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CONFORMATIONAL EFFECTS IN THE INTERACTION OF PHENYLBUTAZONE WITH ALBUMIN STUDIED BY CIRCULAR DICHROISM

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Abstract—The binding of phenylbutazone (PB) to human serum albumin (HSA) at different pH and in the presence of different NaSCN and urea concentrations that alter the conformation of the protein was examined qualitatively on the basis of extrinsic elliptical strength at 288 nm by means of circular dichroism (CD). The values of the binding index expressed as a ratio of $[\theta]^{max}/[\theta]^{pH7.4}_{288}$ at each extrinsic rotational strength in the presence of various concentrations of NaSCN, urea and hydrogen ion were directly proportional to the α-helix content based on the peptide backbone alteration of HSA by NaSCN, urea and hydrogen ion except for the pH range of 5.0 to 10.0. The values in the pH range of 7.4 to 10.0 depended on the concentration of hydrogen ion and not on the α-helix content, showing a significant effect of the hydrogen ion on the tertiary conformation with respect to the binding sites of the amino acid chain rather than the peptide backbone of HSA. The increases in the binding index observed in the pH range of 7.4 to 10.0 were not observed at all in the case of NaSCN and urea at the concentrations studied. It was demonstrated that the binding of PB to HSA increased with the change in the tertiary conformation caused by hydrogen ions but decreased with that in the secondary conformation caused by a concentration change of NaSCN and urea. Thus, the binding was closely associated with skeletal conformational alterations as well as changes in the binding sites of the amino acid chains of the protein.

It is generally supposed that the development of drug action is closely related to the intensity of the interaction of the drug with its receptor protein. To examine this intensity, it would be useful to obtain information on the ability of drug to bind to conformationally altered protein. It has been reported previously that conformational alteration of protein correlates with drug binding ability [1, 2]. It is well known that a non-steroidal antiinflammatory drug binds to specific sites in human serum albumin (HSA†). There have been detailed investigations into the effects of pH and small molecules on the binding of non-steroidal antiinflammatory drugs on the tertiary conformationally altered HSA at physiological pH and on binding sites of the protein by means of equilibrium dialysis [3–8], microparticle [4, 5] and CD methods [5–10]. However, these methods, except for CD, are inadequate for determining the effects of the drug binding on secondary conformationally altered protein, since these methods can not detect the dynamic movement of secondary conformation. Consequently, there is little information [1] available on the binding of drug to HSA that has been conformationally altered in its secondary structure by changes in salt and hydrogen ion concentrations over a pH range of 3.0 to 12.0. HSA represents not only a homogeneous, well-defined and extensively studied protein with receptor-like properties for drug binding: it is also easily subjected to conformational modification in its tertiary and secondary structures by changes in pH and the concentrations of various salts [1, 11, 12]. In addition, the CD spectrum of HSA shows an intrinsic Cotton effect [7–10] depending on the degree of binding when HSA was mixed with PB which does not show any CD absorption. Therefore, it is an excellent model for studying conformational effects on PB–HSA interactions and the molecular aspects of ligand–protein interactions.

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† Abbreviations; PB, phenylbutazone; CD, circular dichroism; HSA, human serum albumin.

MATERIALS AND METHODS

Materials. Redistilled, deionized water was used throughout this work. All the other chemicals used were analytical grade reagents and were employed without further purification. HSA and PB were obtained from the Sigma Chemical Co. (St Louis, MO, U.S.A.). The albumin concentration was determined by the method of Lowry [13] as 13.1 μM, and all solutions were made with 1/15 M phosphate buffer and adjusted to the desired pH with 1 M HCl or 1 M NaOH.

Circular dichroism measurements. CD measurements were made at 25° with a JASCO-600 (Japan) spectropolarimeter calibrated with (+)-D-camphorsulfonic acid ammonium salt and equipped with a computerized data processing system. All spectra were recorded in a square quartz cell with 1.0- or 10.0-mm path length for short or long wavelength using time constant, step resolution and

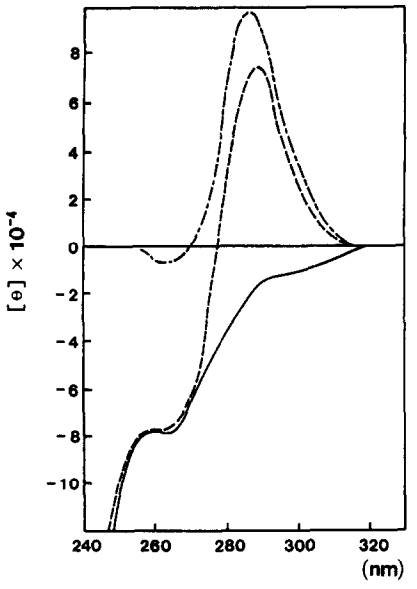

Fig. 1. CD spectra of HSA in the presence or absence of PB and their subtraction. HSA, (——); HSA + PB, (– – –); subtraction, (— — —).

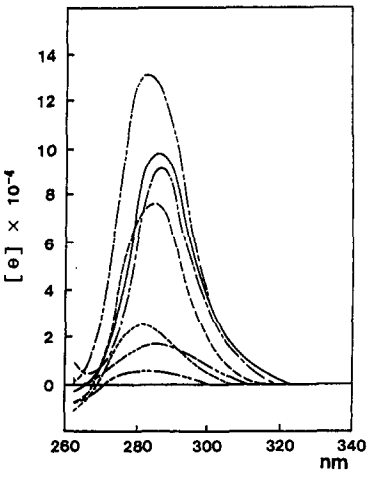

Fig. 2. CD spectra of HSA subtracted from spectra of a mixture of HSA and PB at various pH: 3.0, (— — —); 12.0, (— — —); 3.5, (— — — —); 4.0, (— —); 4.8, (— — —); 7.4, (——); 8.5 and 10.0, (— — —).

band width of 1.0 sec, 0.1 nm and 1.0 nm, respectively. Results are expressed as molar ellipticities, $[\theta]$(deg × cm^2/dmol), calculated with reference to the HSA concentration, using a M_r of 69,000. Each CD spectrum reported is the average of five scans for short or long wavelength. The binding index was calculated by the following equation: index = $[\theta]^{max}/[\theta]_{288}$ where $[\theta]_{288}$ is the molecular ellipticity of 13.1 μM HSA bound to PB at 288 nm in pH 7.4 and $[\theta]^{max}$ is the maximal molecular ellipticity of 13.1 μM HSA bound to PB under various conditions. α-Helix content was estimated from the molecular ellipticity of the 208-nm band as described elsewhere by Greenfield and Fasman [14]. For observations of extrinsic Cotton effect, it has been reported that no difference larger than the experimental error was observed among the various samples of HSA with respect to CD between monomeric or the unchromatographed HSA, although there have been differences reported in rat and rabbit for their binding affinity [15, 16]. Therefore, essentially fatty acid free HSA from Sigma was used throughout the experiments without any further treatment.

RESULTS

The binding of PB to and interaction with conformationally altered HSA caused by changes in the pH and NaSCN and urea concentrations was followed qualitatively by the CD spectropolarimeter on the basis of induced CD spectra in the wavelength region of 240 to 320 nm. Estimation of α-helix was carried out from CD spectra of HSA at 208 nm. Although the CD spectrum of HSA alone showed negative absorption (see Fig. 1), the CD spectrum of HSA solution in the presence of PB showed a strong positive band with a maxima at 288 nm caused by interaction of the two compounds. When PB bound to the protein, the extrinsic Cotton effect due to the absorption at 288 nm was in agreement with previous curves [7–10] and provided evidence for a molecular interaction between HSA and PB. The spectrum of HSA substrated from the spectrum of a mixture of HSA and PB gives the induced spectrum due to the interaction.

Figure 2 shows the induced CD spectra of a mixture of HSA and PB at various pH values. The CD curves at pH 8.5 and 10.0 were stronger than those at pH 7.4, while curves under more acid and alkaline conditions were increasingly weak. Thus, induced CD spectra were greatly modified by change in pH. Accordingly, this induced elliptical strength of the 288-nm band may be used for the calculation of the binding of PB to conformationally altered HSA. The index calculated from the respective maximal ellipticity versus the ellipticity of 288 nm at pH 7.4 might reflect the extent of binding of PB to the HSA molecule with various α-helix contents under the respective conditions. That is to say, the index suggests that the extent of binding of PB to HSA corresponds to the α-helix content which was estimated from the ellipticity at 208 nm of HSA under identical conditions as in the case of the measurement of CD in a mixture of PB and HSA. The elliptical strength at 208 nm, being attributable to α-helix conformation, was used to estimate the α-helix content of HSA at the various pH and with the various concentrations of NaSCN and urea as described previously [1].

The plots of the index calculated from curves in Fig. 2 and of α-helix content versus various pH are presented in Fig. 3. In Fig. 3, the binding index between HSA and PB at various pH can be compared with the α-helix content. The comparison of the curve of α-helix content with that of the index

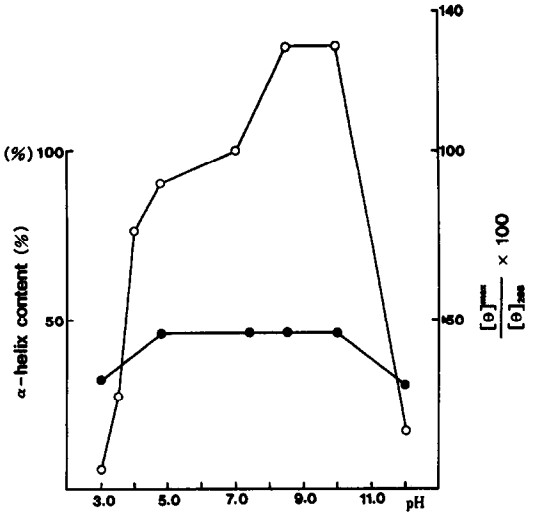

Fig. 3. Plots of α-helix content and binding index versus various pH. Left ordinate (●), α-helix content calculated by the elliptical strength of HSA at 208 nm at various pH. Right ordinate (○), the binding index estimated by a ratio of $[\theta]_{max}/[\theta]_{288}$ at respective ellipticity with variation in pH.

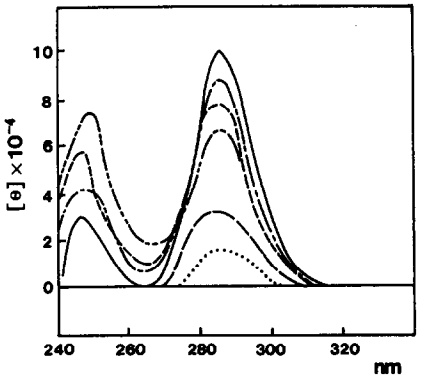

Fig. 4. CD spectra of HSA subtracted from spectra of a mixture of HSA and PB in the presence of various concentrations of NaSCN: 1.0 mM, (——); 5.0 mM, (— — —); 10 mM, (- - - - - - -); 50 mM, (— — — —); 100 mM, (——); 500 mM, (· · · · · · · · ·).

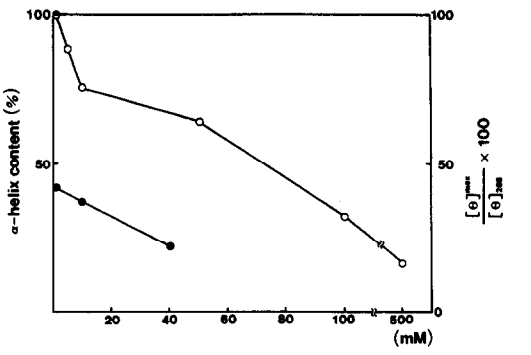

Fig. 5. Plots of α-helix content and binding index versus various concentrations of NaSCN. Left ordinate (●), α-helix content calculated by the elliptical strength of HSA at 208 nm with various NaSCN concentrations. Right ordinate (○), binding index estimated by a ratio of $[\theta]_{max}/[\theta]_{288}$ at respective ellipticity with various concentrations of NaSCN.

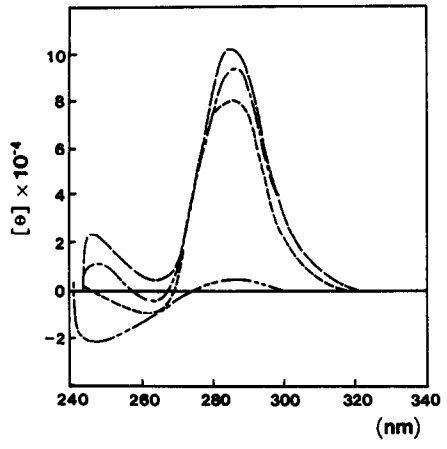

Fig. 6. CD spectra of HSA subtracted from spectra of mixture of HSA and PB in the presence of various concentrations of urea: 0.5 M, (——); 1.0 M, (— — —); 3.0 M, (- - - - - -); 6.0 M, (— — — —).

obtained in ranges of pH from 3.0 to 4.8 and from 10.0 to 12.0 showed a drastic change, i.e. there was a proportional relationship between the index and the α-helix content. In contrast, the binding index over the pH range of 7.4 to 10.0 showed an increase of about 40% in comparison with the index at pH 7.4, although the α-helix content over the range of 4.8 to 10.0 was constant. Thus, no special relationship was observed between the α-helix content and the binding index in the pH range of 7.4 to 10.0.

Figure 4 shows the CD spectra of a mixture of PB and HSA in the presence of various concentrations of NaSCN at pH 7.4 in phosphate buffer. The binding of PB to HSA conformationally altered by NaSCN dropped sharply up to a 10 mM concentration of the salt, and subsequently decreased gradually with the decrease in the α-helix content of HSA, as shown in Fig. 5. Thus, the binding index in the presence of NaSCN showed a curve of two phases dependent on the concentration of the salt. CD measurement of the mixture in the presence of any concentration of NaSCN was used for the estimation of the binding index, but the α-helix content of HSA could not be estimated from the CD ellipticity because the band at 208 nm was disturbed by salt concentrations over 40 mM.

The CD spectra of the mixture of PB and HSA in the presence of various concentrations of urea are shown in Fig. 6. In contrast to the limiting effects

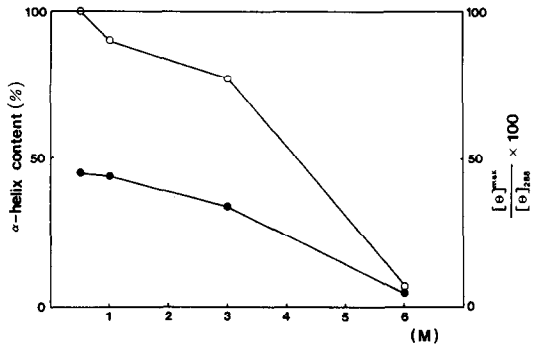

Fig. 7. Plots of α-helix content and binding index versus various concentrations of urea. Left ordinate (●), α-helix contents calculated by the elliptical strength of HSA at 208 nm in various concentrations of urea. Right ordinate (○), the binding index estimated by a ratio of $[\theta]_{max}/[\theta]_{288}$ at respective ellipticity with various concentrations of urea.

of NaSCN with regard to the estimation of the α-helix content, urea allowed the measurement of the α-helix content over a wide range of concentrations, from 0.5 mM to 6.0 M. The curves of both the α-helix content and the binding index calculated from the curves in Fig. 6 versus various concentrations of urea are shown in Fig. 7. The curves are similar in shape which suggests a proportional relationship between the change in secondary conformation and the binding of PB to the protein. The index value above 100, which was seen in the pH range of 8.0 to 10.0 (Fig. 3), was not observed with either NaSCN or urea, even at the lowest concentration of these salts.

DISCUSSION

The conformation of HSA in the pH range of 7.0 to 9.0 is well documented [11, 12] the N form occurring mainly below neutral pH and the B form at higher pH (so-called neutral-to-base or N–B transition). These conformations arose from changes in the tertiary structure and not from the secondary conformation of HSA. The role of the conformational N–B transition on drug binding has been described mainly in the case of warfarin, which probably competes with PB for the same or similar binding sites on HSA [17]. The increase in the binding index in the pH range of 7.4 to 10.0 (see Fig. 2) is in accordance with the results observed for warfarin. That is to say, the B form is favoured as the ionic strength is increased and is responsible for exposing the unmasked hydrophobic interfaces in HSA. It seems possible that the increase in exposed interfaces contributes to the magnitude of the binding index. In contrast to the increased binding index, the α-helix content estimated from ellipticity of CD spectra of HSA in the same pH range and buffer remained constant. This is evidence that the binding of PB to HSA is directly related to a conformational change in the tertiary structure and not to a charge in the secondary conformation in the pH range of 7.4 to 10.0.

The properties of the conformational changes of HSA in the pH ranges of 3.0 to 5.0 and of 10.0 to 12.0 are different from those of that in the pH range 7.0 to 9.0. The conformation in both the acid and alkaline range is attributable to the change in secondary and not tertiary structure, judging from the decrease in α-helix content, as seen in Fig. 2. Therefore, the dramatic decrease in the binding index in these pH ranges provided evidence that PB binding to HSA depends on the α-helix content in the secondary conformation. It is likely that the conformational change in the tertiary structure serves to bind PB to HSA while that in the secondary structure interfaces with the binding.

Hofmeister [18] was the first to recognize specific ion effects on macromolecules and gave the name "chaotropic salts" to the ions concerned. Recently, we have demonstrated that NaSCN caused a major shift in the secondary conformation of HSA [19] and poly-L-lysine [20] at much lower concentrations than other salts such as KCl, SeCl and NaCl. That is to say, the degree of destruction of the α-helix in HSA caused by NaSCN was 100 times greater than that caused by KCl and urea. In addition to these actions of NaSCN, it has been reported that this salt showed an inhibitory effect on normal and neoplastic mammary development through thyroid hormone modification in female mice and rats [21, 22]. Consequently, it would be of interest to obtain information regarding the effect of NaSCN on drug–protein interaction in relation to secondary conformational change in the protein.

The α-helix content of HSA decreased markedly and linearly with an increase in the concentration of NaSCN, although the maximum concentration of the salt that could be used for CD measurement was 40 mM because the CD spectrum of HSA at 208 nm was disturbed by the salt. This secondary conformational change in HSA in the presence of NaSCN contrasts with the observation of a constant value for α-helix content in the pH range of 5.0 to 10.0 (Fig. 2). This difference between the effects of NaSCN and pH on conformation is not only attributable to the change in the secondary and tertiary conformations, respectively, but also seems to be useful for the study of the binding of PB to the different kinds of conformation of HSA. The value of the binding index corresponding to concentrations ranging from 0 to 10 mM of NaSCN dropped sharply and there was then a gradual decrease over concentrations up to 500 mM. Thus, the index in the presence of NaSCN showed a biphasic curve due to a different pattern of binding.

This phenomenon of two phases must be due to the existence of different kinks in the PB binding site HSA as suggested previously [3]. The dramatic decrease in the index was attributable to the fact that a change in tertiary conformation, which was easily modified by a low concentration of NaSCN, serves to remove the PB bound to the surface of the binding sites in the protein. The gradual decrease in the index with increasing salt concentration seems to result from the removal of PB bound to inner binding sites revealed by modifications of the secondary conformation of HSA with high concentrations of the salt.

The curves of the α-helix content and the binding index versus various concentrations of urea were a similar shape suggesting a proportional relationship between the α-helix content in secondary conformation and the binding index of PB to the protein (Fig. 7). The concentration of urea required to produce the same content of α-helix as produced by NaSCN was much higher than that of the salt (Figs 5 and 7). Furthermore, the binding index values corresponding to the same value for the α-helix content in the presence of NaSCN and urea were not identical. Change in the tertiary structure of HSA aid the binding of PB to the protein, but secondary conformational changes caused by NaSCN and urea interfere with the binding of PB.

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