Species Differences of Serum Albumins: I. Drug Binding Sites

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Purpose. The purpose of this study was the classification and identification of drug binding sites on albumins from several species in order to understand species differences of both drug binding properties and drug interaction on protein binding.

Methods. Binding properties and types of drug-drug interaction on the different albumins were examined using typical site I binding drugs, warfarin (WF) and phenylbutazone (PBZ), and site II binding drugs, ibuprofen (IP) and diazepam (DZ) on human albumin. Equilibrium dialysis was carried out for two drugs and the free concentrations of drugs were then treated using the methods of Krug-h-Hansen (Mol. Pharmacol. 34, 160–171, 1988).

Results. Binding affinities of site I drugs to bovine, rabbit and rat albumins were reasonably similar to human albumin. However, interestingly, those to dog albumin were considerably smaller than human albumin. On the other hand, binding parameters of DZ to bovine, rabbit and rat albumins were apparently different from those of human albumin. These differences are best explained by microenvironmental changes in the binding sites resulting from change of size and/or hydrophobicity of the binding pocket, rather than a variation in amino acid residues.

Conclusions. We will propose herein that mammalian serum albumins used in this study contain specific drug binding sites: Rabbit and rat albumins contain a drug binding site, corresponding to site I on human albumin (3). X-ray studies of crystalline human albumin (4) support this view and indicate that site I and site II are located within specialized cavities in subdomain IIA and IIIA, respectively. In addition, the crystallographic structure of equine albumin has also been determined, and the data suggests that there are also two specific drug binding sites on this molecule (5) as well. This suggests that, with respect to binding sites, other mammalian albumins are analogous to human albumin, considering the structural similarities between the molecules. In fact, Panjehshahin pro-

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posed the hypothesis that bovine, dog, horse and sheep albumins contain binding sites for warfarin (WF) and dansylsarcosine, which have properties similar to site I and site II on human albumin (6). This group also implied that rat albumin contains the binding sites different from other albumins (6). This idea, however remains open to question, since they did not measure the free concentration of the fluorescent probes and carry out mutual displacement experiments in their study. If a more detailed characterization of drug binding sites, such as the location and drug binding properties were carried out, this would allow more valid comparisons of drug-drug interactions to be made between animal vis-a-vis human albumins. The present study was undertaken to investigate whether or not animal albumins, including those from rat and dog contain drug binding sites which are equivalent to site I and site II of human albumin. This was carried out by employing WF, phenylbutazone (PBZ), ibuprofen (IP) and diazepam (DZ), as marker ligands, which are known to specifically bind to site I and site II on the human albumin molecule. The study was conducted using rigorous competition experiments, involving all possible combinations of the four marker ligands for four animal albumins, and the data compared to that for human albumin.

MATERIALS AND METHODS

Reagents

Human albumin was donated by the Chemo-Sera-Therapeutic Research Institute (Kumamoto Japan). Bovine, dog, rabbit and rat albumin were purchased from the Sigma Chemical Co. (St Louis, MO, U.S.A.). The samples were defatted with activated charcoal in solution at 0°C, acidified with H2SO4 to pH 3 and then freeze-dried (7). All albumins used in this study showed only one band by SDS-PAGE, and the molecular masses were assumed to be about 66 kDa. Potassium WF (Eisai Co., Tokyo, Japan), IP (Kaken Pharmaceutical Co., Tokyo, Japan) and DZ (Sumitomo Pharmaceutical Co., Osaka, Japan) were obtained from the manufacturers and PBZ was purchased from Nakalai Tesque (Kyoto, Japan). All other chemicals were of analytical grade. 0.067 M Phosphate buffer (pH 7.4) was prepared with dibasic sodium phosphate and monobasic sodium phosphate and used exclusively in this study.

Determination of Binding Parameters

In order to quantitatively analyze the binding mode, binding parameters were determined by equilibrium dialysis. Aliquots (1.5 ml) of ligand-albumin mixture at various ratios were placed in plastic dialysis cells (Sanko, Fukuoka, Japan), whose compartments were separated by Visking cellulose membranes (12 kDa molecular weight cut off), and dialyzed against the same volume of buffer at 25°C for 12 h. After equilibrium was obtained, ligand concentrations as the free ligand (Cf) 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.

Binding parameters were determined by fitting the experimental data to the following Scatchard equation using a non-linear squares program (MULTI program).

$$r = \sum_{i}^{n} \frac{n_i K_i C_i}{1 + K_i C_i}$$  (1)

where $n_i$ is the number of binding sites and $K_i$ is the association constant in the $i$th binding class, and $r$ is the mole of bound ligand per mole of total protein (C/I).  

**Interaction Mode of Two Ligands at High-Affinity Binding Sites on Various Serum Albumins**

In order to simultaneously estimate the interaction mode between two ligands binding to each primary binding site of albumin, the data were treated according to the method of Kragh-Hansen (8). According to this method, a coupling constant ($\chi$) is represented by $\chi = K_{BA}/K_A$ or $\chi = K_{AB}/K_B$, which is capable of distinguishing interaction manner. $K_{BA}$ is the association constant of ligand B in the presence of ligand A, and $K_{AB}$ is that of ligand B in the presence of ligand A. If A and B bind independently to albumin, then $\chi = 1$; i.e., $K_{BA}$ is equal to $K_A$ and $K_{AB}$ to $K_B$. $\chi > 1$ and $0 < \chi < 1$ indicate cooperative and anti-cooperative binding, respectively. In addition, competitive displacement between two ligands is represented by $\chi = 0$. The $\chi$ value was determined as described previously (8).

**RESULTS**

**Determination of Binding Parameters of Four Drugs to Different Albumins**

In order to clarify the species differences of the interaction between the drugs and various albumins, typical site I binding drugs for human albumin, namely, WF and PBZ, and site II binding drugs, IP and DZ, were used as model drugs. Fig. 1 shows scatchard plots of the binding of IP to different albumins as a typical example, and Table 1 shows the obtained binding parameters of WF and PBZ to different albumins. The binding constant of WF to rat albumin was the highest, and those to bovine and rabbit albumins were nearly the same as for human albumin. Interestingly, binding parameters for the primary binding site of WF and PB to dog albumin were not obtained, as opposed to other albumins. In addition, the binding constants of PBZ to rabbit and rat albumins were low, compared with human and bovine albumins.

The binding parameters for site II drugs, IP and DZ, are shown in Table 2. For IP, the numbers of the primary binding site to all albumins were 1, but the numbers for secondary binding site were different depending upon albumin species. The primary binding constants to human and dog albumins were relatively large, compared with rabbit and rat albumins. However, the binding parameters for the primary binding site of DZ for bovine, rabbit and rat albumins were not determined, different from those to human and dog albumins.

Numerous reports have appeared relative to the binding parameters of these drugs to different serum albumins, especially human albumin. The binding parameters listed in Tables 1 and 2 are generally in good agreement with the values reported by many (but not all) of these reports (9–14). The discrepancies may be due to the differences in experimental conditions, such as the albumin lot (difference in fatty acid content and mercapto albumin content), pH, temperature and salt concentration.

**Interaction Between WF, PBZ, IP and DZ Bound to High Affinity Binding Sites on Albumin Molecules**

For identification of drug binding sites for each drug, we examined the interaction of two ligands. Firstly, the interaction between WF and PBZ, site I binding drugs on human, was investigated for the animal serum albumins. Fig. 2 shows the interaction mode of these two ligands on albumins. It is clear from Fig. 2 that WF interacted with PBZ on human albumin in a competitive manner, as has been previously reported (15). A similar phenomenon was observed for rabbit and rat albumins. For the case of bovine albumin, the type of interaction was different from that of human, rabbit and rat albumins. In these cases, the displacements appeared to be anti-cooperative. On the other hand, the interaction mode of WF- and PBZ on dog albumin was not determined because of the lacking of primary binding parameters for WF and PBZ.

Fig. 3 shows interactions of WF or PBZ with IP, specific ligands, which are known to bind to site I and site II on human albumin. As shown in Fig. 3, the interaction of WF and IP with rat albumin appeared to be independent, as expected from the results of human albumin. For the case of the PBZ-IP system, the type of interaction was anti-cooperative, because binding curves constructed using a common value of $\chi = 0.58 \pm 0.02$ agreed with the experimental finding. In addition, both interactions of WF-IP or PBZ-IP systems for rabbit albumin showed anti-cooperative binding. However, no mutual displacements for WF-IP or PBZ-IP were observed in bovine albumin. All the displacement data obtained are summarized in Table 3.

**DISCUSSION**

Although serum albumins are highly homologous, in terms of amino acid sequences (about 80% between human and the species used in this study), drug binding properties differ considerably among the species. As a result, species differences with respect to drug binding to serum albumins have been extensively examined (16–19). However, since these studies were not investigated systematically in terms of drug binding sites, the issue of whether or not there are specific drug binding sites on serum albumins is not clear, nor is the issue of the nature of such sites.

On the other hand, the highly homologous amino acid sequences of serum albumins of other species are useful in studies of active amino acid residues, as "mimics" of human albumin. This is especially useful in the comparison of structures and ligands binding properties among the species which may be useful in determining the functionality of certain amino acid residues.

For the purpose of obtaining information, we attempted to identify drug binding sites on different albumins using typical site I binding drugs on human albumin (WF for the WF binding site and PBZ for the azapropazone binding site), as well as typical site II binding drugs for human albumin (IP and DZ).