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Subdomain IIIA of dog albumin contains a binding site similar to site II of human albumin

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Running Title: Drug binding site on dog albumin

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Abbreviations: KP, ketoprofen; **CNBr**, cyanogen bromide; **DTT**, dithiothreitol; **TFA**, trifluoroacetic acid; **WF**, warfarin; **OCT**, sodium octanoate; **DZP**, diazepam; **IP**, ibuprofen;

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Abstract

Dog albumin contains a specific drug binding site that binds most of the site II ligands of human albumin. This study was undertaken to elucidate the structural configuration of this binding site using photoaffinity labeling technique. Dog albumin and albumins of other animal species were photolabeled with [^{14}C]ketoprofen (KP). The photolabeled albumins were cleaved with cyanogen bromide（CNBr）and analysed autoradiographically after electrophoretic separation. A 11.6 kDa CNBr fragment of the photolabeled dog albumin was found to have incorporated most of the radioactivity. Site II ligands of human albumin inhibited photoincorporation of radioactivity to this fragment. The binding constants of human and dog albumins ranged from 10 to 12×10^5 M^{-1}, at least twice as high as those of rat, rabbit and bovine. Edman degradation was performed to elucidate the amino acid sequence of the photolabeled peptide derived from further digestion of the dog 11.6 kDa CNBr fragment with lysyl endopeptidase (Lys-C). The sequence was XXSESLVXRX, which corresponds to Cys476-Arg485 of dog albumin. Dog albumin contains a binding site that may have similar binding microenvironment of site II on human albumin. Therefore, dog may be a better experimental animal for data extrapolation from animal to human with regard to site II drug-drug interactions.

Introduction

The binding of drugs in human plasma, in most cases, is mainly caused by binding to albumin because albumin is normally present at high concentrations of about 40 mg/ml (0.6 mM) in healthy human subjects (Peters., 1996; Davi et al., 1999) , and also the presence of high affinity binding sites in the protein. In some cases, albumin binding will influence the drug's pharmacodynamic activity, too (Lewis et al., 2006). Determination of the distribution and plasma protein binding of drug for species used in preclinical safety investigations, as well as characterize the interaction of the drug with human albumin, can be critical for its pharmacokinetics and are needed for a comparison of pharmacokinetics across species (Kosa et al., 1997; Weiss et al., 2006). Research on species difference in albumin binding of drugs has been carried out but in most cases only the binding characteristics were investigated, with very few research on the binding sites of albumins of different species (Mizojiri et al., 1997; Nonaka et al., 2003; Acharya et al., 2006). Hence, elucidation of the drug binding sites on albumins of different species will provide useful information when investigating drug interactions with animal models.

Albumins bind endogenous as well as exogenous substances including drugs (Peters., 1996; Petersen et al., 2002; Simard et al., 2006). There are at least two discrete drug binding sites on human albumin, namely, site I and site II, according to Sudlow's classification (Sudlow et al., 1975). Crystallographic analysis of human albumin confirm the locations of the two binding sites I and II at domains II and III, respectively (Petitpas et al., 2003; Ghuman et al., 2005). We have previously reported that rat, rabbit and bovine albumins contain a binding site similar to site I, whereas dog albumin contains a binding site similar to site II of human albumin using ibuprofen and diazepam as the site II probes (Kosa et al., 1997). However, the conclusion was made based on the results of fluorescent probe displacement experiment, which did not address the location of binding site on the protein molecule. In addition, at present only crystallographic structures of human and horse albumins are available (Ho et al., 1993; Curry et al., 1998). Lacking of structural data for albumins of other animal species has, to a certain extent, impeded further assessment of drug binding models.

In a previous study, we had successfully identified the binding site of ketoprofen (KP) on human albumin. KP is a site II ligand of human albumin that can be used as a labeling agent due to its benzophenone moiety. In the study, the binding site structural configuration of site II of human albumin was found to consist of Cys476-Pro499 which forms part of subdomain IIIA of human albumin (Chuang et al., 1999). Hence, in order to identify the location of KP binding site on dog albumin, [^{14}C]KP was used to photolabel dog albumin and amino acid sequence of the photolabeled dog albumin peptide had been determined.

Materials and Methods

Materials. Human, dog, rat, rabbit and bovine albumins were obtained from Sigma. Prior to all experiments, all albumins were defatted with activated charcoal in solution at 4℃, acidified with HCl to pH3 and then lyophilized. The albumins used in this study showed only one band of approximately 66 kDa in SDS-PAGE. [^{14}C]KP (12.95 μCi/mmol) was obtained from Hisamitsu Pharmaceutical Co., Inc., Tosu Laboratories (Saga, Japan). Cyanogen bromide (CNBr), dithiotreitol (DTT), and trifluoroacetic acid (TFA) were obtained from Nacalai Tesque (Kyoto, Japan). Warfarin (WF) was obtained from Eisai Co., Ltd., (Tokyo, Japan). Sodium octanoate (OCT) was obtained from Wako Pure Chemical Industries, Ltd., (Osaka, Japan). Diazepam (DZP) was obtained from Sumitomo Pharmaceuticals Co., Ltd., (Osaka, Japan). Ibuprofen (IP) was obtained from Kaken Pharmaceutical Co., Ltd., (Tokyo, Japan). All other chemicals were of analytical grade.

Photoaffinity Labeling of Albumins with [^{14}C]KP. Albumin (50 μM) was incubated with [^{14}C]KP (25 μM), in the absence and presence of WF, IP, OCT, and DZP (250 μM), in 100 μL of 20 mM Tris buffer, at pH7.4, in a 1.5 mL Eppendorf tube at room temperature in the dark for 60 min. The incubation mixture was then placed on ice and irradiated for 30 min at a wavelength of longer than 320 nm by a 100-W black light/blue lamp (Ultra-Violet Products, Inc., San Gabriel, CA, USA) at a distance of 10 cm. After irradiation, the photolabeled albumins were precipitated by adding 1 mL of acetone, followed by centrifugation at 15,000 rpm for 10 min. The pellet was reductively pyridylethylated.

Reductive Pyridylethylation. 100 μL of the reduction medium (6 M Guanidium HCl, 1 M Tris buffer, pH8.0, 20 mM EDTA and 100 mM DTT) was added to the pellet, which was then incubated under N_2 at 37°C for at least 12 h. After the addition of 1 μL of 4-vinylpyridine, the mixture was incubated under N_2 for an additional 30 min at room temperature in the dark. At the end of the pyridylethylation reaction, 1 mL of acetone and 100 μL of 0.1% TFA was added to the mixture to terminate the reaction. The suspension was then vortexed vigorously followed by a light centrifugation. 1 mL of ethanol was added, the

mixture was further vortexed, to remove the salts and unreacted 4-vinylpyridine, the suspension was centrifuged at 15,000 rpm for 10 min.

CNBr Cleavage and Tricine SDS-PAGE. The pyridylethylated pellet was dissolved in 100 μL of CNBr in 70% formic acid (CNBr:Methionine residues = 200 : 1) and incubated under N_2 for 24 h in the dark at room temperature. 1 mL of milli-Q water was added at the end of the CNBr cleavage to stop the reaction, and the resulting mixture was lyophilized. The lyophilized CNBr fragments were resuspended in 100 μL of 0.1% TFA, and the protein concentration was determined by a Bradford assay 6.5 μg of the fragment mixture was applied to each lane of the gel and separated with tricine SDS-PAGE.

Autoradiographic Analysis. For autoradiographic analysis, the dried SDS-PAGE gel was placed in contact with an imaging plate (BAS III, Fuji Film Co., Tokyo, Japan) in a cassette (BAS cassette 2040, Fuji Film Co., Tokyo, Japan) at room temperature for 48 h. The imaging plate was scanned and analyzed using a Bio-Imaging Analyzer (model BAS FLA-3000 G, Fuji Film Co., Tokyo, Japan), and was then analyzed using L Process V 1.6 software (Fuji Film Science Lab 98, Fuji Film Co., Tokyo, Japan). The incorporation of radioactivity into individual fragments was quantified using Image Gauge V 3.1 software (Fuji Film Co., Tokyo, Japan).

Capillary HPLC Separation and Sequence Analysis. The 11.6 kDa CNBr peptide of dog albumin was eluted from the gel after tricine SDS-PAGE with Electro-Eluter (Model 422, BIO-RAD, California, USA). The peptide was then digested with enzyme lysyl endopeptidase (Lys-C) in 20 mM ammonium bicarbonate at 37°C for 16 h. 10 μL of the digestion sample was then injected onto the ABI 173 A MicroBlotter Capillary HPLC System (Perkin Elmer, Inc., Massachusetts, USA). The sample was manipulated following the manufacturer's instructions. The blotted membrane from the capillary HPLC separation was in contact with an imaging plate for 48 h prior to autoradiographic analysis. The PVDF membrane was positioned with the chromatogram of a peptide map from ABI 173 A MicroBlotter Capillary HPLC System. Portion of the PVDF membrane was excised for sequencing with reference to the autoradiogram. Edman

degradation of the photolabeled peptide was carried out with Procise Sequencer of Applied Biosystems.

Determination of Binding Parameters. In order to quantitatively analyze the binding mode, binding parameters were determined by ultrafiltration. Ultrafiltration was performed using Ultrafree MC (Amicon Division, Danvers, MA, USA). Ligands were added to 400 μL of albumins (10 μM) in 20 mM Tris buffer, at pH7.4, and preincubated at 25°C for 30 min before centrifugation (2000 rpm for 20 min). To determine the free ligand concentrations (C_f) , a 200 μL aliquot of the filtrate was added to 2 mL of scintillation cocktail in a scintillation vial (Pyrex, ASAHI TECHNO GLASS CORPORATION, Chiba, Japan), vortexed, and counted by using a Scintillation Counter (LSC-5121, ALOKA Co., Ltd, Tokyo, Japan). No adsorption of the ligands to membrane or apparatus was detectable.

Binding parameters were determined by fitting the experimental data to the following equation using a nonlinear least squares program (MULTI program)

$$r = \sum_{i=1}^{m} \frac{n_i K_i C_f}{1 + K_i C_f} \qquad (1)$$

where n_i is the number of binding sites and K_i is the binding constant in the *i*th binding class and *r* is the mole of bound ligand per mole of total protein (C_b / P_t)

CD Spectra Measurements in the Presense of KP. CD spectra measurement was performed using a JASCO J-820 spectropolarimeter (JASCO, Tokyo, Japan) at 25°C, using a 10 mm path length cell. The concentration of various albumins was 60 μM and KP concentration of 100 μM, in 20 mM Tris-HCl buffer (pH7.4). The scan speed was adjusted at 10 nm•$min^{-1}$and was average of three scans, with the range of wavelength scanned from 300 to 400 nm. The results were represented as observed ellipticity (θ_{obs}) in mdeg. Induced CD was determined as the CD of the albumin–KP mixture after subtraction of CD of the albumin alone.

Results

Binding parameters of KP to albumins of different species. The number of binding sites and the corresponding binding constants for binding of KP to albumins were estimated using ultrafiltration (Table 1 and Fig.1). The primary binding constants of human and dog albumins ranged from 10 to 12×10^5 M^{-1}, at least twice as high as those of rat, rabbit and bovine which ranged within 2 to 5×10^5 M^{-1}. In human, dog and bovine albumins, KP binds at primary and secondary sites. On the other hand, in rat and rabbit albumins, KP binds to three sites that could not be categorized into primary or secondary level.

CD spectra measurements of albumins in the presence of KP. When KP binds to HSA, a specific Cotton effect will be induced (Dubois et al., 1994; Zandomeneghi, 1995). Fig. 2 shows the CD spectra obtained when KP was added to the different albumins solutions. Binding of KP to human and dog albumins resulted in a negative Cotton effect, with a maximum at about 340 nm. Binding of KP to rabbit albumin led to a decrease in the specific negative Cotton effect as compared with those induced in the human and dog albumins. Meanwhile, the spectrum of bovine albumin-KP binding was appreciably different from those of human, dog and rabbit albumins. Different from other albumins, binding of KP to rat albumin resulted in a positive Cotton effect.

Photolabeling of albumins of various species with [^{14}C]KP. The autoradiogram in Fig. 3 shows that the radioactivity band appeared only upon photoirradiation of albumins with [^{14}C]KP. Absence of a radioactive band in the samples without irradiation indicated that no covalent attachment of KP to albumin occurred in the dark. Human and dog albumins appeared to incorporate radioactivity to a greater extent than the other albumins.

CNBr fragments of albumins of various species photolabeled with [^{14}C]KP. Human and rat albumins have six, while dog and bovine albumins contain four methionine residues. Rabbit albumin contains only one methionine reside (Fig.4). Since amino acid sequence of each albumin is available, CNBr cleavage products of these albumin can be identified by their mobilities on SDS

containing gels in relation to their molecular weights. Thus, it is possible to preview which subdomain is photolabeled via inspection of the radioactivity intensity of each band (Fig.5). Significant radioactivity could be observed for the 11.6 kDa CNBr fragments of the photolabeled human and dog albumins, and 15.3 kDa of bovine albumin was found to have incorporated most of the radioactivity. On the other hand, only a comparatively low level of radioactivity could be observed for the 31.8 kDa CNBr fragments of the photolabeled rat albumin and 36.0 kDa peptide of rabbit albumin.

Photolabeling inhibition by site I and II ligands of human albumin. In order to determine the presence of a binding site, the specificity of the photolabeling of [^{14}C]KP to albumins was further investigated by competition experiments. The peptide containing subdomain IIIA has a molecular weight of 11.6 kDa for human, dog and bovine albumins, 31.8 kDa for rat albumin and 36.0 kDa for rabbit albumin. In the absense of a competitor, the extent of radioactivity incorporation of the peptide containing subdomain IIIA for each albumin was the same as the result in Fig. 5. KP binds primarily to site II of human albumin. Site II ligands, IP, DZP and OCT as well as a site I ligand, WF, were used as competitors in photolabeling albumins with [^{14}C]KP. The extent of photolabeling inhibition was expressed as a percentage of the control. A decrease in the radioactivity of the peptide containing subdomain IIIA reflects the inhibition of photoincorporation of [^{14}C]KP by the competitor. In the presence of DZP and OCT, the intensity of the 11.6 kDa band of human and dog albumins decreased to an extent greater than that in the presence of IP. On the other hand, IP, DZP and OCT reduced the radioactivity intensity of the 11.6 kDa band of bovine albumin to the same extent. WF did not appear to affect the intensity of the band containing subdomain IIIA (Fig.6).

Determination of the photolabeled peptide amino acid sequence of dog albumin. The 11.6 kDa CNBr peptide from dog albumin was subjected to further digestion with Lys-C in order to locate more precisely which region of subdomain IIIA was photolabeled by KP. The peptides generated from second digestion were separated and simultaneously blotted onto a strip of PVDF membrane with a capillary HPLC system. One radioactivity spots corresponding to 1 peak was

obtained (Fig. 7 (A) and (B)). The amino acid sequence of the photolabeled fragment after further digestion with Lys-C was XXSESLVXRX, which corresponds to the sequence Cys476-Arg485 of dog albumin (Fig. 7 (C)).

Discussion

High binding affinity to a plasma protein may cause the drug to be retained in the plasma and not readily distributed to the tissue for therapeutic action to take place. The high degree of binding of UCN-01 to human α_1-acid glycoprotein causing a reduction in the distribution and clearance, resulting in high plasma concentrations in humans has been reported (Fuse et al., 1998). Moreover, we previously reported that species differences observed with phenylbutazone has an impact on the *in vivo* serum protein binding of sulfadimethoxine (Imamura et al., 1986).

In the process of drug development, preclinical animal trials are required to examine the safety and efficacy of the candidate drug. From the animal experiments, drug protein binding and distribution data are significant to compare the pharmacokinetics of drug across species and for interspecies scaling. Such comparison will allow for a meaningful referencing of pharmacokinetic parameters to either blood or plasma concentrations of the drug. In order to achieve this, a further refinement of the animal experimental design via selection of appropriate experimental animal based upon the drug class and the expected interacting proteins is desirable.

In spite of the fact that the primary structure of the serum albumins of various species are highly homologous, (about 80% between human and the species used in this study), drug binding properties differ considerably among the species. We reported that dog albumin is suitable for investigating drug-drug interactions on site II, while rat, rabbit and bovine albumins are suitable for examining the binding of site I drugs (Kosa et al., 1997). Based on the study we attempted elucidating the binding site structural configuration of dog albumin that resembles site II of HSA, using photoaffinity labeling method (Garabedian and Yount, 1991; Chuang et al., 1999; DeSantis et al., 2000; Katsuki et al., 2005).

The usefulness of KP as a photoaffinity labeling agent and a probe for identifying drug binding sites on human albumin has been established previously (Chuang et al., 1999). To our knowledge, this study is the first one ever reporting identification of the presence of binding sites in various experimental animal albumins using photoaffinity labeling technique. The extent of photoincorporation of [^{14}C]KP to each albumin was in accordance with the binding experiment result

obtained with ultrafiltration and CD spectra measurements in the presence of KP (Table 1, Fig. 3). Moreover, these results are in agreement with the binding experiments using IP that we reported previously (Kosa et al., 1997) (Fig. 1, Fig.2, Fig.3 and Table 1). The primary binding constant of KP to human albumin was similar with that reported by Rahman et al. (1993).

Autoradiographic analysis of CNBr fragments of human albumin photolabeled with [^{14}C]KP indicated that a 11.6 kDa peptide that corresponded to Pro447-Met548 contained the highest radioactivity. This sequence was found to form the binding pocket of site II, in subdomain IIIA of human albumin. Dog and bovine albumins will also derive a peptide with a molecular weight of 11.6 kDa upon CNBr cleavage, as could be deduced from the amino acid sequences (Met446 – Met 548) of these two albumins. The CNBr peptides of rat and rabbit containing the sequence with high homology to that of the human albumin 11.6 kDa CNBr peptide were Met264-Met548 (31.8 kDa) and Met264-Glu584 (36 kDa), respectively (Fig. 4). As shown in Fig. 5, only the 11.6 kDa CNBr peptide of dog albumin incorporated radioactivity to an extent comparable to that of human albumin. Interestingly, the corresponding peptide of bovine albumin did not incorporate a significant extent of the radioactivity. Instead, a peptide with an estimated molecular weight of 15.3 kDa exhibited higher level of radioactivity. Edman degradation of this peptide revealed an amino acid sequence starting from Pro447 (data not shown), indicating that the peptide might derived from an incomplete cleavage of CNBr which resulted a peptide with a sequence starting from Pro447-Ala584. It is very likely that Met548 of bovine albumin could have been photolabeled by [^{14}C]KP, resulting in its resistant to CNBr cleavage.

Such discrepancy could be due to a difference in the amino acid sequence that would notably influence the secondary as well as the tertiary structures of the albumins. The induced CD spectra for KP binding (Fig. 2) and the intrinsic CD for albumin themselves showed a difference between human albumin and bovine albumin (data not shown), in agreement with the results reported by Kosa et al (1998). Hence, human, dog and bovine albumins contain a binding site of KP in subdomain IIIA, but the binding site structural configuration of bovine albumin is somewhat different from those of human and dog albumins.

IP, DZP and OCT, site II ligands of human albumin, were able to inhibit the binding of KP to the primary binding sites of dog and bovine albumins. However

the inhibition pattern suggested that KP interacted with dog albumin in a manner similar to human albumin but different from bovine albumin (Fig.6). In a previous study, we reported that bovine albumin has no primary but only secondary binding site for DZP (Kosa et al., 1997). Hence, this secondary binding site of DZP might overlap with the binding site of KP on bovine albumin. The inhibition pattern of human albumin, and hence dog albumin, by IP, DZP and OCT could be explained from the crystal structures of drug-HSA complex (Ghuman et al., 2005). We previously reported that the hydrophobic moiety of R- KP interacts with Arg485 (Chuang et al., 1999). In the IP- human albumin complex crystal structures, IP binds in the site II binding pocket in such a way that its hydrophobic moiety was away from Arg485 in contrast to DZP that binds near to Arg485 (Ghuman et al., 2005). Bhattacharya et al. (2000) proposed that two molecules of medium chain fatty acids, including OCT, bind to site II and one of the two molecules forms hydrogen bond to Arg485. This may provide an explanation for the observation that IP inhibited KP binding to a lesser extent than DZP and OCT.

Amino acid sequence analysis of the photolabeled peptide of dog albumin after further digestion with Lys-C enzyme indicated a sequence corresponding to the sequence Cys476-Pro499 of dog albumin (Fig. 7 (C)). In a previous study, we reported that Cys476-Pro499 in subdomain IIIA (site II) of human albumin was the region photolabeled by KP (Chuang et al., 1999). Sequence homology comparison of this Cys476-Pro499 peptide of human albumin (Chuang et al., 1999) and dog albumin (Fig. 7 (C)), with all other albumins used in the study showed that dog and bovine albumin has the highest homology (91.7%) with human albumin. As shown in table 2, human and dog albumins have Glu492 and Val493, but bovine albumin doesn't have. Moreover, DZP, a site II ligand, binds to dog albumin with high affinity, but only binds to bovine albumin with much lower affinity (Kosa et al., 1997). Thus Glu492 and Val493 in human and dog albumins are important residues that constitute the structure of site II binding site. In addition, the induced CD spectra of human albumin-KP complex agreed with that of dog albumin-KP complex (Fig. 2). The CD spectra of these albumins indicated that the secondary and tertiary structures of dog albumin was almost the same as those of human albumin (data not shown). Therefore, dog albumin contains a binding site located in subdomain IIIA exhibiting similar binding structural configuration to that of site II of human albumin.

In conclusion, in the preclinical animal experiments, dog may be a better candidate animal for examination of the protein binding as well as distribution of site II drugs.

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Legends for Figures

Figure 1. Scatchard plots of the binding of KP to dog (●) and rat (◆) albumins.

Figure 2. CD spectra of different albumins in the presence of KP at 25 °C
The sample solutions contained 100 μM KP and 60 μM albumin in 20 mM Tris-HCl buffer (pH7.4).
Human albumin (———), dog albumin (- - - -), rat albumin (— — —), rabbit albumin (— · — · -) and bovine albumin (·····)

Figure 3. Photolabeling of different albumins（1, 2：human、3, 4：dog、5, 6：rat、7, 8：rabbit、9, 10：bovine）with [^{14}C] KP.
Lane 1, 3, 5, 7, 9：Sample taken just before photoirradiation.
Lane 2, 4, 6, 8, 10：sample taken after 30 min irradiation

Figure 4. The position of methionine residues on different albumins. The numbers above the line are calculated molecular weights. The numbers below "Met" are the positions of methionine residues in the amino acid sequence.

Figure 5. CNBr fragments of different albumins after [^{14}C] KP photoaffinity labeling separated by tricine gel electrophoresis and the corresponding autoradiogram. 1: human (11.6kDa), 2: dog (11.6kDa), 3: rat (31.8kDa), 4: rabbit (36.0kDa), 5: bovine (11.6kDa)

Figure 6. Autoradiogram and relative radioactivity of the bands containing subdomain IIIA after photoirradiation in the presence of various competitors. The

photolabeled albumins were cleaved with CNBr and separated with tricine gel electrophoresis. The final concentration ratio for different albumins, [^{14}C] KP and competitors was 1 : 0.5 : 5.

control (☐), WF(■), OCT(▒), DZP(▥) and IP(▨).

Data are expressed as means ± SD (n = 3-4). *p < 0.01, compared with control.

Figure 7. Chromatogram of capillary HPLC (A), autoradiogram of blotted PVDF membrane of dog albumin digested with endopeptidase Lys-C (B) and N-terminal amino acid sequence analysis by Edman degradation method and amino acid sequence of the photolabeled region of dog albumin(C). (Lys-C peptides) PTH, phenylthiohydantoin.

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Table 1. Binding parameters of KP to different serum albumins at pH7.4

	n_1	K_1 ($\times 10^5 M^{-1}$)	n_2	K_2 ($\times 10^4 M^{-1}$)
human	1.34 ± 0.04	10.02 ± 0.47	4.82 ± 0.16	2.11 ± 0.13
dog	0.91 ± 0.01	11.65 ± 0.21	7.09 ± 0.62	0.66 ± 0.09
bovine	1.72 ± 0.13	4.62 ± 0.48	5.46 ± 0.59	1.16 ± 0.30
		n	K ($\times 10^5 M^{-1}$)	
rat		3.32 ± 0.05	2.45 ± 0.06	
rabbit		2.93 ± 0.05	3.20 ± 0.15	

determined by ultrafiltration

Note: The values represent the mean ± S.D.

Table 2 The amino acid sequence of peptide (476-499) of albumin of different species.

Species	The peptide of 476 - 499	Homology	Primary Binding sites	
Human	CCTES LVNRR PCFSA LEVDE TYVPK		**IP***	**DZP ***
Dog	CC**S**ES LVNRR PCFSG LEVDE TYVPK	**91.7 %**	○	○
Bovine	CCTES LVNRR PCFSA L**TP**DE TYVPK	**91.7 %**	○	✕
Rat	CC**SG**S LV**E**RR PCFSA L**T**VDE TYVPK	**83.3 %**	○	✕
Rabbit	CC**S**ES L**S**NRR PCFSA L**GP**DE TYVPK	**83.3 %**	○	✕

* IP = Ibuprofen, DZP = Diazepam (Kosa et al., 1997).

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Fig. 1

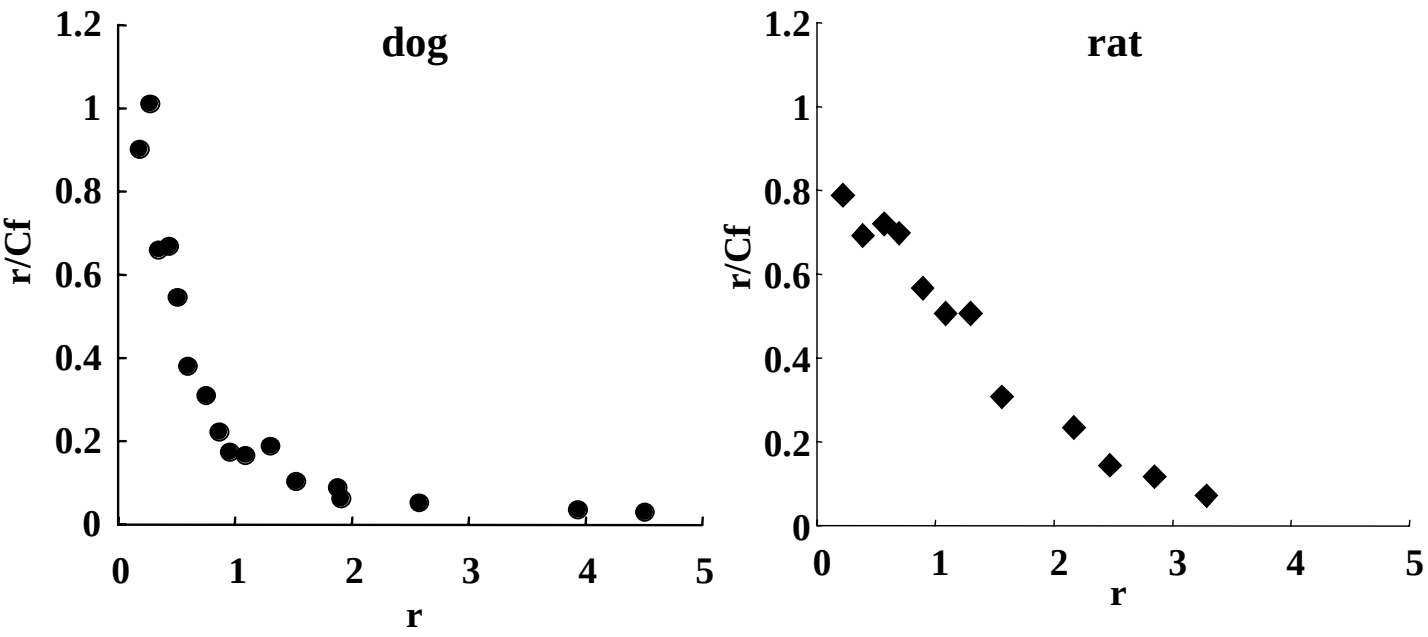

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Fig. 2

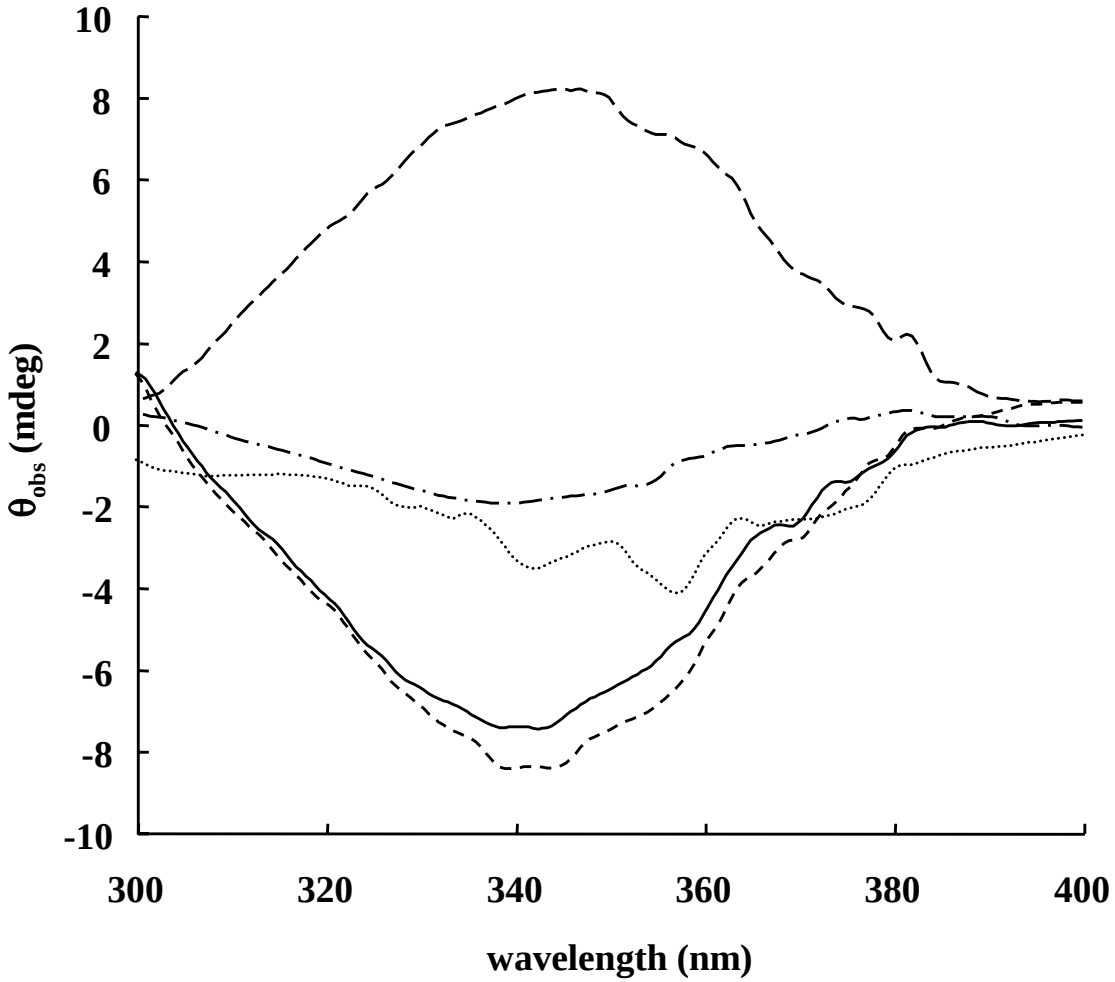

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Fig. 3

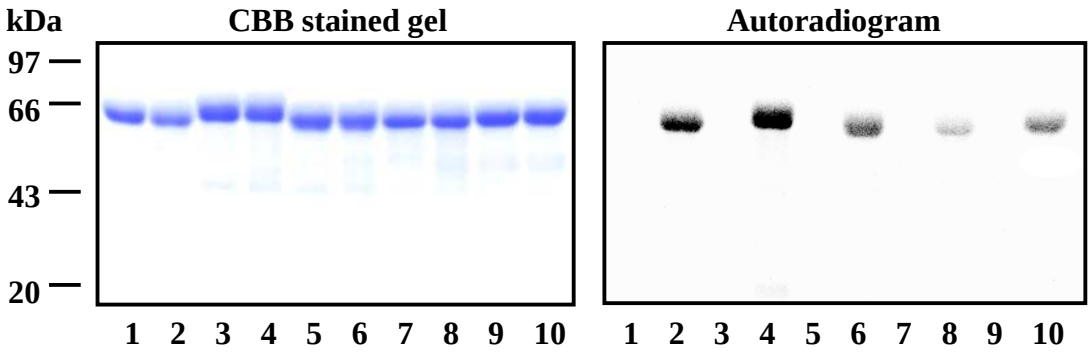

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Fig. 4

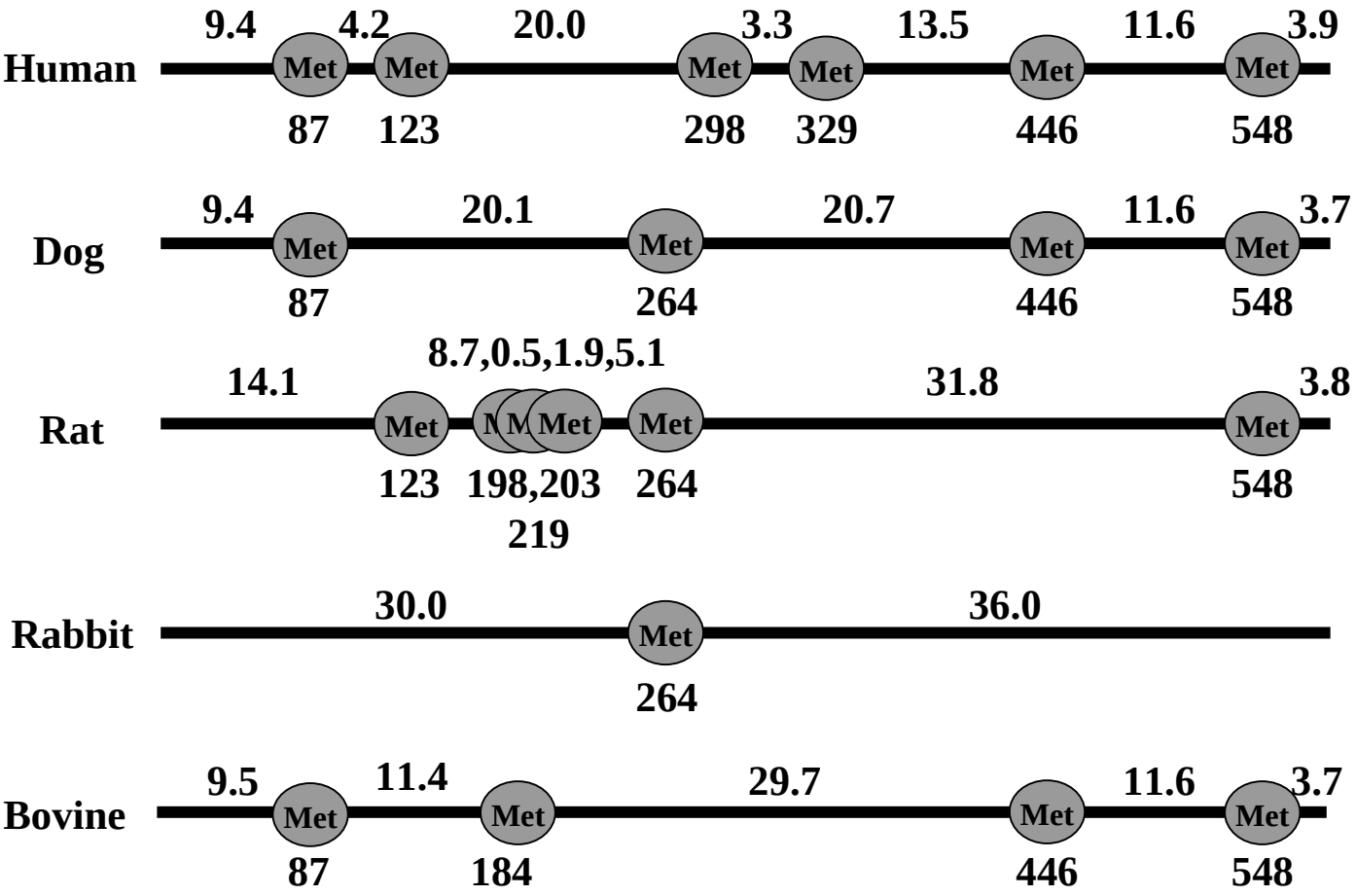

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Fig. 5

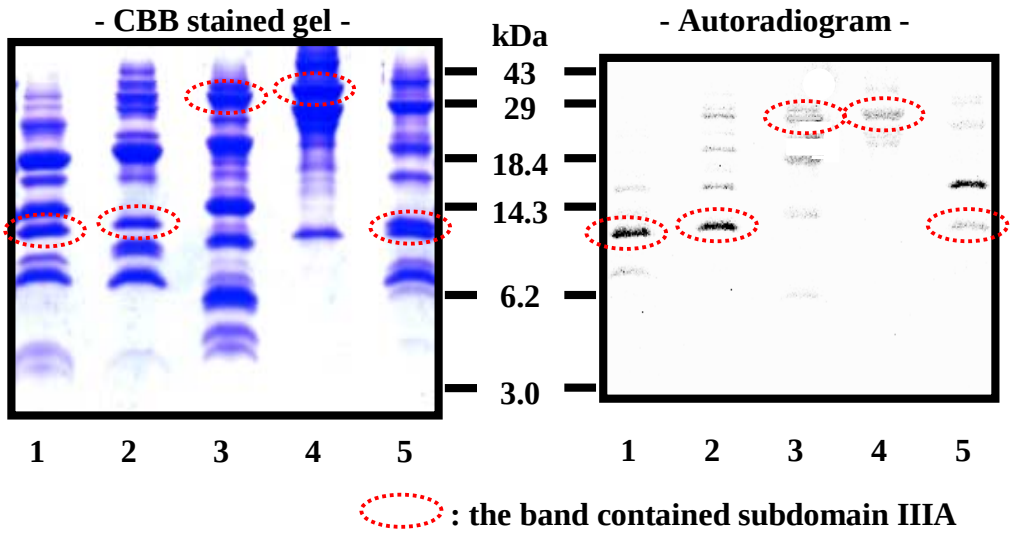

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Fig. 6

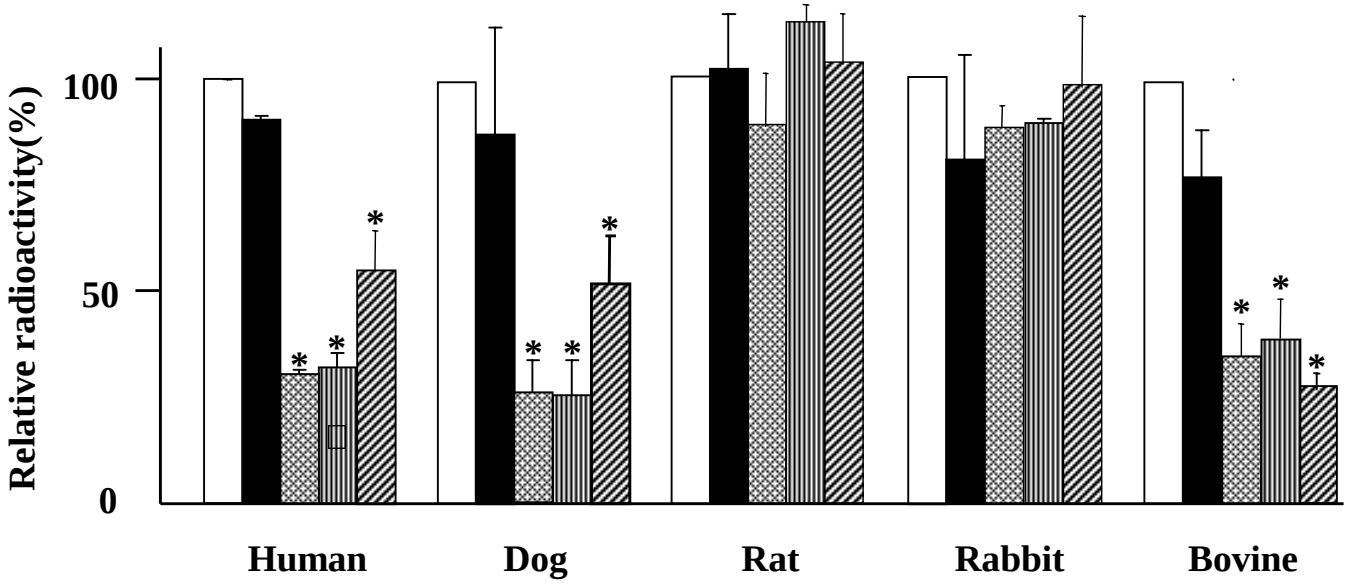

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Fig. 7