Species Differences of Serum Albumins: III. Analysis of Structural Characteristics and Ligand Binding Properties During N-B Transitions[1]

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Purpose. The aim of this study was to investigate the characteristics of the structural transitions and changes in ligand binding properties of different albumins during the pH-dependent structural transition, often referred to as the N-B transition.

Methods. Structural transitions were evaluated by means of spectrometry, differential scanning calorimetry and chemical modification. In addition, ligand binding properties were investigated using typical site-specific bound drugs (warfarin, phenylbutazone, ibuprofen and diazepam).

Results. Conformational changes, including N-B transition, clearly occurred in albumins from all species used in this study. The conformational stabilities of all the albumins were clearly lost in the weakly alkaline pH range. This was probably the result of the destruction of salt bridges between domain I and domain III in the albumin molecule. In addition, the profiles of the ANS-induced fluorescence were different and could be classified into two patterns, suggesting that hydrophobic pockets in the albumin molecules were different for the different species. The data suggest that the amino acid residues responsible for the transitions were some of the His residues located in domain I. Further, the ligand binding properties of the albumins were slightly different but statistically significant.

Conclusions. The overall mechanisms of the N-B transition may be similar for all the albumins, but its impact is considerably different among the species in terms of both structural characteristics and ligand binding properties. Furthermore, the transitions appear to be multi-step transitions.

KEY WORDS: serum albumin; species difference; N-B transition; protein binding; conformational stability; chemical modification.

INTRODUCTION

It is well known that human and bovine serum albumins undergo structural transition in weakly alkaline solutions (2). Because this structural transition, referred to as the N-B transition, occurs within the physiological pH range and involves changes in ligand binding properties, the properties of this transition have been the subject of intense study.

It is well known that serum albumin facilitates ligand uptake into tissues (3–8). This may be due to conformational changes, induced as a result of interactions between biomembrane and albumin (9). In fact, Horie et al., using ESR techniques, concluded that this conformational change was similar to those which occur during the N-B and/or the N-F (the conformational transition occurring in the acidic pH range) transitions (9).

Sudlow et al. (10) demonstrated the presence of at least two very specific and selective binding sites, which were referred to as site I and site II, on the human albumin molecule, which represent binding sites for a large number of tightly bound drugs. The binding properties for site I and the diazepam binding site at site II are known to be influenced by the transition (11–19). Recently, crystallographic structures of human and equine albumins have been reported (20,21). As a result, the positions of the drug binding sites on human albumin could be clearly identified, and the specific amino acids responsible for the binding of some ligands are now well known. Further, it is also clear that these albumins consist of three domains in which each domain is composed of two subdomains.

Very recently, the identification and classification of the drug binding sites on different mammalian serum albumins have been carried out in this laboratory (22): a specific drug binding site, corresponding to site I on human albumin is present on rabbit and rat albumins but not dog albumin, while a site corresponding to site II is present on dog albumin but not bovine, rabbit and rat albumins. These species differences in drug binding sites prompted us to surmise that the pH-dependent ligand binding properties may also show species differences. These, in turn, suggest species differences relative to ligand uptake through the bio-membrane. It is unfortunate that data on the structural transition and ligand binding properties of experimental animal albumins regarding the N-B transition for human and bovine albumins have not yet been obtained.

The present paper describes the structural characteristics of different albumins during the N-B transition by spectroscopic and thermodynamic analysis. The transition was also studied by ^{1}H-NMR and chemical modification techniques. In addition, the ligand binding properties of these albumins during the N-B transition were investigated by employing warfarin (WF), phenylbutazone (PBZ), as site I binding ligands, and ibuprofen (IP) and diazepam (DZ), as site II binding ligands.

MATERIALS AND METHODS

Materials

Human albumin was donated by the Chemo-Sera-Therapeutic Research Institute (Kumamoto Japan). Human, bovine, dog, rabbit and rat albumins were purchased from the Sigma Chemical Co. (St Louis, MO, U.S.A.). Bovine albumin was also obtained from Wako Pure Chemical Industries (Osaka, Japan). The samples were defatted with activated charcoal in solution at 0°C, acidified to pH 3 with dilute H_2SO_4 and then freeze-dried. All albumins used in this study showed only one band by SDS-PAGE, and the molecular masses were assumed to be approximately 66kDa. Potassium WF (Eisai Co., Tokyo, Japan), IP (Kaken Pharmaceutical Co., Tokyo, Japan) and DZ (Sumitomo Pharmaceutical Co., Osaka, Japan) were obtained from the manufactures and PBZ was purchased from Nakalai Tesque (Kyoto, Japan). All other chemicals were of analytical

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grade. 0.067M phosphate buffer was prepared with dibasic sodium phosphate and monobasic sodium phosphate and was used exclusively in this study.

Circular Dichroism and Fluorescence

For estimating the tertiary structural properties of the different albumins, samples containing each albumin were prepared, and the ellipticities at about 292 nm, which reflect the change in tertiary structure were monitored with a JASCO J-720 type spectropolarimeter (JASCO, Tokyo, Japan). To estimate the tertiary structural properties in the vicinity of the Trp residue(s) on different albumins, fluorescence measurements of these samples were performed with a JASCO FP-770 type fluorescence spectrometer (JASCO, Tokyo, Japan). A fluorescence excitation wavelength of 295 nm was employed.

Differential Scanning Calorimetry

For thermodynamically estimating the pH-dependent structural transition, differential scanning calorimetry (DSC) was carried out on a MicroCal MC-2 ultrasensitive DSC (MicroCal Inc., Northampton, MA) at heating rates of 1 K/min, using sample concentrations of 0.1 mM. The obtained DSC data were applied to nonlinear fitting algorithms to calculate the thermodynamic parameters, thermal denaturation temperature (Tm) and calorimetric enthalpy (ΔH), from the temperature dependence of excess molar heat capacity, C_p, by employing the Using Origin™ scientific plotting software.

^{1}H-NMR Spectroscopy

Chemical shifts of the C2 proton signal of His residues were monitored by high-resolution 500 MHz ^{1}H-NMR spectra. The experiments were carried out, essentially as described previously (23). In short, a spectral width of 6000 Hz and an 8K data points collection, giving an acquisition time of 0.68s, and an applied pulse angle 60° were used. The number of accumulated transients per spectrum varied from 600 to 6000. The pD (pH*) values are uncorrected, i.e., the values given by pH meter were used, as obtained.

Chemical Modification of His Residues

Chemical modification of His residues on different albumins was carried out following the generally accepted methods (24) with modification of some conditions, e.g., low concentrations of modifying reagent (diethyl pyrocarbonate (DEP)) were used. The modifications of multiple His residues were confirmed as described previously (24), and were found to be 2, 3, 2, 2 and 2 for human, bovine, dog, rabbit and rat, respectively. Removal of the modifying reagent from the His-modified albumin was also done according to generally accepted methods. Briefly, hydroxylamine hydrochloride, dissolved in acetonitrile was added to the solutions of His-modified albumin (about 2 mg/mL) to a final concentration of 1M. After the samples had reacted for 10 min at room temperature, excess reagent was removed using a sephadex G-25 column, and then freeze-dried. The obtained samples (DEP-free albumins) were used in the fluorescence experiments.

Equilibrium Dialysis and Determination by HPLC

Equilibrium dialysis was carried out, in order to analyze the effect of pH on the fraction which contained bound WF, PBZ, IP and DZ. Aliquots (1.5 ml), containing various ratios of ligand-albumin mixtures were placed in plastic dialysis cells (Sanko, Fukuoka, Japan), where the compartments were separated by Visking cellulose membranes (12 kDa molecular weight cut off), and were dialyzed against the same volume of buffer at 25°C for 12 h. After equilibrium was obtained, the free ligand concentrations (C_f) were determined by HPLC.

The HPLC system consisted of a Hitachi 655A-11 pump and a Hitachi L-4000 type UV detector or Hitachi L-7480 type fluorescence detector. An Inertsil ODS-2 column (5 μM, 4.6×150 mm) was used as the stationary phase. The mobile phase consisted of 0.1 M acetate buffer (pH 4.5)-acetonitrile (40:60, v/v) for WF, PBZ and IP, and water-acetonitrile (40:60, v/v) for DZ. PBZ and DZ were detected at 270 nm and 230 nm using a UV monitor and WF and IP using a fluorescence monitor. The excitation/emission wavelengths were 320 nm/390 nm and 263 nm/293 nm for WF and IP, respectively. The bound fractions of each ligand was determined using the following equation:

$$bound\ fraction = \frac{C_b}{C_f + C_b}$$

where C_b is the concentration of bound ligand.

RESULTS

Structural Characteristics of the Different Albumins During the N-B Transition, as Estimated by Fluorescence, CD, and DSC

Fig. 1A shows intrinsic fluorescence intensity of the single Trp residue on human, dog, rabbit and rat albumins and the double Trp residues on bovine albumin. The intensity is the highest for bovine albumin, which reflects the difference in the number of Trp residue. The intensities were higher for human and rat albumins than for dog and rabbit albumins. The pH profiles of these intensities showed nearly same tendency: almost no change or a slight increase of intensities was observed when proceeding from pH 6 to 7.4 and a clear decrease was

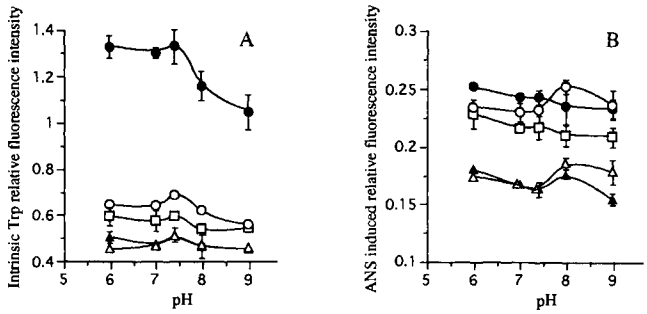

Fig. 1. Intrinsic-Trp (A), and ANS induced (B), pH-dependent fluorescences for different albumins at 25°C. The concentrations of albumins and ANS are 1 μM and 30 μM, respectively. The fluorescence intensities are represented as mean values of three separate experiments. The symbols correspond to the following albumins: (○) human, (●) bovine, (△) dog, (▲) rabbit, (□) rat.

observed from pH 7.4 to 8.8. For all species, wavelengths at maximal intensities were shifted to shorter wavelengths while going from pH 6 to 7.4, and were further shifted to shorter wavelengths while going from pH 8 to 8.8 (data not shown). Fig. 1B shows the pH profiles as a function of the fluorescence intensity induced by 1-anilino-8-naphthalene sulfonate (ANS) on the different albumins. These intensities, which reflect hydrophobicity on the protein surface, were also generally higher for human, bovine and rat albumins than for dog and rabbit albumins. The pH profiles of these intensities for the bovine and rat albumins showed a slight decrease over the pH range from 6 to 8.8, although those for human, dog and rabbit albumins showed maximal intensities at pH 8.

Fig. 2 shows intrinsic ellipticities around 292 nm of the different albumins. The intrinsic CD at this wavelength mainly monitored the aromatic amino acids, such as Tyr and Trp. Hence, these values reflect the properties of the tertiary structures sensitively. The fact that the observed ellipticities for each albumin were different, suggests the existence of environmental differences in tertiary structures. Further, the degree of these changes is clearly different among the species, being relatively small for human and bovine albumins as has been reported earlier, rather large for dog and especially rabbit albumins, and, in contrast, nearly unchange for the rat albumin.

Table 1 indicates the pH-dependent thermodynamic parameters for different albumins, as estimated by DSC. The values of both transition temperature, *Tm*, and calorimetric enthalpy, $\Delta Hcal$, tended to reach a maximum at pH 7.0–7.4 for all species, and were highest for human and dog albumins. The broad thermograms obtained for dog and rabbit albumins, which were previously reported to be biphisic transitions (1), also showed broad peaks over the range of pH from 7.4 to 8.8, although, over the range from 6.0 to 7.4, in contrast, sharper peaks were observed. In addition, at certain pH values, the thermograms for bovine and rat albumins showed two distinct peaks. Thus, the albumins for all species used in this study appear to possess a pH optimum for thermal stability. In particular, in the high pH region (B structure), the albumins lose most of their conformational stability.

Physicochemical properties related to the N-B transition are known to be different among different sources of human albumins (25). Therefore, these differences were investigated using human albumin (from Sigma Co.) and bovine albumin (from Wako Industries). Differences were observed from one lot to another (data not shown). However, these differences were slight as compared to the species differences. This suggests that the differences reported herein are due to species differences.

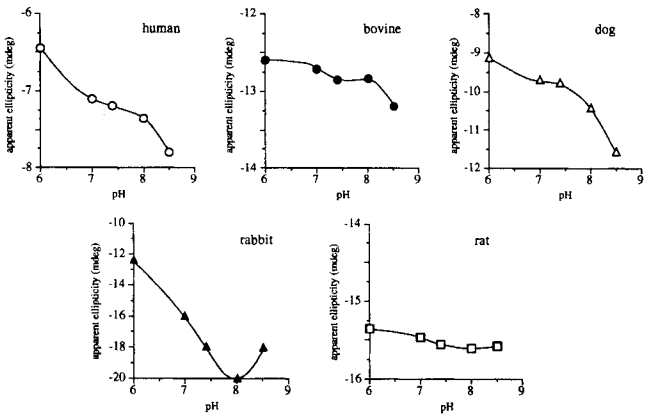

Fig. 2. pH-dependent intrinsic ellipticities depending on pH for different albumins at 292 nm and 25°C. The concentration of albumins is 0.1 mM. The values are represented as mean values of three separate experiments. The deviations are too small to be shown.

Involvement of His Residues in N-B Transition, as Evidenced by ^{1}NMR-spectroscopy and Chemical Modification

The above-mentioned conformational changes may originate from the His residues, which possess groups which dissociate around this neutral pH. We therefore examined the role of His residues in the conformational changes during the N-B transition, along with species differences.

Fig. 3 shows the ^{1}H-NMR spectrum for the C2 proton of the His residues on rabbit albumin at various pD values, because, among the animal albumins used in this study, it clearly shows on N-B transition. It has already been reported that some of the C2 proton signals of His residues on human albumin appear in the range from 7.0 to 8.6 ppm, and that some of these are shifted with pH change (23). Such sensitive His residues might exist in an environment which is relatively exposed to solvent, i.e., near the surface of the albumin molecule, and the chemical shift, which is probably due to a change of dissociation state, of those residues may be largely related to conformational change. It is clear from Fig. 3 that some of the signals, reflecting numerous His residues, were also shifted as observed in the case of human albumin. Such shifts were also observed in the other three species of albumins (data not shown). These results clearly show that the dissociation states of some of the His residues are altered in a pH-dependent manner, similar to the case of human albumin, and that the His residues might play an important role in affecting conformational change during the N-B transition, not only for human and rabbit albumins, but for the other albumins as well.

Fig. 4 shows the effect of the chemical modification of His residues on conformational changes during the N-B transition for different albumins. Although only two or three His residues were chemically modified for each albumin (see "*MATERIALS AND METHODS*"), the changes in the intrinsic Trp fluorescence during the transition were clearly diminished. Furthermore, after removing the modifying reagent by treatment with hydroxylamine hydrochloride, the properties were nearly recovered, as evidenced by the intrinsic-Trp fluorescence (For example, the dotted lines and closed squares for bovine albumin in Fig.4). These results strongly suggest that certain His residues are very important for the N-B transition.

Ligand Binding Properties of Different Albumins During N-B Transition

Fig. 5 illustrates the bound fractions of the site specific ligands of different albumins as a function of pH. Fig. 5A, 5B, 5C and 5D represent the bound fractions of WF, PBZ, IP and DZ, respectively. It is clear from Fig. 5A and 5B, that the bound fractions of WF and PBZ for dog albumin were quite low, reflecting their low binding parameters (22). Interestingly, although the bound fraction of WF for human albumin increased

Table 1. Thermodynamic Parameters of Different Albumins Depending on pH Obtained from Differential Scanning Calorimetry

Species	pH				
	6.0	7.0	7.4	8.0	8.8
Tm(°C)					
human	60.77 ± 0.52	61.13 ± 0.08	59.65 ± 0.05	58.49 ± 0.01	59.13 ± 0.01
bovine	61.51 ± 0.02	59.56 ± 0.25	56.80 ± 0.27	53.40 ± 0.44[a]	50.68 ± 0.16
				61.04 ± 0.40[b]	59.89 ± 0.14
dog	58.58 ± 0.12	60.68 ± 0.27	59.50 ± 0.10[c]	58.15 ± 0.13	57.66 ± 0.03
rabbit	57.55 ± 0.05	59.75 ± 0.08	57.78 ± 0.36[c]	57.21 ± 0.37	59.04 ± 0.62
rat	57.14 ± 0.08[a]	58.87 ± 0.73[a]	57.63 ± 0.37	57.00 ± 0.06[a]	—[d]
	69.59 ± 0.07[b]	69.34 ± 0.43[b]		68.85 ± 0.05[b]	
Δ*Hcal*(*kcal/M*)					
human	133.5 ± 12.0	176.0 ± 14.1	166.3 ± 3.2	127.0 ± 5.7	130.5 ± 4.9
bovine	135.5 ± 3.5	157.0 ± 9.9	152.3 ± 9.9	75.5 ± 5.4	50.4 ± 5.4
dog	111.5 ± 4.9	171.0 ± 5.3	168.7 ± 8.1	113.0 ± 4.2	84.9 ± 3.5
rabbit	144.5 ± 0.7	182.0 ± 1.0	164.5 ± 14.2	123.5 ± 29.0	60.2 ± 5.2
rat	95.0 ± 9.4	119.0 ± 17.8	132.0 ± 4.4	129.0 ± 4.2	—[d]

[a] Lower Tm value of two peaks.
[b] Higher Tm value of second peak to appear.
[c] The apparent value of one broad peak: see text.
[d] Not determined: accurate baselines were not obtained.

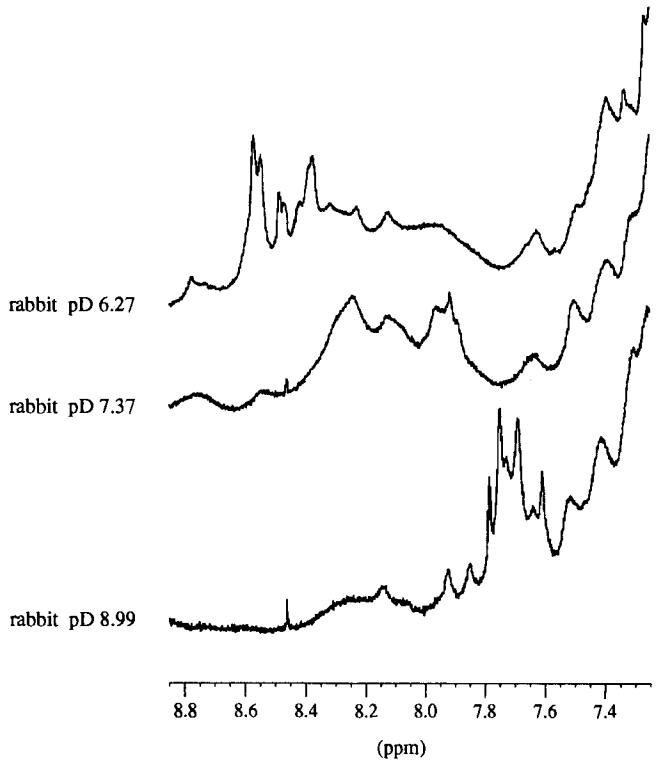

Fig. 3. ^{1}H-NMR His C2H resonances of rabbit albumin at various pD and 25°C. Other details of the conditions are given in *"Materials and Methods."*

in a pH-dependent manner as reported previously (11,13,18), except for rat, those for the other albumins were significantly decreased over the pH range from 8 to 8.8. The PBZ bound fractions for human, bovine, and rat albumins decreased slightly during the pH transition. In sharp contrast, the fraction for dog and rabbit albumins decreased significantly over the pH range from 6 to 7.4, but for the pH range from 7.4 to 8.8, they again increased. As shown in Fig. 5C, the bound fraction of IP was relatively high for all species, reflecting its high binding constant (22). The bound fraction of IP decreased slightly during the pH transition, but the changes for human and bovine albumins were negligibles. In contrast, the bound fraction of DZ differed significantly among the species (Fig. 5D). Human and dog albumins with relatively highly bound fractions, showed a slight increase during pH transition, while rabbit, rat and especially bovine albumins with a low bound fraction, showed a considerable increase as a function of pH change.

DISCUSSION

It is clear from the results obtained in this study that all albumins showed structural transitions, which were similar to the N-B transition for human albumin. These transitions involve changes in ellipticities at 292 nm (Fig. 2). Since the ellipticities at this wavelength sensitively reflect the environment of the aromatic amino acid residues, it can be concluded that larger conformational transitions occur in dog and rabbit albumins than in human, bovine and rat albumins. The intrinsic Trp fluorescences are significantly decreased (Fig. 1A) and maximal wavelengths shifted to shorter wavelengths (data not shown) at pH values higher than 7.4. This indicates that Trp residues are buried in hydrophobic environments but that fluctuation of the residue increased at this pH range. A considerable decrease in *Tm* and Δ*Hcal* were observed over the same pH range (Table 1), suggesting that the thermal stabilities were decreased considerably at weakly alkaline pH. The above data suggest that the fluctuations of the main chain of the B form are increased, compared to the N form (albumin structure at neutral pH). In fact, the B form (the structure of albumin at weakly alkaline pH) of human albumin is generally thought to cause an increase in fluctuation, based on findings obtained from the analysis of its reaction with iodine (26) as well as the H-D (hydrogen-deuterium) exchange of amide protons (27,28).

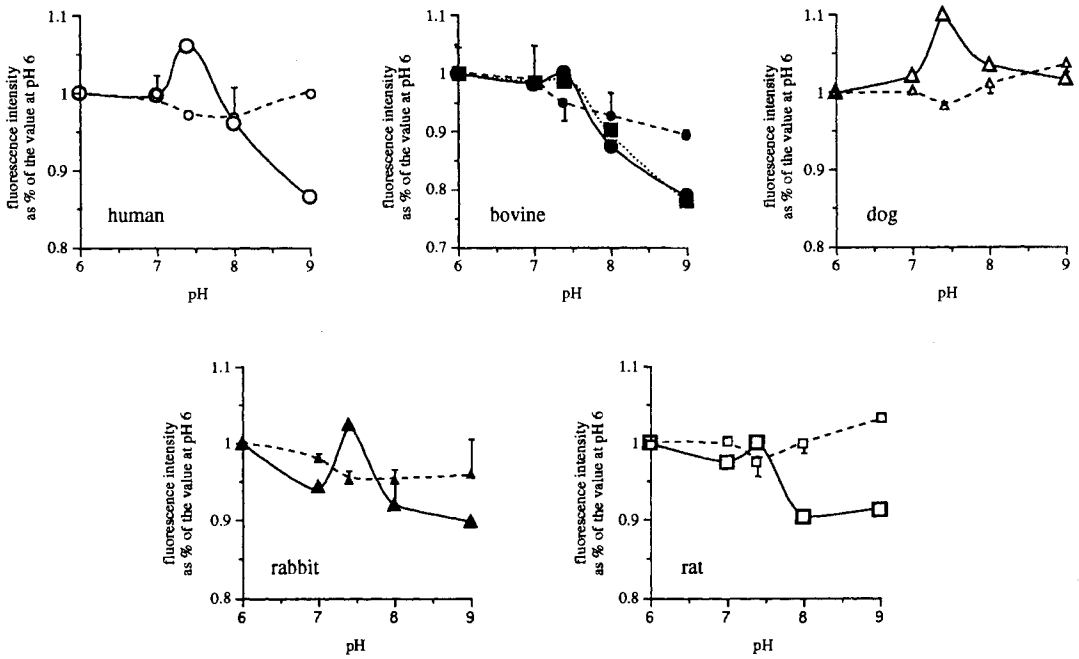

Fig. 4. Intrinsic Trp fluorescences of native (big symbols and solid lines) and His-modified (small symbols and broken lines) albumins at 25°C. Dotted line and closed square for bovine albumin show intrinsic Trp fluorescences of DEP removed from bovine albumin by treatment with hydroxylamine hydrochloride (see detail: "*Materials and Methods*" and "*Results*"). The values are represented as a % of the values at pH 6. The concentration of albumins is 1 μM. The symbols correspond to the following albumins: (○) human, (●) bovine, (△) dog, (▲) rabbit, (□) rat.

The structural transitions may originate from His residues on the albumin molecule, since the residues have a pKa within the pH range where these transitions are observed. In the case of human albumin, it has already been reported that chemical shifts of the C2 proton for some His residues can be observed (23). Such shifts were also observed in all other species (only the case of rabbit albumin is shown, Fig. 3). This conclusion is supported by the fact that the chemical modification of His residues on each albumin dramatically reduced the transition (Fig. 4). His proton 3 could dissociate in the same pH range as the transition occurs, so chemical modification by DEP at this position prevents the transition. Furthermore, the bovine albumin was restored to its original state after removal of DEP. These facts strongly suggest that the N-B transition originates from the dissociation of the third protons of selected His residues on albumin molecules. It is also possible that some of the His residues in domain I, which are involved in salt bridges between domain I and domain III, were chemically modified, and further restrained the transition.

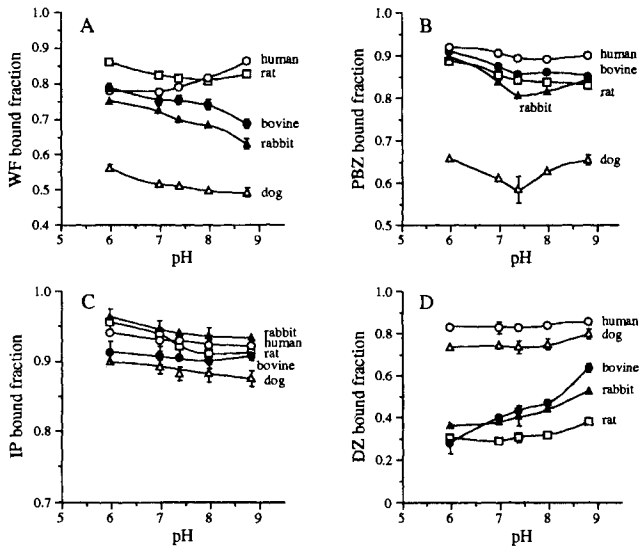

Fig. 5. Effects of pH on the binding of site-specific bound drugs (WF(A), PBZ(B), IP(C), DZ(D)) at 25°C. The concentrations of albumins and drugs was 20 μM. The values are represented as bound fractions, and are shown as mean values of three or four separate experiments. The symbols are the same as in Fig. 1.

It has been reported that the N-B transition involves the destruction of salt bridges between domain I and domain III (29). These salt-bridges originate from the delayed deprotonation of five His residues in domain I. Although dog albumin contains only 11 His residues, in contrast with 16 for human albumin and 23 for rabbit albumin, it showed a similar or larger degree of transition (Fig. 1, 2 and Table 1), compared with human albumin. This indicates that the His residues which are necessary for the N-B transition are present in dog albumin as well as the others. In domain I, four His residues are conserved among the species examined in this study. It is therefore reasonable to conclude that these residues are responsible for the N-B transition. Furthermore, since only two or three chemically modified His residues were found on each albumin molecule, some of the modified residues might exist in domain I, and play an important role for the transition. The positions of the modified residues will be identified by amino acid analysis in the near future.

Thus, domain I and, partially, domain III appear to be important for the stabilities and complete structures of albumins. However, this rational is not completely supported in terms of ligand binding. Most of the site II binding drugs on human albumin are not generally influenced by the N-B transition (18), although the affinity of diazepam or nitrazepam (a site II binding drug) is pH-dependent (16,17,19). In contrast, site I drugs are strongly influenced by this transition. This suggests that domain II is more strongly influenced by the transition than is domain III. These results are fundamentally the same for other species (Fig. 5). However, the extent of the change was considerably different among species. For example, the extent of the change in the binding of DZ was significantly different among species. The large changes for bovine, rabbit and rat albumins may be explained as follows: the conformation which is suitable for DZ binding via minimizing steric hindrance and/or optimizing the size of the binding pocket for DZ may occur during the N-B transition. The binding of WF increases for human but decreases for the other species with increasing pH. These facts suggest that the slight conformational change, as the result of the dissociation of His residues in domain II and III, causes microenvironmental changes in the vicinity of the drug binding sites on each albumin during the N-B transition.

It is also of interest that all the data for Trp intrinsic fluorescence, ANS induced fluorescence, thermal stability and the bound fraction of PBZ are consistent with the occurrence of distinct transitions with a boundary at around pH 7.4. These results suggest that structural transitions of albumins can be explained by consecutive transitions, as reported by t' Hart (16), rather than a two state transition between the N form and the B form.

ANS mainly binds to a hydrophobic area on albumin molecule such as site I and site II. The driving force for ligand binding to site I is hydrophobic interactions, although that for site II is mainly electrostatic and partially hydrophobic interactions (20). Therefore, the hydrophobicity of the site I binding pocket is important for drug binding. The ANS induced fluorescence was clearly different among the species (Fig. 1B). The species differences in the pH-dependent binding of PBZ can be largely explained by the ANS binding properties. The hydrophobicity within the binding pocket, which corresponds to site I on these albumins could increase, as the result of the N-B transition, and ligand binding at the azapropazone binding site in site I could be enhanced. These phenomena are supported by the data for intrinsic Trp fluorescence as described above. In any event, although the N-B transition in all species originates from His residues on the albumin molecules (Figs. 3 and 4), the dissociation of these residues also cause conformational changes in albumins (Fig. 1 and 2). However, this conformational change is not large but, rather, mild (Table 1). Further, specific drug binding sites might still exist and allow relatively strong drug binding (Fig. 5) even if the dissociation of His residues affect the microenvironment of drug binding sites.

The differences in ligand binding as the result of the N-B transition observed here may indicate that species differences in ligand uptake into tissues across the bio-membrane exist. Since the vicinity of bio-membrane is weakly acidic and negatively charged, interactions between albumin and bio-membrane are conceivable. For example, weakly acidic conditions cause a conformational change in albumin and then generates hydrophobic interactions with the bio-membrane. Otherwise, positively charged amino acid residues such as His, Arg and Lys on the albumin molecule would interact with negatively charged portions of the bio-membrane, causing a conformational change in albumin. In any event, it is possible that the N-B transition plays an important role in membrane transport of ligands. Structural information relative to the interaction of albumin with bio-membrane is limited to ESR spectral studies (9). A single conformational change, such as the N-B transition cannot be proposed as the real conformational change which occurs during interactions between albumins and bio-membrane. However, the results obtained in this study provide useful information relative to conformations of albumin molecules for several species.

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