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Binding of rose bengal onto bovine serum albumin

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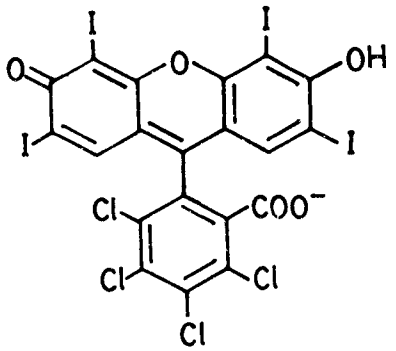
Abstract. The binding of rose bengal (RB) to bovine serum albumin (BSA) occurs with both the folded (at *p*H 7·4) and the unfolded (*p*H 12·7) forms of BSA. Absorption spectroscopy has revealed an identical red-shift of 15 nm in λ_{max} of RB in presence of BSA both at *p*H 7·4 and 12·7. The affinity constants (K) at *p*H 12·7 have been reduced only by 50% in magnitude from those at *p*H 7·4. These lead us to infer that neither disulphide loops nor buried residues are involved but that the binding of RB occurs at the sites near the surface of BSA. Moreover, the drastic alterations in the near-UV circular dichroism suggest tertiary structural changes induced by RB on binding to BSA. The conformational changes at the binding sites of BSA at *p*H 7·4 and the affinity of RB particularly towards the exposed residues in BSA at *p*H 12·7 are the significant factors in the binding of RB to BSA.

Keywords. Bovine serum albumin; rose bengal; binding; exposed residues; near-UV CD; conformational changes.

1. Introduction

Rose bengal (RB) is a fluorescein dye and has recently found application as a model organic anion for the liver plasma membrances (Yachi *et al* 1989). Binding studies with RB are of interest as the compound is found to exhibit photo-oxidizing property (Westhead 1965). However, there has not been any well-documented report on its interaction with proteins to suggest its role as a photo-oxidant for proteins. This is



Rose Bengal (RB)

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unlike the cases of methylene blue (MB) (Weil 1965; Spikes and MacKnight 1970; Knowles and Gurnani 1972; Nilsson *et al* 1972; Straight and Spikes 1985) and riboflavin (RF) (Kinsey and Frohman 1951; Zigler and Goosey 1981) which act as photo-oxidants for proteins. There are a number of reports illustrating the utility of MB and RF as sensitizers in understanding the mechanism of photo-oxidation especially of eye-lens proteins, namely crystallins (Bose *et al* 1985, 1986; Chakrabarti *et al* 1986; Mandal *et al* 1986). Moreover, the eye-lens proteins undergo conformational changes during photo-oxidation (Harding 1981; Liang and Chakrabarti 1981; Andley *et al* 1984; Liang *et al* 1985). The facile photo-oxidation of the exposed amino-acid residues as compared to that of buried ones has also been the subject of investigation recently (Mandal *et al* 1986; Walker and Borkman 1989).

In the light of these reports, the present study of RB–BSA interaction gains significance as the binding affinities of RB to two distinct conformational forms of BSA have been compared. Also, the conformational changes in BSA, if any, caused by RB have been monitored by circular dichroism. Though the present investigation does not analyse the photo-oxidation processes, this study can serve as a preliminary report for developing RB as a sensitizer in future investigations.

2. Experimental

Bovine serum albumin (BSA) (fatty acid-free) was obtained from the Sigma Chemical Co., USA. A pure sample of dye rose bengal (RB) (BDH, England) was used. Solutions of BSA were prepared based on the molecular weight of 69,000 and the concentrations were checked using $A^{1\%}_{1\,cm} = 6{\cdot}6$ (Janatova *et al* 1968). Experiments were carried out at *p*H 7·4 in 0·05 M Na_2HPO_4–KH_2PO_4 buffer and at *p*H 12·7 in 0·05 M Na_3PO_4 buffer.

Spectrophotometric experiments were carried out using Carl-Zeiss Specord UV-Vis spectrophotometer. For the experiments, constant [BSA] and varying [RB] were employed. Constant [BSA] of 2·0, 8·0 and 40·0 μm at *p*H 7·4 and constant [BSA] of 4·0, 10·0, and 20·0 μM at *p*H 12·7 were employed. [RB] were varied from 4·0 to 24·0 μM for each of these [BSA] at both the *p*H values. The absorbance measurements were made at 555 nm and the data were analysed by the Lineweaver–Burk method (Laidler 1978) for the determination of binding affinities.

Near-UV circular dichroism (CD) spectra at *p*H 7·4 were recorded on a Jasco J-500A spectropolarimeter linked to a data processor DP-501N. The studies have been made at constant [BSA] of 20·0 μM and varying [RB] of 10·0 to 90·0 μM. Several scans were taken to obtain reproducible CD spectra. Cell path lengths of 5 mm were used and results were expressed as molar ellipticities [θ] (deg cm^2 dmol^{-1}), calculated with reference to the concentration of BSA.

3. Results and discussion

3·1 *Spectrophotometric studies*

RB exhibits λ_{max} at 540 nm and 542 nm, respectively, at *p*H 7·4 and 12·7 in absence of BSA. Figures 1A and 1B show the corresponding changes caused by increasing

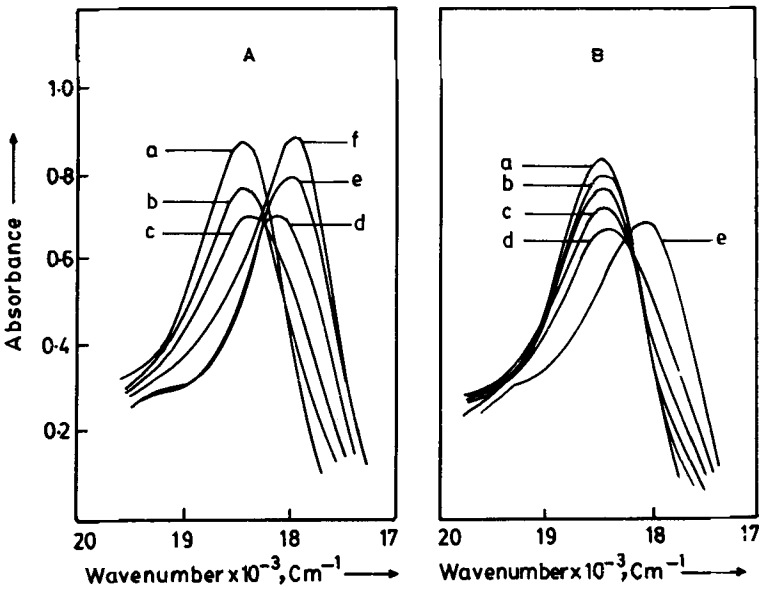

Figure 1. Absorption spectra of RB–BSA complex at *p*H 7·4 (**A**) and 12·7 (**B**). Spectra in 20·0 μM of RB only (a), and in presence of BSA (b)–(f). [BSA] (in μM) in (A) = 0·5(b), 1·0 (c), 2·0 (d), 4·0 (e), 20·0 (f) and in (B) = 0·5 (b), 2·0 (c), 4·0 (d), 20·0 (e).

[BSA] at these two *p*H values. The RB–BSA interaction results in the red-shift of the λ_{max} of RB to 555 nm at both the *p*H levels. As the absorption of RB in this region originates because of the n-π^* transitions, the red-shift indicates the presence of dye chromophore in more nonpolar environment on binding. This suggests that RB binds predominantly at the hydrophobic environment in BSA.

It is interesting to note that the red-shift arising from the interaction is identical at both *p*H 7·4 and 12·7 (figures 1A and B). It is well-known that at *p*H 7·4, BSA is in folded conformation and at extreme alkaline conditions (*p*H 12·7) BSA is found to exist in unfolded form (Peters 1985). Moreover, during this reversible unfolding of BSA at *p*H 12.7, several structural modulations have been known to occur in BSA, one of them being the exposure of hidden tyrosyl residues (Steinhardt and Stocker 1973). This exposure is also accompanied, by breaking of some of the S-S linkages in BSA (Noel and Hunter 1972) and making the hidden loops more accessible to the solvent. Therefore, the observed formation of the RB complex with unfolded BSA at *p*H 12·7 invariably suggests that the binding sites are not buried deep inside the BSA and also give credence to the fact that the disulphide loops present do not participate in the binding. That is, the binding sites are only at or near the surface of BSA, as otherwise, the binding would cease to exist at *p*H 12.7 if the sites happen to be in the interior of BSA. The absence of binding to BSA at *p*H 12.7 has been noticed for the dyes Bromocresol Green (BCG) and Eosine Blue (EB) (Kishore 1989), due to the presence of the binding sites of these two dyes in the interior of BSA.

Figure 2 shows the representative Lineweaver–Burk plots (Laidler 1978) at a single [BSA] for the binding of RB to BSA at both *p*H values. The affinity constants (i.e., K_M^{-1}) at both the *p*H levels, obtained from these plots, are given in table 1. It can

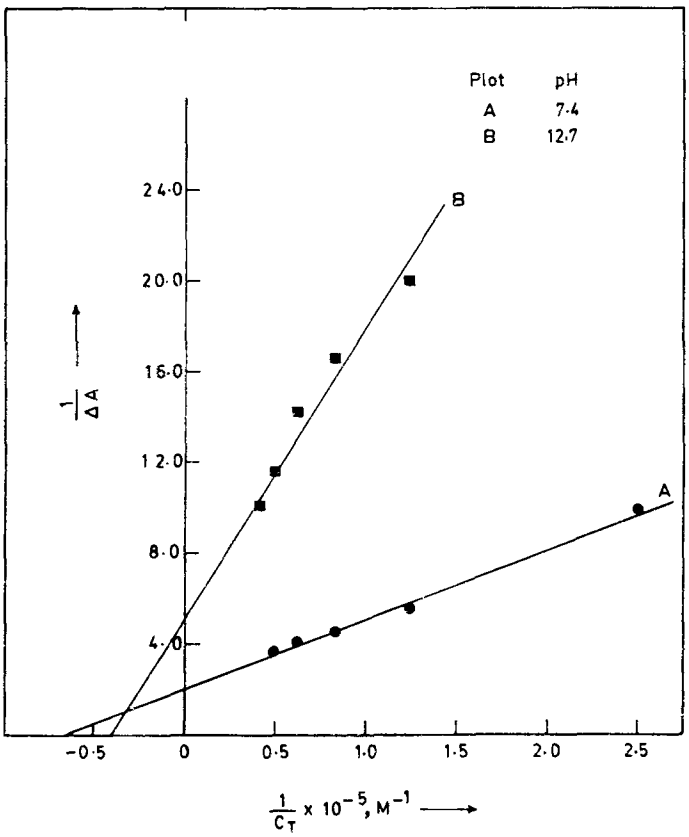

Figure 2. Lineweaver-Burk plots for the RB–BSA interaction at *p*H 7·4 and 12·7. [BSA] = 2·0 and 4·0 μM, respectively, for plots A and B.

Table 1. Affinity constants for RB–BSA interactions at *p*H 7·4 and 12·7.
Temperature = 25°C [RB] = 4·0 to 24·0 μM

*p*H	[BSA](μM)	$K \times 10^{-4}(M^{-1})^a$
7·4	2·0	6·7
	8·0	2·0
	40·0	1·0
12·7	4·0	3·7
	10·0	1·3
	20·0	0·5

[a] *K* values have been obtained from Lineweaver–Burk plots

be seen that the affinity constant, *K*, is inversely dependent on [BSA] at both the *p*H levels. A similar inverse dependence of binding constant on [albumin] has also been observed in an earlier investigation of dye 2-(4′-hydroxyphenylazo) benzoic acid binding to human serum albumin (Bowmer and Lindup 1980). However, the reasons for the inverse dependence have been discussed elsewhere (Scholtan 1962) and it stems

from the possibility of the mutual interactions of the side-chains of amino-acid residues. Consequently, the mutual interactions of the side-chains of amino-acid residues being diminished at lower [BSA] and becoming larger at higher [BSA] so as to mask the binding sites would be the probable explanation for the observance of decreasing K with increase in [BSA] in the present study. Further, as the mutual interactions of the side-chains of amino-acid residues would tend to affect the sites, especially when they happen to be present in the exterior, it leads to confirmation of the sites of RB being present only at or near the surface of BSA.

The presence of binding sites near the surface can be further substantiated by comparison of the K values at *p*H 7·4 and 12·7 (table 1). Inspection of K values at all three [BSA] indicates that there is reduction in magnitude of K values by only 50% when BSA is in unfolded form at *p*H 12·7 from those at *p*H 7·4 when BSA is in folded form. That is, the exposure of hidden residues at *p*H 12·7 does not affect the binding very drastically and indeed establishes that RB–BSA complex formation would not be in the interior but at the hydrophobic sites near the surface. Since the sites are at the surface, the complex would not be very rigid and this is also discernible from the observed K values which vary from $\simeq 10^4$ to $10^3\ M^{-1}$ depending upon the [BSA] at both *p*H values for the RB binding (table 1). This is because the K values have been observed to be of the order of 10^5–$10^6\ M^{-1}$ as determined in the cases of ligands exhibiting rigid complex formation and strong binding to albumins (Peters 1985). The photoreactions are often favoured by factors such as noncovalent pre-binding of the sensitizer (the dye) to the proteins (Jori and Spikes 1981) and exposure of amino acid residues (Pigault and Gerard 1984, 1988; Chenchal Rao *et al* 1990). From the foregoing discussions, it is evident that RB exhibits affinity towards unfolded BSA as well as to BSA in an exposed condition (at low [BSA]), and thus the dye can be an effective photo-oxidizing agent.

3·2 *Circular dichroism studies*

Circular dichroism (CD) studies have been carried out at *p*H 7·4. The dye RB is not optically active and does not show ellipticity in the region 250–400 nm. On the other hand, the CD spectrum of BSA exhibits negative ellipticity below 300 nm. Upon addition of increasing [RB] to BSA, negative bands having ellipticity maxima at $\approx$ 346 nm appear and changes in CD signals in the 250–270 nm region are also noticed (figure 3). Both these changes are concentration-dependent, occurring on account of addition of increasing [RB] to BSA. The generation of negative bands at 346 nm can be either intrinsic (due to protein) or extrinsic (due to the binding of RB to BSA) in nature. But BSA shows neither CD nor UV absorption above 300 nm; whereas RB shows UV absorption with absorption maximum at 306 nm (figures not shown). Consequently, the observance of a negative band with ellipticity maximum at 346 nm would arise due to the perturbations of electronic transitions of the RB chromophore by BSA. This indicates the induction of optical activity in RB and this can happen only if RB binds at the asymmetric locus of BSA. Thus the band at 346 nm is extrinsic in nature and is caused by the binding of RB at the asymmetric environment in BSA. However, as the molar ellipticity $[\theta]$ reaches a saturation value of $-26 \times 10^3\ \text{deg cm}^2\ \text{dmol}^{-1}$ at [RB]/[BSA] = 2·0 (figure 3) it leads to the suggestion that binding to only two sites in BSA generates the extrinsic cotton effect at 346 nm.

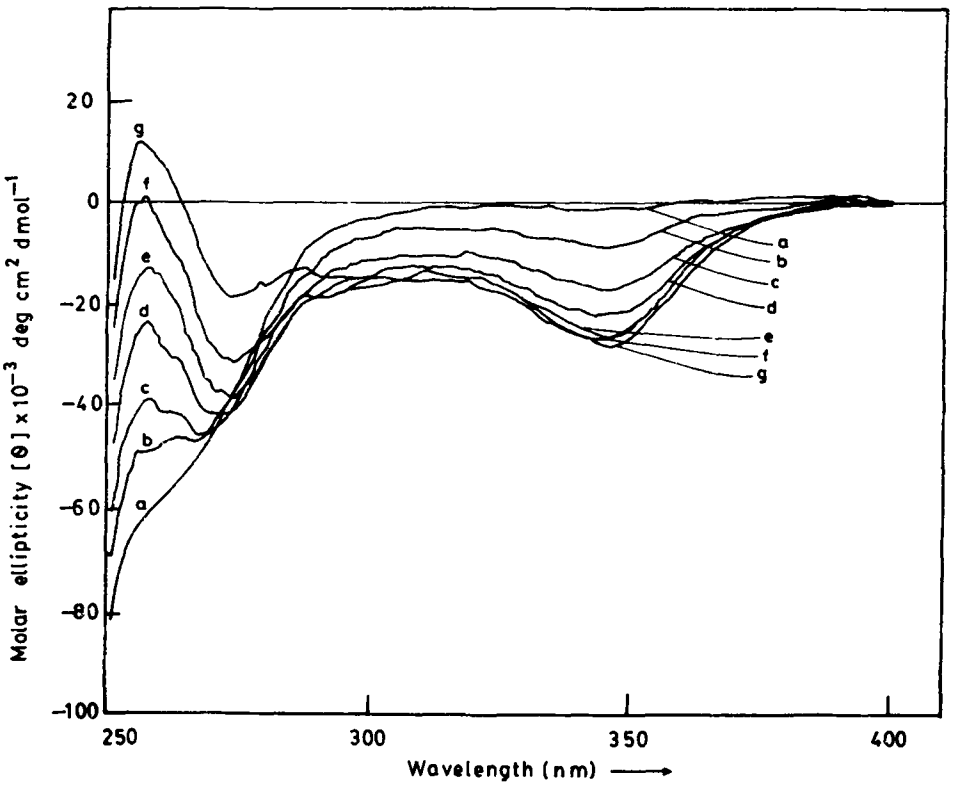

Figure 3. Circular dichroism spectra at *p*H 7·4 for RB–BSA interaction. (a) 20·0 μM of BSA only, (b)–(g): BSA + RB. [RB] (in μM) = 10·0 (b), 20·0 (c), 30·0 (d), 40·0 (e), 50·0 (f) and 70·0 (g).

Here it is to be mentioned that the binding of RB to more than two sites in BSA is possible as observed previously (Meenakshi and Kishore 1993).

Considering the changes in the near-UV CD region of 250–270 nm, the broad negative elliptical shoulder of BSA starts exhibiting characteristic peaks on addition of RB. The changes in this region can be considered intrinsic in origin as BSA has strong negative CD and also RB does not show any characteristic absorption maxima in this region. Moreover, the optical densities are kept below 0·2 for the range of concentrations of RB employed. Therefore, the prominent well-structured 257 nm peak observed (figure 3) on addition of RB is intrinsic in nature and occurs due to alterations in the tertiary structure of BSA caused by the binding of RB. Further, it can also be noticed that the well-structured 257 nm band does not show saturation like the negative band at 346 nm.

This 257 nm band can be attributed to the phenylalanine (Phe) transitions in BSA as assigned for crystallin proteins elsewhere (Liang and Chakrabarti 1981). This prominent band is also comparable to that of near-UV CD of denatured horseradish peroxidase (Strickland *et al* 1970). Hence it leads to the inference that the appearance of the well-structured 257 nm band could be due to gross conformational changes caused by RB which can be explained by the unfolding of BSA. But it has been established that only amino acid residues like cysteine, histidine, tryptophan and tyrosine undergo photo-oxidation in presence of sensitizers (Creed 1984). Hence, the appearance of the 257 band does not necessarily involve conformational modulations

involving Phe residues. Instead it is the disruptions in the orientations of residues like tryptophan (Trp) and tyrosine (Tyr) in BSA that has caused the Phe transitions and led to the emergence of the prominent band. An identical explanation has been offered by Strickland (1974) for the observed changes in the Phe transitions in proteins. Consequently, it has been possible to suggest tertiary structural changes caused by RB during its binding to BSA.

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