J. Pharm. Pharmacol. 1981, 33: 697–700
Received October 1, 1980

0022–3573/81/110697–04 $02.50/0

Circular dichroic examination of the interaction of some planar acidic drugs with tryptophan-modified human serum albumin

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The single tryptophan region in human serum albumin was investigated for its involvement in binding planar hydrophobic and acidic drug molecules. The lone tryptophan was alkylated with a selective and specific agent, 2-hydroxy-5 nitrobenzyl bromide. The subsequent interaction of four drug molecules with tryptophan modified, and normal albumin was examined by circular dichroism (CD). In all cases the CD signal of tryptophan modified albumin was perturbed at low drug concentrations. The predominantly α-helical structure of the albumin remained intact. It is suggested that the primary binding site of these four acidic planar drugs does involve the tryptophan region.

A principle region of binding of planar hydrophobic and acidic drug molecules to human serum albumin is thought to be around the single tryptophan residue. Swaney & Klotz (1970) have established the amino acid sequence of this region and Thorp (1971) has suggested that this region provides an ideal microenvironment for the binding of a large variety of ligands.

It has previously been shown that optically-inactive drug molecules can become optically active upon binding to albumin (Chignell 1964; Rosen 1970; Perrin & Nelson 1972; Sjoholm & Sjodin 1972). These extrinsic Cotton effects have been shown to be a quantitative measurement of the complex formation and sensitive to the binding environment on the protein (Perrin et al 1975; Gambhir et al 1975; Szekerke & Howath 1976), and so may be a sensitive probe for the region of the binding site.

Assessment of the binding site location on human serum albumin and whether such a site may be common for chemically related drug molecules is of considerable interest. Drug displacement from mutual binding sites may play a role in adverse clinical symptoms. Recently Fehske et al (1978) have examined the lone tryptophan residue of human serum albumin in order to assess its involvement in binding indole and benzodiazepine compounds. We additionally wish to report, in work conducted several years ago (Vallner 1974), the results obtained after modification of the single tryptophan in albumin with Koshlands reagent (2-hydroxy-5-nitrobenzyl bromide).

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Alkylation of the lone tryptophan in albumin will alter the shape of the binding site and therefore the binding pattern of small molecules binding at this site should also be altered. If induced Cotton effects originate from this site, then alkylation should alter the characteristics of these effects. In this modification study the induced optical activities of phenylbutazone, flufenamic acid, sulfaethidole and dicumarol have been investigated following their binding to alkylated and non-alkylated human serum albumin.

MATERIALS AND METHODS

Sulfaethidole (N^1-5-(ethyl 1-1, 3, 4-thiazole-2-yl) sulphanilamide) was obtained from Smith, Kline and French, (Philadelphia, Pa) and was recrystallized twice from water to give a melting point of 185–186 °C. Phenylbutazone, Geigy (Ardsley, N.Y.) dicumarol, Abbot (North Chicago, Ill) and flufenamic acid, Parke Davis (Detroit, Mich.) were used as supplied. Human serum albumin fraction V was purchased from Armour (Kankakee, Ill.). 2-hydroxy-5-nitrobenzyl bromide (Koshland reagent), grade II, Sigma (St Louis, Mi.) and Sephadex G-25, fine grade, Pharmacia (Piscataway, N.J.) were purchased from their respective suppliers and all other chemicals were of reagent grade.

Modification of albumin

The alkylated albumin was prepared by dissolving 0·10 gm HSA V in 8 M urea at pH 2·7 and incubating at 37 °C overnight. This was reacted, with vigorous stirring, with 0·1 ml of a freshly prepared 0·2 M 2-hydroxy-5-nitrobenzyl bromide solution in dry acetone at room temperature (20 °C) and in the

dark. After 10 min the alkylated albumin was separated from excess reagent and acetone using a Sephadex G 25 column and eluted with a physiological phosphate buffer of pH 7·4. Quantitative elution was assumed, and a fraction assayed after adjustment of the pH to 10·2 and the absorbance read at 410 nm. At a molar absorptivity of 18450 (Loudon & Koshland 1970), samples of reacted albumin containing 0·98 to 1·05 molecules reagent per mole albumin were considered satisfactory for further investigation. For the circular dichroism measurements the concentration was adjusted to $1{\cdot}45 \times 10^{-5}$ M before an equal volume of drug solution was added. All solutions were in 0·054 M sodium phosphate of pH 7·4 made isotonic with sodium chloride; deionized water was used throughout. The CD spectra were obtained from 6002 attachment to a Cary 60 spectropolarimeter using a slit programmed for a half-band width of 15 Å and 1- or 2-cm pathlength cells. The ellipticities are reported in the figures as induced ellipticities. These are defined as observed ellipticities of drug plus albumin minus the observed ellipticity of the same concentration of albumin alone at the given wavelength. The signal-to-noise ratio was never less than 15 to 1.

The CD spectra of the drug-non-alkylated albumin complex were obtained using albumins that had been subjected to the above treatment but without the reaction with the alkylating agent.

RESULTS AND DISCUSSION

To obtain a satisfactory modification of HSA, the methods given above had to be adhered to rigidly. Two-thirds of albumin consists of mercaptalbumin (Hughes 1947), containing one free SH group. Under the acidic conditions used this free SH should not react with 2-hydroxy-5-nitrobenzyl bromide (Barmen & Koshland 1967). The u.v. absorbance at 250 nm of alkylated albumin, containing one mole of reagent per mole of protein, and non-alkylated HSA were compared after reaction with *p*-chloromercuribenzoate (Boyer 1954; Riordan & Vallee 1972) to assess the integrity of the SH groups. These measurements confirmed that none of the free SH groups has been alkylated by Koshland's reagent.

Comparison of the u.v. spectra of alkylated and non-alkylated albumin at pH 7·4 showed that alkylation reduced the absorbance by 3 to 5%. Similarly the ellipticity of the albumin through the aromatic region was reduced 3 to 5% on alkylation. At lower wavelengths (200–240 nm) the alkylated preparations were consistently of 5% lower ellipticity than the unreacted albumins, although the shape of the curve was unaltered from that normally associated with a predominantly α-helical structure. The shape and magnitude of the CD curves did not alter over the 48 h after alkylation.

The treatment of tryptophan residues with 2-hydroxy-5-nitrobenzyl bromide increases the hydrophobic nature of the tryptophan residue in the albumin and also increases the area for hydrophobic interaction and so may enhance binding to this region of the protein. However, the shape of the binding site is altered, and the fit of the small molecule into the site may be affected, as well as the local conformation of the protein. A small local conformational change would not be detected by the CD technique under these experimental conditions. The induced CD spectra obtained from the association of four acidic drugs with alkylated and non-alkylated albumin have been compared to determine whether their spectra originated from the tryptophan region of the albumin.

Sulfaethiodole

Bovine serum albumin (BSA) has been found to have a single site capable of inducing optical activity into the sulfaethidole molecule, whereas dialysis clearly showed two classes of binding sites (Perrin & Nelson 1972). The CD data showed only a single site and the CD curves, which had a negative peak at 278·8 nm and a positive peak at 256·8 nm, were shown to have a shape independent of concentration. Investigations with HSA showed similar CD spectra and again one single site. CD investigations of the competition of a variety of acidic drugs, including salicylates, barbiturates, coumarins, and sulphonamides (Perrin & Nelson 1973), have suggested that these drugs share the same primary binding site on bovine serum albumin sulfaethidole.

Following alkylation of the albumin, the shape of the CD curve on binding of sulfaethidole was unaltered (Fig. 1), however, there was a substantial reduction in the intensity of the signal at both peaks. As there was no wavelength change and the percentage change was similar at both CD peaks, it appears that the decrease is due to decreased binding rather than a change in the perturbations necessary for the induced optical activity.

Flufenamic acid

Flufenamic acid has been reported to give a positive peak at 296 nm and a smaller negative peak at 345 nm on binding to HSA at pH 7·4 (Chignell 1964). The current investigations show the peak near

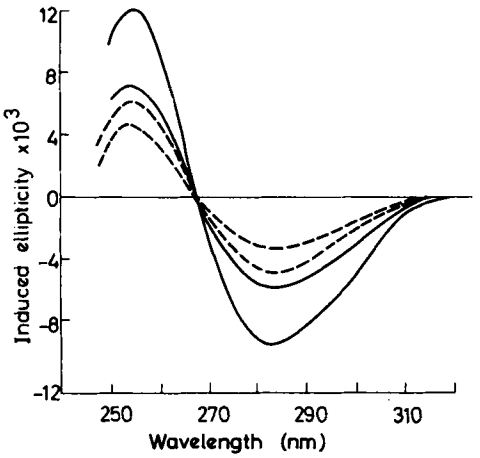

FIG. 1. Extrinsic Cotton effects following the binding of sulfaethidole to 7·25 × 10⁻⁶ M HSA V in 1 cm cells. Drug concentrations 1·26 × 10⁻⁵ and 6·3 × 10⁻⁶ M. - - - - alkylated HSA, —— HSA.

296 nm, however, the negative peak at higher wavelengths appeared only at drug-to-protein ratios greater than one (Fig. 2 and insert). The new perturbation found at high drug-to-protein ratios was probably a result of the binding of the drug to another binding site of lower affinity. Alklylation again decreased the size of the peaks, particularly at high drug-to-protein ratios, but there were also associated peak shifts and at low drug-to-protein ratios a slight shoulder appeared at 305–310 nm. The data suggest that following alkylation, new perturbations in the chromophores are involved. The wavelengths of the induced Cotton effects are characteristic of the drug molecule, therefore a different mode of binding for the drug with alkylated albumin is indicated.

Phenylbutazone

Phenylbutazone has been shown to have an induced CD peak at 287 nm following binding to HSA (Chignell 1964). Rosen (1970) found a peak at 285 nm and that the induced CD came from a single site of high affinity and two additional sites of lower affinity. Here a smaller and very concentration-dependent peak was found in the region of 258–268 nm. Unfortunately, because of absorption problems, the small ellipticity involved, and the large contribution of the albumin to the circular dichroism, good quantitation at this lower wavelength was impossible. Alkylation of the albumin caused an increase in the ellipticity at higher wavelengths (Fig. 3 and insert) and a slight reduction at the lower wavelengths. At higher drug-to-protein ratios the alkylation reduced the peak height at 287 nm; at lower wavelengths the effect was impossible to measure quantitatively. Earlier, Rosen (1970) had shown that the induced CD results from two types of binding sites and the single tryptophan can only be involved in one binding site; however, it is possible that the second binding site is in close proximity to the first site and that the incorporation of the hydroxy nitrobenzyl group at one of the sites alters the geometry of the second site so interfering with the binding indirectly.

Dicumarol

Dicumarol was shown to have induced Cotton effects exhibiting strong concentration dependence (Perrin

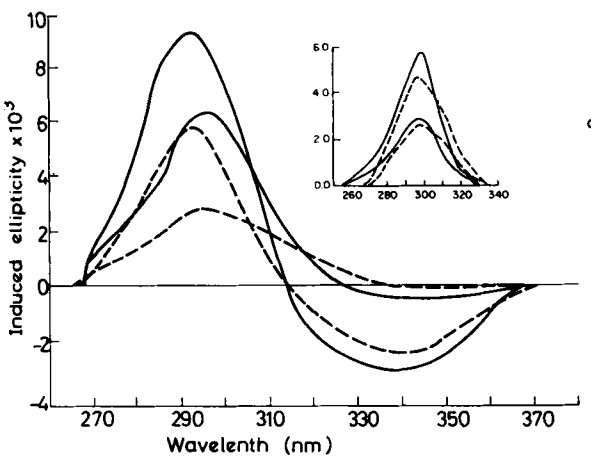

FIG. 2. Extrinsic Cotton effects following the binding of flufenamic acid to 7·25 × 10⁻⁶ M HSA V. —— HSA and - - - alkylated HSA. Drug concentrations 1 × 10⁻⁵ and 5 × 10⁻⁵ M. 1 cm cells. *Insert* Drug concentrations 4·44 × 10⁻⁶ M and 8·88 × 10⁻⁶ M. 2 cm cells.

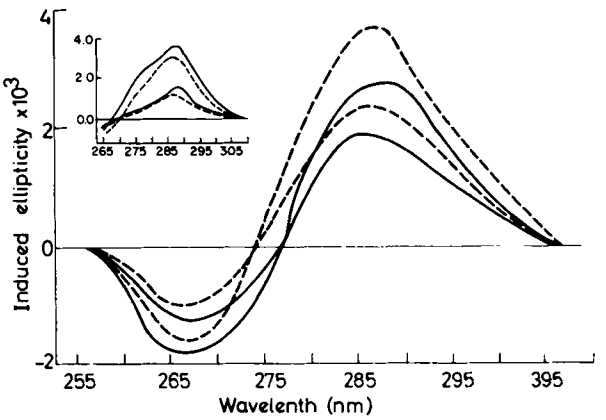

FIG. 3. Extrinsic Cotton effects following the binding of phenylbutazone to 7·25 × 10⁻⁶ M HSA V. —— HSA and - - - alkylated HSA. Drug concentrations 1·25 × 10⁻⁶ and 2·5 × 10⁻⁶ M. 1 cm cells. *Insert* Drug concentrations 4·06 × 10⁻⁶ and 8·1 × 10⁻⁶ M. 2 cm cells.

et al 1975). At lower concentrations a peak or shoulder was present around 305 nm, whereas at higher concentrations a pronounced peak occurred at 317 nm. The induced CD was shown to be from two non-equivalent classes of sites, each class containing a single site. Alkylation (Fig. 4 and insert) did not give any clear picture although the shapes of the induced curves were altered. At low concentrations the peak at lower wavelengths was virtually obliterated and in this region the induced ellipticity was enhanced. At higher concentrations the ellipticities diminished following alkylation, and at the drug-to-protein ratio of approximately 3 to 1 the peak at lower wavelengths was obliterated, as with HSA. It is apparent that alkylation interferes with at least one of the two binding sites either directly or indirectly.

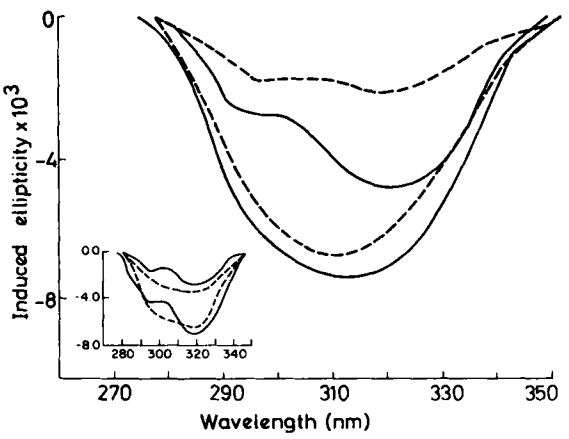

FIG. 4. Extrinsic Cotton effects following the binding of dicumarol to $7{\cdot}25 \times 10^{-6}$ M HSA V. —— HSA and - - - alkylated HSA. Drug concentrations $1{\cdot}12 \times 10^{-5}$ and $2{\cdot}23 \times 10^{-5}$ M. 1 cm cells. *Insert* Drug concentrations $3{\cdot}73 \times 10^{-6}$ and $7{\cdot}45 \times 10^{-6}$ M. 2 cm cells.

In summary, the treatment of HSA with Koshland's reagent did not appear to significantly alter the predominantly α-helical structure of the albumin; however, it should be realized that any localized modification of structure which may be significant in hiding or exposing binding sites would not be detected by the Cary 6002 spectropolarimeter.

In all cases investigated, the CD signal was modified at low drug concentrations, suggesting that the primary binding site of the four drugs did involve the tryptophan residue or region; in the case of phenylbutazone the binding was also modified at higher drug-to-protein ratios, suggesting a proximity of a secondary site to the primary site or a conformational change.The modification of the CD spectra following alkylation may arise from changes in the extent of binding or from changes in the perturbations arising from the interaction of drug and the region involving the alkylated tryptophan.

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