CORE
Metadata, citation and similar papers at core.ac.uk
Provided by Elsevier - Publisher Connector

THE USE OF REPORTER GROUP CIRCULAR DICHROISM IN THE STUDY OF CONFORMATIONAL TRANSITIONS IN BOVINE SERUM ALBUMIN

D.D. WHITE, Sheena STEWART and G.C. WOOD
Department of Pharmaceutical Chemistry, University of Strathclyde, Glasgow, G1 1XW, Scotland

Received 3 May 1973

1. Introduction

While the pronounced conformational changes which occur in serum albumin at $4 > pH > 10$ have been intensively studied [1], relatively little information is available about structural changes between pH 5 and pH 9. While hydrodynamic studies [2] show that the bovine serum albumin (BSA) molecule remains compact in this pH range, the binding of ions and non-ionic compounds [3, 4] is markedly pH-dependent. ORD studies [5–7] indicate that while the α-helical content does not change appreciably between pH 4 and pH 10 there may be subtle conformational changes between pH 6 and pH 9.

In order to obtain more detailed information about pH-dependent conformational changes in BSA, we have determined: i) circular dichroism (CD) spectra of the protein in the near-UV range as a function of pH, and ii) extrinsic CD spectra, induced in a covalently-bound chromophore by the dissymmetric protein molecule as a function of pH. Kagan and Vallee [8] have shown that the extrinsic CD of azo-chromophores introduced into various proteins depends on the conformation of the proteins and may be used to monitor minor changes of conformation. Dowben and Orkin [9] and Schechter [10] have described the optical activity of mercurifluorescein derivatives bound to the single – SH group of BSA and have shown that its magnitude depends on pH. In this study we have used a different mercurial reagent to introduce a CD reporter group in the same position in the protein molecule and present a more detailed study of the pH-dependence of its optical activity.

2. Materials and methods

Crystalline BSA was obtained from Sigma U.K. Ltd. Its –SH content (determined with Ellman's reagent [11]) was 0.64 moles per mole of protein (assumed mol. wt. 69 000). Mercaptalbumin-rich fractions of BSA (0.90–0.94 moles –SH per mole of protein) were prepared by chromatography on SP-Sephadex C-50 using a procedure based on that of Hagenmaier and Foster [12].

4-(*p*-Dimethylaminobenzeneazo) phenylmercuric acetate was prepared according to Jacobs and Heidelberger [13]. To prepare modified BSA a solution of the protein (50 ml, 5×10^{-5} M or 5×10^{-4} M in 0.067 M sodium phosphate buffer, pH 7.5) was cautiously stirred with excess of the water-insoluble reagent for 24 hr at 4°C. The solution then contained approx. 1.5 moles mercurial per mole of protein. In order to remove non-covalently bound dye the solution was stirred gently with charcoal (Darco M, 0.75 g per g protein) for 1 hr and centrifuged (20 000 *g*, 0.5 hr). In some experiments the solution was dialysed against distilled water or KCl solution at 4°C. Modified BSA contained 0.65–0.70 moles dye per mole of protein. When BSA in which the –SH groups had been blocked by treatment with *N*-ethylmaleimide was subjected to the same treatment the product contained 0.035 moles dye per mole of protein. Thus more than 95% of the dye molecules in modified BSA were located at the –SH group. Modified mercaptalbumin contained 0.85–0.88 moles dye per mole protein.

CD spectra were determined at 27°C using a Cary Model 60 recording spectropolarimeter with a 6003

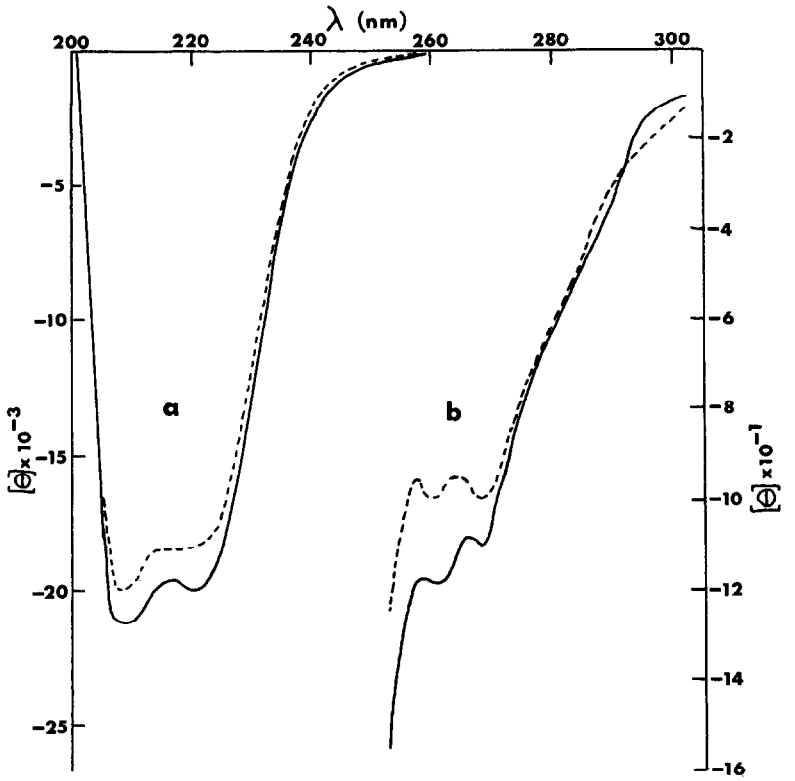

Fig. 1. Circular dichroism spectra of bovine serum albumin in 0.1 M KCl; (a) 8×10^{-6} M, (——) pH 5.40, (– – –) pH 9.50; (b) 4×10^{-5} M, (——) pH 5.30, (– – –) pH 9.35. Ordinate – mean residue ellipticity, [θ], deg. cm^2 $dmol^{-1}$.

CD attachment, calibrated with D-10-camphorsulphonic acid. Each CD spectrum was the average of at least two successive scans. To investigate the pH-dependence of CD spectra, protein solution (2.8 ml) was placed in a polarimeter cell and CD spectra determined after adding successive volumes of 0.5 N HCl or 0.5 N NaOH required to bring the solution to predetermined pH values.

The dye content of protein solutions was estimated from their mercury contents, determined by a cold vapour method [14] using an EEL Model 140 atomic absorption spectrophotometer. Protein concentrations were determined by a modification of the method of Miller [15].

3. Results and discussion

Apart from the large change below pH 4.5, the CD spectrum of BSA in the region of peptide bond absorption (e.g., 220 nm) changed little with pH (figs. 1 and 2). More pronounced pH-dependence was observed in the near-UV region, particularly in the region of tryptophan absorption (295 nm) where the relative change was largest. The CD spectrum of modified BSA between 200 nm and 300 nm did not differ significantly from that of the unmodified protein.

The absorption spectrum at pH 7.5 of the dye covalently bound to BSA contained two unresolved absorption bonds (fig. 3). Both were rendered optically active by association with the protein, giving rise to two CD peaks of approximately equal magnitude but opposite sign at 390 nm and 450 nm (fig. 3). Molecular CD spectra were independent of protein concentration between 5×10^{-5} M and 5×10^{-4} M. CD spectra of modified mercaptalbumin were identical with those of modified BSA. Since cysteine modified with the mercurial showed no CD in this region the optical activity of the protein-bound dye arises from interaction of the chromophore with regions of the BSA

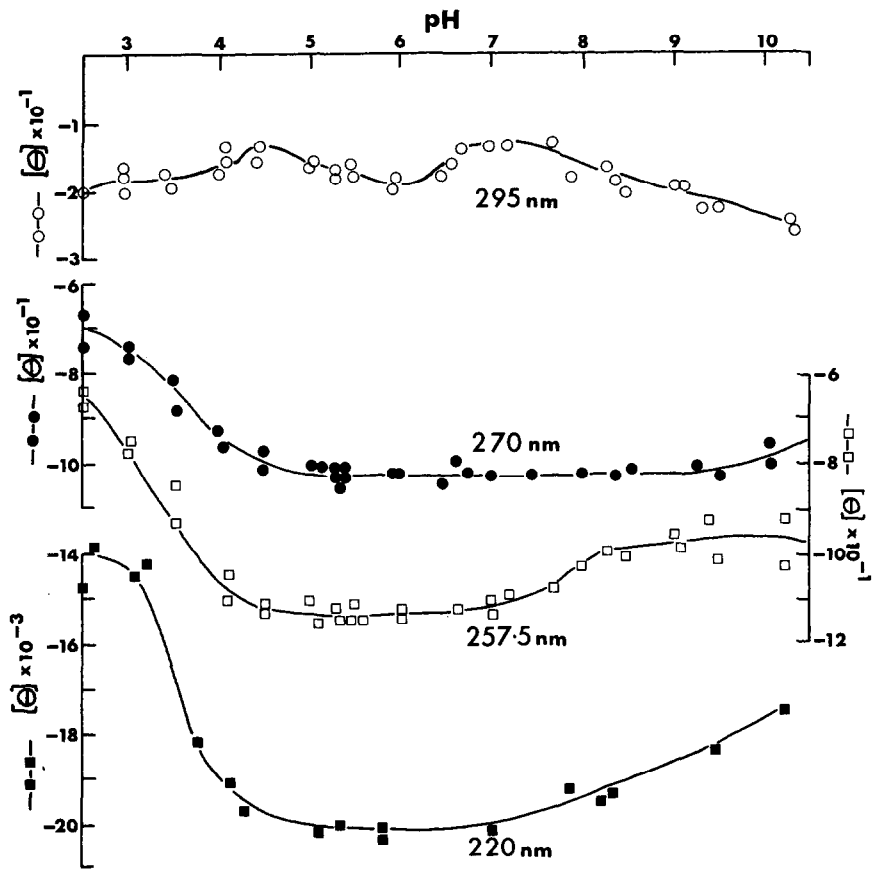

Fig. 2. Variation of the circular dichroism of bovine serum albumin with pH. Conditions as for fig. 1. Ordinate – mean residue ellipticity, $[\theta]$, deg. cm^2 $dmol^{-1}$.

molecule near the cysteine residue to which it is attached. Comparison of the CD spectra of modified protein before and after charcoal treatment enables one to calculate tha approximate CD spectrum of dye bound non-covalently to covalently-labelled BSA or mercaptalbumin. This differed from the CD spectrum of the covalently bound dye both in magnitude and in the location of the peaks (fig. 3), indicating that the environments of the covalently and non-covalently bound chromophores are different. Presumably the non-covalently bound dye has access to binding sites which is denied to the dye which is tethered to the cysteine residue. Subsequent work was concerned only with the covalently-labelled protein.

When the CD spectrum of a solution of modified BSA (2.5×10^{-5} M) was determined in the presence of 6 M urea marked changes in CD at $\lambda < 290$ nm were observed, as expected in view of the disruption of the organised protein structure that 6 M urea causes. In addition, the 390 nm CD peak of the covalently bound dye was abolished and the magnitude of the 450 nm peak reduced by 27%. When dithiothreitol (1.8×10^{-2} M) was present in addition to the urea the 450 nm peak too was reduced by 90%. Similar results were obtained with modified mercaptalbumin. Low concentrations of sodium dodecyl sulphate (<10 moles per mole of protein) are believed to cause minor changes in the tertiary structure of BSA [16]. When the CD spectrum of modified BSA (2.5×10^{-5} M) was determined in the presence of sodium dodecyl sulphate (8 moles/mole BSA) the 390 nm peak was absent and the 450 nm peak reduced to about 20% of its value in the absence of detergent. It appears therefore that the induced optical activity of the dye may

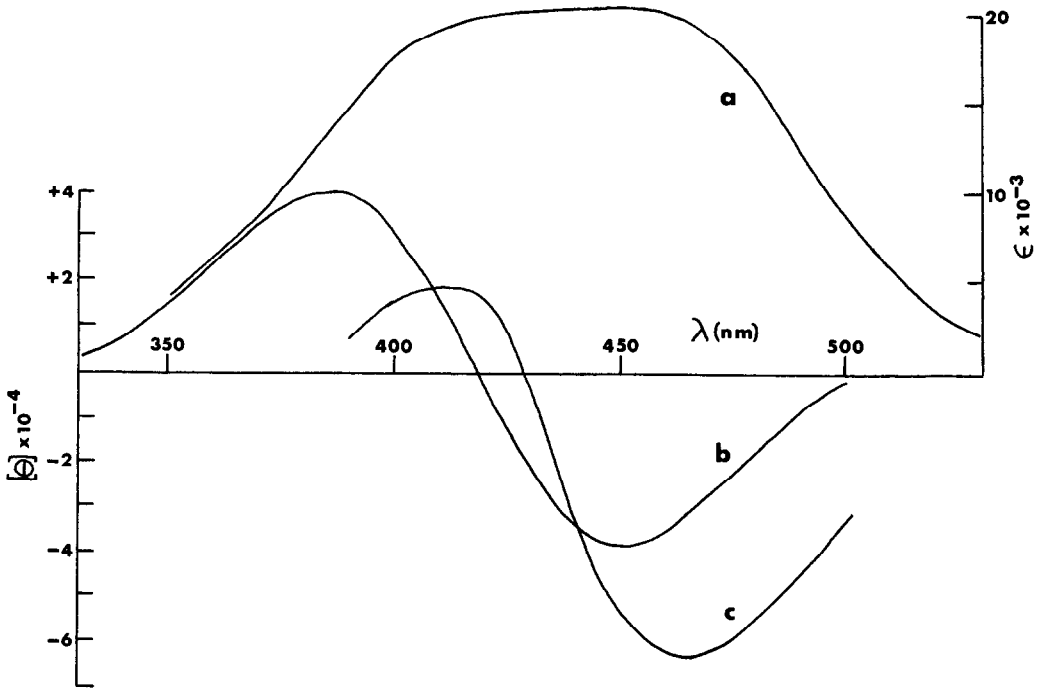

Fig. 3. Absorption and circular dichroism spectra of 4-(*p*-dimethylaminobenzeneazo)-phenylmercuric acetate bound to bovine serum albumin. a) Absorption spectrum of covalently bound dye. b) CD spectrum of covalently bound dye. c) CD spectrum of non-covalently bound dye, 0.45 moles/mole albumin. Protein concn. 5×10^{-5} M in 0.067 M sodium phosphate buffer, pH 7.5. Molecular ellipticity $[\theta]$ expressed with respect to the dye, deg. cm^2 $dmol^{-1}$.

be affected by minor as well as major protein conformational changes.

The absorption spectrum of covalently bound dye varied little with pH. The absorbance at 430 nm (A_{430}) was constant between pH 5 and pH 10 but A_{450}/A_{390} increased from 1.22 at pH 10 to 1.45 at pH 4.5. With modified albumin in water the general shape of the CD spectrum was as shown in fig. 3, between pH 4.5 and pH 10. Below pH 4.5 the shape of the CD spectrum changed somewhat, partly due to conformational changes in the protein and partly due to ionisation of the dimethylamino group of the dye (pK_a = 1.8, [17]). Above pH 4.5, where the dye chromophore is uncharged and the protein molecule remains compact, the magnitude of the CD peaks was markedly pH-dependent (fig. 4). The shapes of the $[\theta]_{max}$ *vs* pH curves indicate two transitions; one between pH 6 and pH 7.5 with a mid-point at about pH 6.7 and one between pH 7.5 and pH 9 with a mid-point at about pH 8.2. The low-pH transition was completely reversible between pH 7.5 and pH 5.5. The high-pH transition was 95% reversible between pH 7.5 and pH 9.3. In the presence of 0.15 M KCl similar reversible transitions were observed, but the high-pH transition was shifted to higher pH. Similar transitions were observed with modified mercaptalbumin. Comparison of fig. 2 with fig. 4 suggests that changes corresponding to those in the CD of the bound dye are observed in the CD spectrum of native BSA at 257.5 nm and at 295 nm, although their magnitudes are very much smaller. This indicates that the transitions revealed by the bound dye are not peculiar to the modified protein but are probably a property of the native protein itself.

Dowben and Orkin [9] and Schechter [10] both studied the pH-dependence of the induced optical activity of mercurifluorescein derivatives covalently bound to BSA at the same site as our reporter group. Both observed marked variation of rotational strength at pH > 6 which is probably related to the low-pH transition which we observed. No evidence for more than one transition above pH 6 was presented but

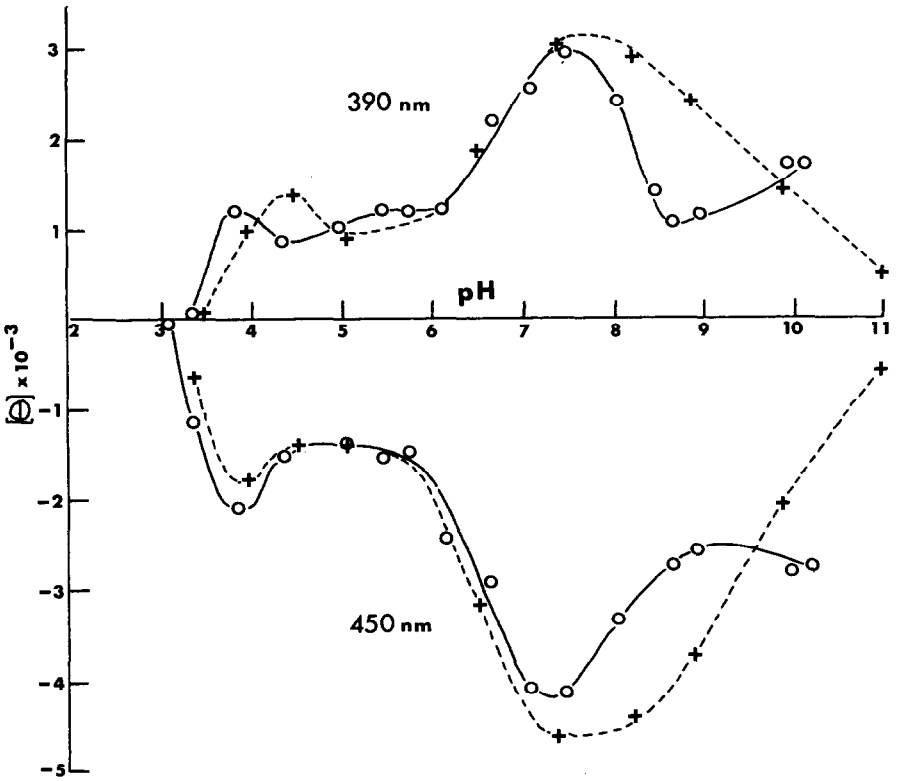

Fig. 4. Variation of extrinsic CD of dye, covalently bound to bovine serum albumin, with pH; (o—o—o) in water; (+---+---+) in 0.15 M KCl. Protein concn. 5×10^{-5} M. Ordinate, molecular ellipticity of dye, $[\theta]$, deg. cm^2 $dmol^{-1}$.

their limited data do not preclude this. Schechter reported a second transition between pH 4.2 and pH 5.5 which finds no equivalent in our observations; it seems likely that his data in this pH range are due to alteration of the mode of interaction of the fluorescein derivative with the protein, resulting from ionisation of its carboxyl groups, rather than to any conformational change in the protein itself.

Table 1
Molecular ellipticities (deg. cm^2 $dmol^{-1}$) of dye covalently bound to bovine serum albumin (5×10^{-5} M) in KCl or $CaCl_2$.

pH	$[\theta]_{390} \times 10^{-3}$		$[\theta]_{450} \times 10^{-3}$	
	KCl	$CaCl_2$	KCl	$CaCl_2$
5.0	+ 9.3	+ 6.9	−19.2	−19.0
6.2	–	+ 20.2	–	−32.2
7.5	+ 31.4	+ 16.2	−50.0	−26.6
8.5	–	+ 13.6	–	−21.9

Ionic strength 0.06.

The pH-dependent conformational transitions which have been suggested for native BSA on the basis of measurement of binding of butane [4], fluorescence [18] and optical rotation at 313 nm [6,7] occur between pH 7 and pH 9 and may therefore be associated with the high-pH transition we have observed. In hydrogen–deuterium exchange studies also [19,20], pH-dependence is most marked in this range.

The low-pH transition (mid-point about pH 6.7) is probably connected with dissociation (pK_a 6.9) of at least some of the histidine residues of BSA. According to Harmsen et al. [7] a transition involving a small (~ 4%) change in $[\alpha]_{313}$ (mid-point at pH 8.1) which they observed in solutions of BSA in 0.06 M KCl, is associated with dissociation of histidine residues with abnormally high pK_a. They also observed that when the solvent was $CaCl_2$, ionic strength 0.06, the transition was shifted to lower pH (mid-point pH 7.25), an

effect which they associate with the reversion of the pK_a of the abnormal histidine residues to normal values. If our high-pH transition is the same as theirs and if we are correct in interpreting our data in terms of two transitions, then the peak near pH 7.5 in our $[\theta]$ *vs* pH curves should be very much reduced in magnitude in the presence of $CaCl_2$. Table 1 shows that this is in fact the case, providing strong evidence for the identity of our high-pH transition with that of Harmsen et al. These data suggest that the low-pH transition also may be shifted slightly to lower pH in the presence of $CaCl_2$.

Reporter group CD may thus be a powerful tool in monitoring minor pH-dependent conformational changes, not readily detectable by other means, in BSA and possibly other proteins. The results with sodium dodecyl sulphate also suggest that it may be useful in detecting changes induced by the binding of small molecules to BSA.

Acknowledgements

We thank the Nuffield Foundation and the Medical Research Council for financial support and the Wellcome Trust for a grand to permit the purchase of the spectropolarimeter.

References

[1] J. Steinhardt and J.A. Reynolds, Multiple equilibria in proteins (Academic Press, New York, 1969) p. 117.
[2] C. Tanford and J.G. Buzzell, J. Phys. Chem. 60 (1956) 225.
[3] I.M. Klotz, R.K. Burkhard and J.M. Urquhart, J. Am. Chem. Soc. 74 (1952) 202.
[4] D.B. Wetlaufer and R. Lovrien, J. Biol. Chem. 239 (1964) 596.
[5] E.S. Benson, B.E. Hallaway and R.W. Lumry, J. Biol. Chem. 239 (1964) 122.
[6] W.J. Leonard, K.K. Vijai and J.F. Foster, J. Biol. Chem. 238 (1963) 1984.
[7] B.J.M. Harmsen, S.H. DeBruin, L.H.M. Janssen, J.F. Rodrigues De Miranda and G.A.J. Van Os, Biochemistry 10 (1971) 3217.
[8] H.M. Kagan and B.L. Vallee, Biochemistry 8 (1969) 4223.
[9] R.M. Dowben and S.H. Orkin, Proc. Natl. Acad. Sci. U.S. 58 (1967) 2051.
[10] E. Schechter, European J. Biochem. 10 (1969) 274.
[11] J.F. Robyt, R.J. Ackerman and C.G. Chittenden, Arch. Biochem. Biophys. 147 (1971) 262.
[12] R.D. Hagenmaier and J.F. Foster, Biochemistry 10 (1971) 637.
[13] W.A. Jacobs and M. Heidelberger, J. Biol. Chem. 20 (1915) 513.
[14] R. Braun and A.P. Husbands, Spectrovision 26 (1971) 2.
[15] G.L. Miller, Analyt. Chem. 31 (1959) 964.
[16] H. Polet and J. Steinhardt, Biochemistry 7 (1968) 1348.
[17] I.M. Klotz and J. Ayers, J. Am. Chem. Soc. 79 (1957) 4078.
[18] R.F. Steiner and H. Edelhoch, Biochim. Biophys. Acta 66 (1963) 341.
[19] E.S. Benson and B.E. Hallaway, J. Biol. Chem. 245 (1970) 4144.
[20] A. Hvidt and W. Knut, J. Biol. Chem. 247 (1972) 1530.