme proteins (table 4). However, according to Hirsch *et al* (1993), Hb in EPP exhibits increased oxygen affinity. This variation may be a result of difference in the state of aggregation of the porphyrins, because in this disease condition PP level in erythrocytes is increased to as high as 90–270 μM, whereas the normal erythrocyte PP level is approximately 2 μM. Oxygen release from Hb and Mb due to interactions with other drugs namely, phenothiazines – chlorpromazine and trifluoperazine have also been reported from this laboratory (Bhattacharyya *et al* 1996). The porphyrin-induced oxygen release from Hb and Mb can be of significance in case of PDT of cancer treatment. As the oxygen gets released, a hypoxic atmosphere may be created in RBC and muscle cells, which will become more dependent on glycolysis for energy supply. This leads to increased production of lactic acid (via anaerobic glycosis), thereby reducing the intracellular pH. As acidic environment favours localizaion of porphyrin within cells (Jain 1992), the released oxygen may facilitate enhanced accumulation of the porphyrin within the cells. Simultaneously, the porphyrins will get lesser amount of oxygen for generation of $^1O_2$, the prime functionally reactive species of porphyrin-induced PDT. Thus the PP-induced release of oxygen from Hb and Mb probably facilitates enhanced porphyrin accumulation within the cells, but reduces their efficiency.

Table 5. Effect of PP on peroxidase activities of Hb and Mb.

Reaction mixture	Rate of o-dianisidine oxidation (ΔOD_{450nm}/min)	Rate of NADH oxidation (ΔOD_{340nm}/min)
PP (control)	0·005 ± 0·002	0·003 ± 0·001
Hb (control)	0·222 ± 0·015	0·156 ± 0·010
Hb + PP (5·0 μM)	0·267 ± 0·030	0·175 ± 0·012
Hb + PP (10·0 μM)	0·301 ± 0·021	0·226 ± 0·015
Hb + SOD (3·0 units)	0·259 ± 0·020	0·185 ± 0·010
Mb (control)	0·195 ± 0·012	0·042 ± 0·003
Mb + PP (5·0 μM)	0·229 ± 0·015	0·058 ± 0·005
Mb + PP (10·0 μM)	0·247 ± 0·020	0·067 ± 0·004
Mb + SOD (3·0 units)	0·268 ± 0·018	0·057 ± 0·004

Hb concentrations used were 1·50 μM and 2.30 μM for estimation of o-dianisidine oxidation and NADH oxidation, respectively. Mb concentrations used were 0·43 μM and 1·9 μM for estimation of o-dianisidine and NADH oxidation, respectively. 10·0 μM PP was used in the control experiment. The values are mean of three experiments ± SD.

Hb and Mb possess peroxidase activities (Everse *et al* 1994; Giardina *et al* 1995; Patel *et al* 1996). The enhanced peroxidase activities of Hb and Mb by PP (table 5) may be due to the porphyrin-induced altered conformation of the proteins, because these two processes are positively correlated. However, there is another possibility for porphyrin-induced enhanced enzyme activity. Hb and Mb auto-oxidise, particularly at low pH, to generate superoxide radicals, which may inhibit the peroxidase activities (Balagopalkrishna *et al* 1998). It is probable that porphyrin inhibits the formation of superoxide radicals, thereby enhancing the peroxidase activities of the proteins. To verify this possibility, peroxidase activities of Hb and Mb were also measured in the presence of SOD. SOD also enhanced the enzyme activity (table 5), suggesting removal of superoxide radicals might be associated with potentiation effect of peroxidase activities of Hb and Mb. This view is also supported by porphyrin-induced potentiation of horseradish peroxidase (HRP)-catalysed oxidation of NAD(P)H (van Steveninck *et al* 1987, 1988; Sil and Chakraborti 1997). Superoxide radicals are generated in HRP/H_2O_2/NAD(P)H system (Takayama and Nakano 1977) and these radicals may inhibit the peroxidase activity of HRP (Fridovich 1986). van Steveninck *et al* (1987, 1988) have postulated that superoxide radical generation is prevented by the porphyrin hematoporphyrin derivative leading to the potentiation of HRP activity. Potentiation of HRP-catalysed NAD(P)H oxidation by SOD strengthens this view. It is not yet known how porphyrins remove or inhibit generation of superoxide radicals in HRP system. Interaction of PP with HRP as demonstrated from this laboratory (Sil and Chakraborti 1997) may be involved in this process, because potentiation of HRP activity has a positive correlation with the extent of binding of the protein with the porphyrin. Similar argument may also hold for PP-induced enhanced peroxidase activities of Hb and Mb. This increased activity may be hazardous and can cause enhanced lipid peroxidation, carbohydrate degradation or cross linking of proteins (Everse *et al* 1994).

Thus PP enters into the cells and interacts with Hb and Mb leading to structural and functional modifications of the proteins, which may be important in understanding the pathophysiological conditions such as EPP. The findings of this study should also be considered in establishing efficacy of therapeutic uses of porphyrins as well as in elucidating their mechanisms of action as therapeutic agents.

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