Biochemical Pharmacology, Vol. 27, pp. 1043–1047. Pergamon Press, 1978. Printed in Great Britain

OPTICAL ACTIVITY STUDIES OF DRUG–PROTEIN COMPLEXES. THE INTERACTION OF ACETYLSALICYLIC ACID WITH HUMAN SERUM ALBUMIN AND MYELOMA IMMUNOGLOBULIN

Y. Y. THOMAS SU and BRUNO JIRGENSONS

Department of Biochemistry, The University of Texas System Cancer Center, M. D. Anderson Hospital and Tumor Institute, Houston, TX 77030, U.S.A.

(*Received* 26 *March* 1977; *accepted* 3 *August* 1977)

Abstract—The binding of acetylsalicylic acid (ASA) to human serum albumin (HSA) and myeloma immunoglobulin (IgG) has been studied. The heterogeneity of the binding sites on both proteins was indicated by the non-linearity of the Scatchard plots. The association constants and the number of binding sites were determined. Near-UV circular dichroism (CD) spectrum of ASA–HSA complex showed a broad peak at 300 nm, which originated from the perturbed ASA molecule. This agrees with previous suggestion that electrostatic interactions played an important role in the binding. IgG–ASA complex showed CD spectrum changes between 300 nm and 260 nm. These changes suggested that, besides electrostatic interactions, the side chain chromophores of IgG also took part in the binding by hydrophobic interactions.

The interactions of a drug with proteins of blood or tissues may be responsible for the distribution and the transport of the drug and may limit its activity or metabolism. The binding constant is of fundamental importance for the concentration of free drug, which in turn determines the pharmacodynamic activity of the drug and the rate of inactivation and elimination [1].

In 1946 Lester *et al.* [2] and Smith *et al.* [3] in their extensive studies of salicylates in body fluids concluded that at low salicylate levels (< 5 mg/100 ml) more than 90 per cent of the salicylate was bound to plasma proteins and that the percentage of binding decreased as the level of drug increased or as the concentration of protein was decreased. Lester *et al.* [2] also demonstrated the presence of acetylsalicylic acid in the serum after its oral administration. In the preliminary binding experiments they found that at a 12 mg/100 ml concentration about 33 per cent of acetylsalicylic acid (ASA) was in the bound form. Quantitative experiment to study the binding of ASA by human serum albumin (HSA) was carried out using ^{14}C-labeled ASA and gel filtration to separate the free from bound form [4]. The results demonstrated that the binding increased to about 70 per cent after 40 hr. Another finding from this experiment was the displacement of salicylate from its binding site on albumin by ASA. However the association constant and the conformation of HSA–ASA complex are still unknown.

The interaction of IgG* and ASA has not been studied yet, though Haberland *et al.* [5] in their pharmacological studies on the mode of action of antiphlogistic agents found that ASA was capable of inactivating antibodies. They proposed that ASA might interact with antibodies and that complexes were thus formed. However, the details of the interactions between the molecules was still unexplored.

In present report we attempt to estimate the association constants for the formation of ASA–HSA and ASA–IgG complexes by the equilibrium dialysis and to determine the conformation of these complexes by CD analysis.

MATERIALS AND METHODS

Four-times crystallized HSA was purchased from Nutritional Biochemicals Corporation, Cleveland, OH. Human myeloma immunoglobulin was the same specimen which was described in a previous publication [6]. Acetylsalicylic acid, U.S.P. crystals, was obtained from General Chemical Company, NY. All other chemicals were reagent grade. Deionized water was used for the solution preparation.

Protein solutions, with their concentration at about 0.1 per cent, were prepared on 0.1 M phosphate buffer of pH 7. The accurate protein concentrations were determined from extinction coefficients $E_1^{\frac{per\ cent}{cm}}$ at 280 nm of 5.8 and 14.0 for HSA and IgG, respectively [7, 6]. The aspirin stock solution (6.8×10^{-4} M) was also prepared in the same phosphate buffer. Serial diluted drug solutions were made from this stock solution.

The bags for dialysis were prepared from cellulose tubing obtained from Fisher Scientific Company, Pittsburgh, PA. The tubing was boiled in diluted EDTA solution for 2 hr to eliminate substances which might absorb u.v. light and interfere with measurements of low concentrations of the drug. The tubing was rinsed with distilled water several times then soaked in phosphate buffer until used.

The extent of binding of drug to protein was

* *Abbreviations*—IgG, Myeloma immunoglobulin; and CD, circular dichroism.

determined by the equilibrium dialysis. Exactly 5 ml of protein solution were pipetted into a dialysis bag. The bag was immersed in 20 ml ASA solution and placed in a cold room. Buffer and protein blanks were run simultaneously. The bag was removed after 72 hr and the external solution was analyzed spectrophotometrically. Sample with the same initial ASA concentration was repeated at least twice. Blanks with buffer in the bags showed that about 10 per cent of the free drug was bound by the bags.

Acetylation of HSA was carried out by using ASA as a reagent. The method of Hawkins *et al.* [8] was used to acetylate HSA with ASA. In this procedure the protein (0.1 mM) was incubated at 37° for 24 hr with ASA (0.5 mM) then dialyzed at 4° for 48 hr against multiple changes of 0.15 M NaCl containing 0.05 M sodium phosphate buffer at pH 7.4, and finally against distilled water.

CD spectra of the complexes were measured on a Durrum–Jasco model CD–SP dichrograph, improved by D. P. Sproul of Sproul Scientific Instruments, Tucson, AZ. The sensitivity scale setting was 5×10^{-5} dichroic absorption per 1 cm on the recorder chart. Cell with 1.0 cm path length was used in the measurement. The data were expressed in terms of mean residue ellipticities, $[\theta]$, in degree·cm^2·*d*mole^{-1}. The ellipticity curves were obtained from at least two recordings.

RESULTS AND DISCUSSION

The equilibrium dialysis of proteins in aspirin. The binding of aspirin was investigated over the concentration range of 6.5×10^{-4} M to 3.0×10^{-6} M after equilibration. The results of binding experiments were illustrated in Scatchard plots [9]. Figure 1 represents the equilibrium dialysis of HSA in aspirin, and Fig. 2 represents that of IgG. Each point in these Figs is the average of at least two samples. The nature of the curves indicates that at least two sites of binding exist, each representing a protein–drug interaction of a separate type. In this case the number of drug molecules bound per molecule of protein, r, could be represented as [10]

$$r/C = \frac{n_1 K_1}{1+K_1 C} + \frac{n_2 K_2}{1+K_2 C}$$

where n_1 and n_2 are numbers of binding sites of two types with their association constants K_1 and K_2,

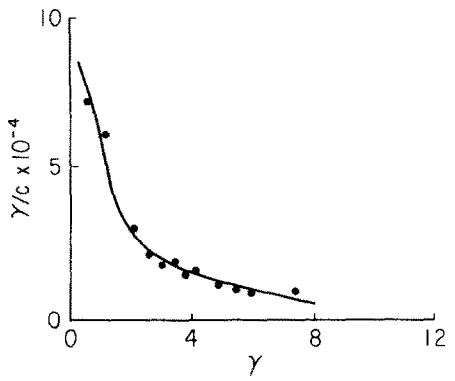

Fig. 1. Scatchard plot of the binding of acetylsalicylic acid to HSA. All measurements were made in the presence of 0.1 M sodium phosphate buffer, pH 7.4. r = number of moles of acetylsalicylic acid bound per mole of HSA; C = molar concentration of free acetylsalicylic acid

and C is the free drug concentration. The approximate values for association constants and the number of binding sites at each type of the binding on these two proteins could be obtained by assigning values for these parameters into the equation. The estimated values of these parameters are summarized in Table 1. Those parameters for bovine serum albumin (BSA) are also included in this Table [11]. It is evident that all three proteins exhibit two types of binding sites—one with high affinity but small in number, the other of low affinity but in greater number.

Davison and Smith [11], in their studies of the binding of salicylic acid and related substances to BSA, have investigated the nature of the binding

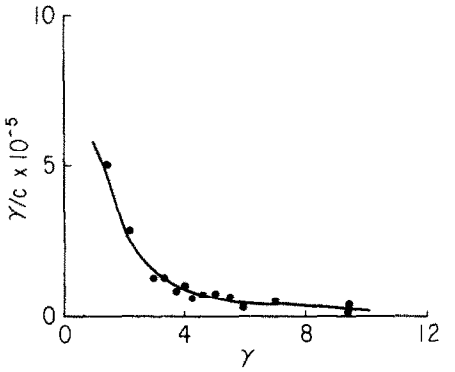

Fig. 2. Scatchard plot of the binding of acetylsalicylic acid to IgG. Conditions and units are the same as in Fig. 1.

Table 1. Binding of acetylsalicylic acid to 0.1% protein solutions in 0.1 M sodium phosphate buffer (pH 7.4) at 4°

Compound	No. of binding sites n_1	Association constant $K_1 \times 10^{-4}$	No. of binding sites n_2	Association constant $K_2 \times 10^{-3}$
IgG	3	22.0	8	9.8
HSA	2	4.62	7	0.56
BSA*	0.04	3.50	4.2	0.20

* From Reference 11, 0.69% BSA in sodium acetate buffer (pH 5.4), ionic strength 0.05, 4°. The difference in the number of binding sites between HSA and BSA may be due to the pH and ionic strength.

sites by the use of changes in pH, and of chemically modified proteins. Their studies showed little difference in binding with changes of pH, or with modification of tyrosine, histidine, tryptophan residues or cystein residues of protein. However, the binding was completely abolished by acetylation or by treatment with formaldehyde and acetamide. All these facts demonstrated that the binding appeared to be predominantly to the ϵ-amino and possibly the guanidino groups of the protein molecule.

There are 57 ϵ-amino groups, 22 guanidino and 1 end amino group in BSA [12]. HSA has essentially the same composition and conformation, and is expected to bind salicylate in a rather similar manner [11]. For IgG there are 104 ϵ-amino groups, 41 guanidino and 4 end amino groups [6]. However, the conformation of IgG is completely different from that of HSA [13]. It is possible that some other mode of interaction may also be involved in the formation of the aspirin–protein complex.

Hawkins *et al.* [8] have reported that ASA can acetylate HSA both *in vivo* and *in vitro*. According to their studies, at pH 7.3 and 37°, about 1.2 acetyl groups were covalently bound per molecule of HSA. In our present study, although the reaction system was kept at lower temperature (4°), the possibility of acetylation can not be fully eliminated. These workers also found that the acetylated HSA had an increased capacity to bind anionic drugs, such as acetrizoate [14]. In order to know the effect of acetylated HSA, the equilibrium dialysis of acetylated HSA in ASA solution was studied. However, the results showed no significant changes from those of unacetylated HSA. The acetylated HSA seems to have the same affinity to ASA as unacetylated HSA.

CD studies of the HSA–ASA and IgG–ASA complexes. CD spectra for the HSA–ASA complex at several bound drug ratios are illustrated in Fig. 3. Since the free drug itself did not exhibit any CD in solution, the spectra showed in Fig. 3 were obtained directly from the measurement. The major CD peaks of HSA–ASA complex are the ones centered around 300 nm and those between 270–250 nm. These peaks below 270 nm are very similar to those of drug-free HSA. A difference spectrum, curve 4 in Fig. 3, also shows only one broad positive peak with the maximum around 300 nm. The mean residue ellipticities of the HSA–ASA complex at 300 nm vs the mole ratio of bound drug to HSA, r, are shown in Fig. 4. The magnitude of the positive ellipticity leveled off at an r value of about 2. This suggests that only two of the binding sites on HSA generated strong CD effects while other binding sites gave rise to much weaker ellipticities.

In order to know whether the spectrum changes are due to the formation of HSA–ASA complex or to the acetylated HSA, the spectrum of the latter was also recorded. The CD spectrum of acetylated HSA, however, was exactly the same as that of the native protein. The changes in the CD spectrum, therefore, come from the binding of ASA rather than from acetylated HSA.

The CD spectra of the IgG–ASA complex at several bound drug ratios are shown in Fig. 5. The spectrum of the complex shows two new peaks between 280–270 nm. The rest of the peaks are

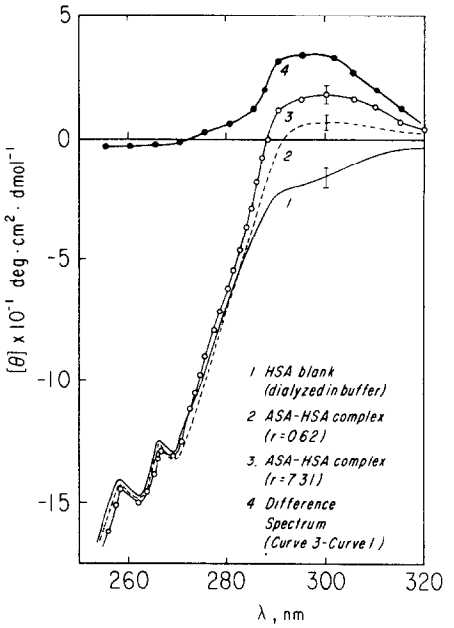

Fig. 3. CD spectra of HSA–acetylsalicylic acid complex. Curve 1, HSA blank (HSA dialyzed in buffer); curve 2, HSA–acetylsalicylic acid complex with $r = 0.62$; curve 3, HSA–acetylsalicylic acid complex with $r = 7.31$; curve 4, difference spectrum (HSA–drug complex–HSA blank). The bars represent S.D.

very similar to those of the drug-free protein though the intensity of the peaks was increased. Therefore the spectrum itself did not provide any direct evidence of conformational changes. A difference spectrum, obtained from the difference between the spectrum of the complex and that of protein, however, showed very distinctive changes which exhibited six positive peaks (Fig. 5), a broad one centered at 300 nm and five others located between 295–260 nm.

A plot of the difference ellipticity at 300 nm vs r is shown in Fig. 6. The curve leveled off at $r = 3$. This indicates that three of the binding sites gave the major CD changes in the complex.

The binding of a symmetric chromophoric drug molecule to a protein might generate extrinsic and/or intrinsic Cotton effects in the normal CD

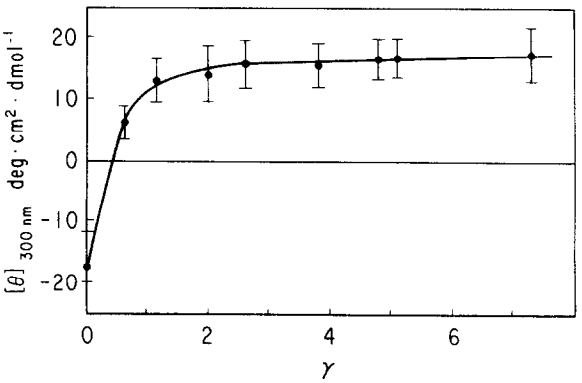

Fig. 4. Relationship between mean residue ellipticities of HSA–acetylsalicylic acid complex measured at 300 nm and the number of moles of drug bound per mole of HSA. The bars represent S.D.

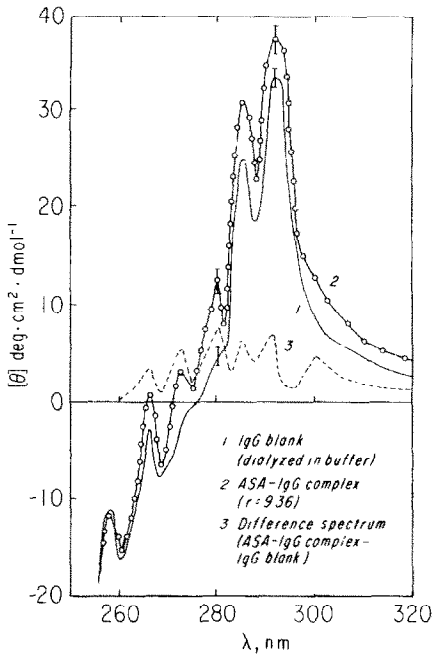

Fig. 5. CD spectra of IgG–acetylsalicylic acid complex. Curve 1, IgG blank (IgG dialyzed in buffer); curve 2, IgG–drug complex with $\gamma = 9.36$; curve 3, difference spectrum (IgG–drug complex–IgG blank). The bars represent S.D.

spectrum of the protein [15, 16]. Extrinsic Cotton effects arise when the asymmetrical center and the perturbed chromophore were not part of the same molecule and the intrinsic Cotton effects are due to conformational changes of the side chain chromophores in the protein molecule.

Extrinsic Cotton effects result from the perturbation of the electronic transitions in a drug chromophore by electrostatic forces associated with the asymmetrical locus at the binding sites [17]. The maximal ellipticity of an extrinsic Cotton effect is expected to occur in the same wavelength region where the perturbed chromophore absorbs light [18]. The sign of an extrinsic Cotton effect is determined by the configuration of the asymmetrical center and its spatial relationship to the perturbed chromophore [17]. The rigidity of the complex is an important factor to determine whether optical activity could be observed or not. A loose complex allows the ligand to move easily so that the asymmetrical center could move into either positive or negative regions. Under these conditions no optical activity would be observed. The magnitude of an extrinsic Cotton effect depends on the distance between the perturbed chromophore and the asymmetrical center as well as on the rigidity of the complex. Extrinsic Cotton effects thus reflect the characteristics of specific asymmetrical sites in proteins.

The CD spectrum of HSA–ASA complex showed a broad positive peak centered at 300 nm (Fig. 3). This band is located very close to the u.v. absorption maximum of ASA (at 296 nm). The binding of ASA to HSA thus generated extrinsic Cotton effects. The difference spectrum shown in Fig. 5 illustrates that both extrinsic and intrinsic Cotton were observed in IgG–ASA complex. The presence of intrinsic Cotton effects indicates that tryptophan, tyrosine and phenylalanine groups from IgG took part in the binding of ASA by hydrophobic interactions.

There is no doubt that electrostatic interactions play an important role in the binding of anionic drugs to HSA [19]. In accord with the investigation of Davison and Smith [11] the ϵ-amino group of lysine may be the point of attachment. However, a one-point electrostatic attachment of ASA to the proteins would allow the drug molecule to move freely, precluding the generation of extrinsic Cotton effects. It has been shown that the extrinsic Cotton effects by binding phenylbutazone to HSA depend not only on electrostatic interactions but also on van der Waals attraction between the phenyl groups of the drug and hydrophobic sites on protein [20]. The extrinsic Cotton effects of HSA–ASA and IgG–ASA complexes appeared at 300 nm which were about 4 nm toward red from ASA absorption maximum. This red-shift of the spectra demonstrates that the drug chromophores were in a hydrophobic environment.

Many different methods have been used for studying protein–drug complexes, e.g. equilibrium dialysis, gel filtration and NMR [2, 11, 21]. CD studies seem to be an attractive alternative because drugs often contain chromophores, and when these chromophores are bound to proteins induced Cotton

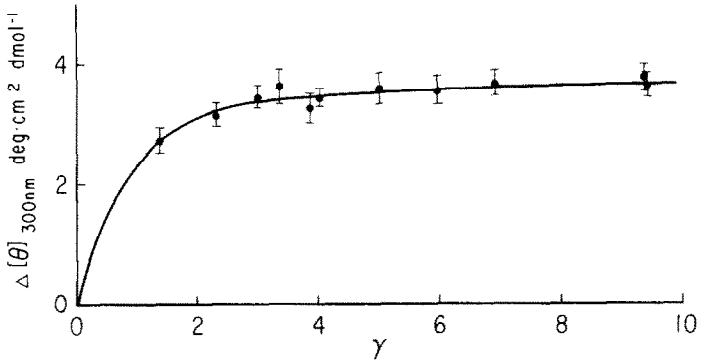

Fig. 6. Difference mean residue ellipticities measured at 300 nm vs the number of moles of drug bound per mole of IgG. The bars represent S.D.

effects can be observed in the normal CD spectrum of the protein [22, 23]. These induced effects can be used to determine the concentration of the drug–protein complex and the number of ligands bound [24]. Moreover, they also give information about binding site characteristics [24].

Acknowledgements—This work was supported by a grant G-051 from The Robert A. Welch Foundation, Houston, Texas.

REFERENCES

1. B. K. Martin, *Nature, Lond.* **207**, 274 (1965).
2. D. Lester, G. Lolli and L. A. Greenberg, *J. Pharmac. exp. Ther.* **87**, 329 (1946).
3. P. K. Smith, H. L. Gleason, C. G. Stoll and S. Ogarzalek, *J. Pharmac. exp. Ther.* **87**, 237 (1946).
4. M. A. Ali and J. Routh, *J. clin. Chem.* **15**, 1027 (1969).
5. G. L. Haberland, L. Busch, W. Fink and H. Friedrich, *Z. Rheumaforsch.* **18**, 220 (1959).
6. D. L. Ross and B. Jirgensons, *J. biol. Chem.* **243**, 2820 (1968).
7. H. E. Schultze, N. Heimburger and Z. Frank, *Biochem. Z.* **336**, 388 (1962).
8. D. Hawkins, R. N. Pinckard and R. S. Farr, *Science, N.Y.*, **160**, 780 (1968).
9. G. Scatchard, *Ann. N.Y. Acad. Sci.* **51**, 660 (1949).
10. F. Karush, *J. Am. chem. Soc.* **72**, 2705 (1950).
11. C. Davison and P. K. Smith, *J. Pharmac. exp. Ther.* **133**, 161 (1961).
12. C. Tanford, S. A. Swanson and W. S. Shore, *J. Am. chem. Soc.* **77**, 6414 (1955).
13. B. Jirgensons, *Optical Activity of Proteins and Other Macromolecules*, 2nd Edn, pp. 77–99, 115–121. Springer, Berlin. (1973).
14. R. N. Pinckard, D. Hawkins and R. S. Farr, *Ann. N.Y. Acad. Sci.* **226**, 341 (1973).
15. E. R. Blout, *Biopolymers*, Symp. No. 1, 394 (1964).
16. I. Sjöholm and T. Sjödin, *Biochem. Pharmac.* **21**, 3041 (1972).
17. J. A. Schellman, *J. chem. Phys.* **44**, 55 (1968).
18. D. D. Ulmer and B. L. Vallee, *Adv. Enzymol.* **27**, 37 (1965).
19. I. M. Klotz, R. K. Burkhard and J. M. Urquart, *J. Phys. Chem. Ithaca*, **56**, 77 (1952).
20. C. F. Chignell, *Molec. Pharmac.* **5**, 244 (1969).
21. C. M. Meyer and D. E. Guttman, *J. Pharm. Sci.* **57**, 895 (1968).
22. C. F. Chignell, *Adv. Drug Res.* **5**, 55 (1970).
23. A. Rosen, *Biochem. Pharmac.* **19**, 2075 (1970).
24. K. Ikeda and K. Hamaguchi, *J. Biochem., Tokyo* **66**, 513 (1969).