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PJBS

ISSN 1028-8880

Pakistan Journal of Biological Sciences

ANSInet
Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

Pakistan Journal of Biological Sciences 11 (20): 2418-2422, 2008
ISSN 1028-8880

Bromophenol Blue Binding to Mammalian Albumins and Displacement of Albumin-Bound Bilirubin

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Abstract: Interaction of bromophenol blue (BPB) with serum albumins from different mammalian species, namely, human (HSA), bovine (BSA), goat (GSA), sheep (SSA), rabbit (RbSA), porcine (PSA) and dog (DSA) was studied using absorption and absorption difference spectroscopy. BPB-albumin complexes showed significant differences in the spectral characteristics, i.e., extent of bathochromic shift and hypochromism relative to the spectral features of free BPB. Absorption difference spectra of these complexes also showed variations in the position of maxima and absorption difference (ΔAbs.) values. Absorption difference spectra of different bilirubin (BR)-albumin complexes showed a significant blue shift accompanied by decrease in ΔAbs. values in presence of BPB which were indicative of the displacement of bound BR from its binding site in BR-albumin complexes. These changes in the difference spectral characteristics of BR-albumin complexes were more marked at higher BPB concentration. However, the extent of these changes was different for different BR-albumin complexes. Taken together, all these results suggest that BPB partially shares BR binding site on albumin and different mammalian albumins show differences in the microenvironment of the BR/BPB binding site.

Key words: Bilirubin displacement, bromophenol blue, ligand binding, mammalian species, serum albumin

INTRODUCTION

Serum albumin is the most abundant protein in mammalian circulatory system with transport function (Peters, 1996). Its ability to bind a wide variety of ligands suits well to its structural flexibility (Carter and Ho, 1994). It protects the newborns from the toxic effects of bilirubin (BR), the end product of heme catabolism, by transporting it to the liver for further conjugation and excretion (Ostrow *et al*., 1994). The interaction of anionic BR to serum albumin at its primary binding site, among others, involves participation of positively charged lysine residues through electrostatic interactions with the carboxyl groups of BR (Khan and Tayyab, 2000, 2001). Several anionic ligands including drugs and dyes bind to the same site on albumin, where, BR binds and thus compete with BR and displace it (Tsutsumi *et al*., 1999; Fung *et al*., 2000; Faizul *et al*., 2008). Binding of one ligand influences the binding of other ligands on the same protein and affects their distribution in blood and tissues (Honore *et al*., 1983). Bromophenol blue (BPB), an anionic dye at moderate pH, has been shown to bind to human serum albumin (HSA) on one site less in the presence of BR (Bjerrum, 1968). It appears that sites for these two ligands overlap. Studies on the binding of such competitor ligand to albumin can give better understanding about the microenvironment around BR binding site on albumin.

Decrease in BR binding capacity of albumin may account for the development of hyperbilirubinemia in newborn infants. If untreated, excessive BR deposits in brain and leads to a permanent neurological disorder, kernicterus, which may result in infant death (Gourley, 1997). Studies on ligand-induced BR displacement are required in developing preventive measures against fatal kernicterus in jaundiced newborn infants (Brodersen *et al*., 1983). These studies need to be carried out on animals before being practiced on human system. Selection of a suitable animal model is a prerequisite for such studies and this requires testing of animal albumins towards their ligand binding properties including BR binding and ligand-induced BR displacement. Extrapolation of results of these studies to human is only useful when the animal albumin binds BR as well as the other competitor ligands in the same manner as human albumin does. Differences have been noticed in

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the BR binding properties of various mammalian albumins (Frandsen and Brodersen, 1986; Rashid *et al*., 1998; Tayyab *et al*., 2003). More studies are required to obtain an insight into interaction of serum albumin with competitor ligands and ligand-induced BR displacement. Though binding of BPB to HSA or bovine serum albumin (BSA) has been studied earlier by Wei *et al*. (1996), Bertsch *et al*. (2003) and Halim *et al*. (2008), other animal albumins have not been tested for such binding studies so far. In view of this, seven mammalian albumins from human (HSA), bovine (BSA), dog (DSA), goat (GSA), porcine (PSA) rabbit (RbSA) and sheep (SSA), respectively were selected and their BPB binding properties and BPB-induced BR displacement were studied.

MATERIALS AND METHODS

Materials: BR (lot 055K0919), BPB (lot 63H3607) and serum albumins from different mammalian species viz. BSA (lot 015K0591), GSA (lot 025H9318), HSA (lot 095K7570), PSA (lot 084K7636), RbSA (lot 104K7560) and SSA (lot 118H7607) were purchased from Sigma Chemical Co. (St. Louis, USA). DSA (lot 316411/1) was the product of Fluka Chemie (Switzerland). All other chemicals used were of analytical grade. These reagents were used as such without any further purification.

Determination of protein concentration: Protein concentration was determined by the method of Lowry *et al*. (1951) using BSA as the standard. Absorbance was recorded on a Shimadzu double beam spectrophotometer, model UV-2450 using quartz cuvettes of 1 cm path length.

Preparation of solutions: BR solution was prepared just before use by dissolving 2.4 mg of BR crystals in 1 mL of 0.5 N NaOH containing 1 mM EDTA and immediately diluting it to 50 mL with 5 mM sodium phosphate buffer, pH 7.4 containing 0.14 M NaCl (0.15PB7.4-NaCl). The solution was centrifuged at 5000 rpm for 10 min to remove insoluble gradients if any. The concentration of BR was determined spectrophotometrically using a molar extinction coefficient of 47 500 M^{-1} cm^{-1} (Jacobsen and Wennberg, 1974). The final concentration of stock BR solution was 80 μM. The BR solution was kept in dark and used within 1 h of preparation. All experiments involving BR were carried out under dim light in order to avoid photodegradation of BR.

Stock solutions (80 μM) of various albumins were prepared by dissolving 134 mg of protein in 25 mL of 0.15 PB7.4-NaCl. BPB stock solution (160 μM) was also prepared in 0.15PB7.4-NaCl by dissolving 53.6 mg of solid BPB in 500 mL of it. This solution was used within a month.

BPB-albumin binding studies: Binding of BPB to different serum albumins was studied by absorption and absorption difference spectroscopy. BPB-albumin binding experiments were performed by adding 0.5 mL of stock (160 μM) BPB solution to 0.5 mL of stock (80 μM) albumin solution from different mammalian species in a total volume of 5.0 mL which was made with 0.15PB7.4-NaCl. The final concentrations of BPB and albumin were 16 and 8 μM, respectively. Free BPB solution was prepared in the same way except that buffer was used instead of protein solution. After incubation for 10 min at room temperature, absorption spectra of both free BPB and BPB-albumin complexes were recorded against buffer in the wavelength range, 520-700 nm on Shimadzu double beam spectrophotometer, model UV-2450. Absorption difference spectra of BPB-albumin complexes were recorded against free BPB in the wavelength range, 520-700 nm.

BR displacement studies: BR-albumin complexes were prepared by adding 0.5 mL of stock (80 μM) BR solution to 0.5 mL of stock (80 μM) protein solution to get a BR/albumin molar ratio as 1:1 and the total volume was made to 5.0 mL with 0.15PB7.4-NaCl. The BR-albumin mixtures (final concentration, 8 μM each) were incubated in dark for 10 min. Albumin free BR solution was prepared by adding 0.5 mL of stock BR solution to the buffer in a total volume of 5.0 mL. The absorption difference spectra of BR-albumin mixtures were recorded against free BR solution in the wavelength range, 400-670 nm.

For another two sets, 1:1 BR-albumin complexes were prepared by mixing 0.5 mL of stock (80 μM) BR solution with 0.5 mL of stock (80 μM) protein solution and incubating these mixtures for 10 min in dark. Then 0.5 and 1.0 mL of stock (160 μM) BPB solution (final concentration as 16 and 32 μM, respectively) were added to these complexes in separate sets and the total volume was made to 5.0 mL with 0.15PB7.4-NaCl. Absorption difference spectra were recorded in the wavelength range, 400-670 nm using suitable blanks without albumin but containing same concentrations of BR and BPB. BR displacement was determined from the shift in the λ_{max} and decrease in Δ Abs. values of BR-albumin complexes.

RESULTS AND DISCUSSION

BPB-albumin interaction: Absorption spectrum of free BPB showed an absorption maximum (λ_{max}) at 591 nm in 0.15PB7.4-NaCl, which was shifted towards higher

wavelength side (red shift) with concomitant decrease in absorbance (hypochromism) upon addition of different mammalian albumins. The spectral changes characterized by a large bathochromic shift and significant hypochromism were indicative of complexation of BPB with these albumins. Similar characteristics of the absorption spectrum of BPB upon binding to BSA have been reported earlier by Tayyab and Qasim (1990) and Bertsch *et al.* (2003). The spectral characteristics of various BPB-albumin complexes (2:1) in terms of λ_{max} and absorbance values (Abs.) at λ_{max} are given in Table 1. The extent of red shift (λ_{max} shift) and percentage decrease in absorbance at λ_{max} (% hypochromism) of different BPB-albumin complexes were calculated relative to free BPB (λ_{max} = 591 nm; Abs. = 1.335) and values are given in the fourth and fifth columns of Table 1. A comparison of the spectral data obtained with different BPB-albumin mixtures (Table 1) suggests significant differences in the extent of red shift and hypochromism for different BPB-albumin complexes. For example, a complex of BPB with GSA showed maximum red shift of 12 nm with its λ_{max} at 603 nm followed by SSA (11 nm) and HSA (10 nm) with their λ_{max} occurring at 602 and 601 nm, respectively. On the other hand, BPB-BSA complex produced a relatively smaller red shift of 6 nm. The percentage decrease in Abs. in these complexes ranged between 15.7 and 26.3% with PSA showing the maximum hypochromism (26.3%) while minimum hypochromism (15.7%) was observed with DSA (Table 1). These changes in the absorption characteristics of BPB on binding to albumin can be ascribed to the change in the polarity of its environment in the bound form. A hydrophobic environment in the dye binding site has been suggested to be responsible for the alteration of a dye's absorption spectrum upon binding to protein (Turner and Brand, 1968). Since absorption maximum (λ_{max}) depends on the energy difference between ground and excited states, a shift in λ_{max} can be attributed to the change in the energy difference of these states. Placement of two negative charges of BPB in a non polar site (hydrophobic environment) of protein destabilizes the ground state and reduces the energy difference between ground and excited states, thus leads to an increase in λ_{max} or red shift (Bertsch *et al.*, 2003). In view of this, it appears that BPB binding site on these albumins differs in terms of hydrophobicity.

The absorption difference spectra of BPB-albumin complexes (2:1) against BPB were characterized by the presence of a positive peak in the higher wavelength (600-670 nm) region and a negative peak in the lower wavelength (520-600 nm) range (Figure omitted for brevity). Though least differences were observed in the wavelength of minimum (~585 nm) among different BPB-albumin complexes, wavelength of maximum showed significant differences. Furthermore, values of ΔAbs. at λ_{max} were also different. Values of absorption maximum along with ΔAbs. at this λ_{max} are given in the last two columns of Table 1. A comparison of these values shows that absorption maximum occurred in the range, 616-620 nm while ΔAbs. values varied in the range, 0.323-0.447 for different BPB-albumin complexes. The slight difference in the value of absorption maximum for BPB-BSA complex (618 nm) with the reported value of 620 nm (Bertsch *et al.*, 2003) can be ascribed to the slight change in pH and composition of the buffer used. These changes in difference spectral characteristics of various BPB-albumin complexes suggest differences in the microenvironment of BPB binding sites on these albumins.

BPB-induced BR displacement: Absorption difference spectra of BR-albumin (1:1) complexes were characterized by the presence of a positive peak in the range, 425-525 nm. These spectra were qualitatively similar but varied significantly from each other in the position of maximum (λ_{max}) and magnitude of difference spectral change (ΔAbs.) as shown in Table 2. Whereas a maximum at 485 nm was produced by BR-HSA complex, BR-DSA complex showed a maximum at 476 nm. BR-BSA, -GSA and -RbSA complexes had similar maxima, occurring at 484 nm. A maximum of 481 nm was observed with complexes of BR with both PSA and SSA. These results were in agreement with those reported earlier by Rashid *et al.* (1998) and Tayyab *et al.* (2003). The magnitude of difference spectral

Table 1: Absorption and absorption difference spectral characteristics of various BPB-albnmin complexes

	Absorption spectra				Absorption difference spectra	
Albumin	λ_{max} (nm)	Abs.	Red shift* (nm)	Hypochromism (%)	Maximnm (nm)	Δ Abs.
HSA	601	1.053	10	21.1	616	0.358
GSA	603	1.014	12	24.0	619	0.447
BSA	597	1.061	6	20.5	618	0.323
PSA	600	0.984	9	26.3	620	0.387
RbSA	600	1.077	9	19.3	617	0.388
DSA	599	1.125	8	15.7	616	0.398
SSA	602	0.998	11	25.2	619	0.398

*Red shift of different BPB-albnmin complexes was calculated relative to free BPB which had λ_{max} at 591 nm (Abs. = 1.335)

Table 2: BPB-induced BR displacement from albumin as determined from absorption difference spectroscopy

	BR-albumin complex		BR + albumin + BPB mixture (Molar ratio) 1:1:2				1:1:4			
Albumin	λ_{max} (um)	ΔAbs.	λ_{max} (nm)	ΔAbs.	Blue shift (nm)	*%-ΔΔAbs.	λ_{max} (um)	ΔAbs.	Blue shift (nm)	*%-ΔΔAbs.
HSA	485	0.150	483	0.118	2	22	481	0.101	4	34
GSA	484	0.252	481	0.214	3	6	480	0.196	4	24
BSA	484	0.197	482	0.160	2	9	480	0.144	4	29
PSA	481	0.199	477	0.172	4	6	475	0.152	6	28
RbSA	484	0.112	480	0.073	4	7	478	0.055	6	55
DSA	476	0.208	472	0.174	4	8	469	0.145	7	35
SSA	481	0.238	479	0.202	2	16	476	0.168	5	33

*% -ΔΔAbs. Represents decrease in ΔAbs. of BR-albumin complex upon addition of BPB

change was found highest with BR-GSA complex and lowest with BR-RbSA complex (Table 2). A slight variation in the microenvironment around BR binding site in these albumins may account for the differences observed in the difference spectral characteristics of these complexes.

Displacement of BR from BR-albumin complexes upon addition of BPB was reflected from the absorption difference spectra of BR-albumin (1:1) complexes in presence of 2 and 4 molar excess of BPB. The spectra were characterized by the presence of two maxima and one minimum. Presence of a maximum at lower wavelength (469-483 nm) was characteristic of BR binding to albumins (Table 2) whereas occurrence of a minimum around 585 nm and a maximum around 616-620 nm were indicative of BPB binding to albumins (Table 1). Though difference spectral characteristics of BR-albumin complexes in presence of BPB at lower wavelength range (400-525 nm) were qualitatively similar to those observed in the absence of BPB, significant differences were noted in the values of λ_{max} and ΔAbs. of these complexes in presence of BPB. Values of λ_{max} and ΔAbs of different BR-albumin complexes in presence of 2 and 4 molar excess of BPB along with shift in λ_{max} (blue shift) and change in ΔAbs (%-ΔΔAbs.) are given in Table 2. As can be clearly seen from the Table 2, changes in difference spectral characteristics (blue shift and decrease in ΔAbs.) indicated displacement of bound BR from BR-albumin complexes in presence of BPB. These changes were more marked in presence of 4 molar excess of BPB compared to those observed with 2 molar excess of BPB. The extent of blue shift and percentage decrease in ΔAbs. among different BR-albumin complexes were 4-7 nm and 24-55%, respectively at 4 molar excess of BPB against 2-4 nm and 06-22%, respectively at 2 molar excess of BPB (Table 2). The blue shift was more significant (6-7 nm) in complexes of BR with DSA, PSA and RbSA (Table 2). In BR-HSA complex, the blue shift was relatively smaller (4 nm) but decrease in ΔAbs was quite large (34%). Based on changes in spectral characteristics of BR-albumin complexes upon addition of BPB, it can be said that BR binding site on albumin is also shared partially by BPB. Differences in the spectral characteristics among different BR-albumin complexes produced by BPB can be ascribed to the changes in the microenvironment of these sites in different albumins. Further studies are in progress to characterize BPB binding sites on these albumins.

ACKNOWLEDGMENTS

We gratefully acknowledge financial support from the University of Malaya (Grant No. FP075/2005C). Facilities provided by the Institute of Biological Sciences, University of Malaya to carry out this study are also acknowledged.

REFERENCES

Bertsch, M., A.L. Mayburd and R.J. Kassner, 2003. The identification of hydrophobic sites on the surface of proteins using absorption difference spectroscopy of bromophenol blue. Anal. Biochem., 313: 187-195.

Bjerrum, O.J., 1968. Interaction of bromophenol blue and bilirubin with bovine and human serum albumin determined by gel filtration. Scand. J. Clin. Lab. Invest., 22: 41-48.

Brodersen, R., B. Friis-Hansen and L. Stern, 1983. Drug-induced displacement of bilirubin from albumin in the newborn. Dev. Pharmacol. Ther., 6: 217-229.

Carter, D.C. and J.X. Ho, 1994. Structure of serum albumin. Adv. Protein Chem., 45: 153-203.

Faizul, F.M., H.A. Kadir and S. Tayyab, 2008. Spectroscopic studies on the binding of bromocresol purple to different serum albumins and its bilirubin displacing action. J. Photochem. Photobiol. B., 90: 1-7.

Frandsen, P.C. and R. Brodersen, 1986. Bilirubin/rat serum albumin interaction. Acta Chem. Scand., 40: 55-59.

Fung, Y.S., D.X. Sun and C.Y. Yeung, 2000. Capillary electrophoresis for determination of free and albumin-bound bilirubin and the investigation of drug interaction with bilirubin-bound albumin. Electrophoresis, 21: 403-410.

Gourley, G.R., 1997. Bilirubin metabolism and kernicterus. Adv. Pediatr., 44: 173-229.

Halim, A.A., H.A. Kadir and S. Tayyab, 2008. Bromophenol blue binding as a probe to study urea and guanidine hydrochloride denaturation of bovine serum albumin. J. Biochem., 144: 33-38.

Honore, B., R. Brodersen and A. Robertson, 1983. Interaction of indomethacin with adult human albumin and neonatal serum. Dev. Pharmacol. Ther., 6: 347-355.

Jacobsen, J. and R.P. Wennberg, 1974. Determination of unbound bilirubin in the serum of newborns. Clin. Chem., 20: 783-789.

Khan, M.M. and S. Tayyab, 2000. On the modulation of photoinduced fluorescence enhancement and conformational stability of albumin-bound bilirubin: Effect of ε-NH_2 groups blocking and chloroform binding. Biochim. Biophys. Acta, 1523: 147-153.

Khan, M.M. and S. Tayyab, 2001. Understanding the role of internal lysine residues of serum albumins in conformational stability and bilirubin binding. Biochim. Biophys. Acta, 1545: 263-277.

Lowry, O.H., N.J. Rosebrough, A.L. Farr and R.J. Randall, 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem., 193: 265-275.

Ostrow, J.D., P. Mukerjee and C. Tiribelli, 1994. Structure and binding of unconjugated bilirubin: Relevance for physiological and pathophysiological function. J. Lipid Res., 35: 1715-1737.

Peters, T. Jr., 1996. All About Albumin: Biochemistry, Genetics and Medical Applications. 1st Edn., Academic Press, New York, ISBN: 978-0125521109.

Rashid, H., S. Muzammil and S. Tayyab, 1998. Comparison of bilirubin binding and other molecular properties of the serum albumin of several mammalian species. Biochem. Mol. Biol. Int., 44: 165-173.

Tayyab, S. and M.A. Qasim, 1990. Binding of bromophenol blue to bovine serum albumin and its succinylated forms. Int. J. Biol. Macromol., 12: 55-58.

Tayyab, S., N.J. Khan, M.A. Khan and Y. Kumar, 2003. Behavior of various mammalian albumins towards bilirubin binding and photochemical properties of different bilirubin-albumin complexes. Int. J. Biol. Macromol., 31: 187-193.

Tsutsumi, Y., T. Maruyama, A. Takadate, M. Goto, H. Matsunaga and M. Otagiri, 1999. Interaction between two dicarboxylate endogenous substances, bilirubin and an uremic toxin, 3-carboxy-4-methyl-5-propyl-2-furanpropanoic acid, on human serum albumin. Pharm. Res., 16: 916-923.

Turner, D.C. and L. Brand, 1968. Quantitative estimation of protein binding site polarity. Fluorescence of N- arylaminonaphthalenesulfonates. Biochemistry, 7: 3381-3390.

Wei, Y., K. Li and S. Tong, 1996. The interaction of bromophenol blue with proteins in acidic solution. Talanta, 43: 1-10.