Functional Analysis of Recombinant Human Serum Albumin Domains for Pharmaceutical Applications

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Purpose. Functional analysis of the three recombinant human serum albumin (rHSA) domains and their potential as stand-alone proteins for use as drug delivery protein carriers.

Methods. Protein structure was examined by fluorescence and CD spectroscopy. Ligand binding was estimated by ultrafiltration. Antioxidant activity was estimated by measuring the quenching of dihydrorhodamine 123. Esterase-like activity and enolase-like activity were estimated from the rate of hydrolysis of *p*-nitrophenyl acetate and conversion of dihydrotestosterone from the 3-keto to 3-enol form, respectively. The domains of human serum albumin (HSA) were radiolabeled with ^{111}In to evaluate their pharmacokinetics.

Results. The ligand binding ability of subsites Ia and Ib could not be detected in domain II. However, the binding of ligands to subsite Ic and site II were preserved in domain II and domain III, respectively. Domain III retained about 45% of its esterase-like activity, and weaker esterase-like activity was also observed in domain I. All domains showed low enolase-like activity in a pH 7.4 phosphate buffer, but domain II had higher activity in a pH 9.2 carbonate buffer. Domain I exhibited antioxidant activity comparable to that of rHSA. All three of the ^{111}In-radiolabeled domains were rapidly eliminated from HSA, with high accumulation in the kidneys.

Conclusion. Domain I of HSA has great potential for further development as a drug delivery protein carrier, due to its favorable properties and the presence of a free cysteine residue.

KEY WORDS: antioxidant activity; domain; enzyme activity; human serum albumin; pharmacokinetics.

INTRODUCTION

Human serum albumin (HSA) is a biological macromolecule that has potential for a wide range of pharmaceutical applications. Due to its remarkably long half-life, its wide *in vivo* distribution, and its lack of substantial immunogenicity, HSA is an ideal carrier for therapeutic peptides and proteins that interact with cellular or molecular components in the vascular and interstitial compartments (1). One strategy for overcoming lack of specificity of a drug toward target tissues is to covalently couple the drug to a suitable carrier. Drug carriers should have high rates of accumulation in the target tissue, low rates of uptake by normal tissue, and low toxicity. They should be easily linked to the drug, and should readily release the drug in the target tissue. Also, they should be readily available and affordable. Several studies have shown that HSA fulfills these requirements for delivery of drugs to tumors (2,3). HSA also appears to be suitable for delivering drugs such as methotrexate to neoplastic tissue and to inflamed joints of patients with rheumatoid arthritis (4). Also, studies have shown that genetic fusion of bioactive peptides to HSA can produce therapeutic HSA derivatives with appropriate pharmacokinetic properties. Therapeutic proteins genetically fused to albumin have been found to have longer circulating half-lives and improved stability. Examples of such genetically fused proteins include interferon-rHSA (Albuferon), human growth hormone-rHSA (Albutropin), recombinant granulocyte colony stimulating factor (rG-CSF)-rHSA (Albugranin), hirudin-albumin fusion protein and serum albumin-CD4 genetic conjugate (5–8).

HSA is a monomeric non-glycosylated polypeptide with a heart-shaped structure that is approximately 67% α-helix and contains no β-sheet. It is composed of three homologous domains (I-III), and each domain has two subdomains (A and B) that possess common structural elements (1). In addition to its remarkable ligand binding properties, HSA has been shown to possess antioxidant activity, esterase-like activity and enolase-like activity (9–13). Expression of recombinant HSA (rHSA) and its recombinant domains has been reported, and the structural basis of their ligand interactions has been examined using methods including X-ray crystallography (14–16). Examination of the crystal structure of ligand-albumin complexes has revealed positions of binding sites and tertiary structure locations of reactive amino acid residues such as Cys-34, which is involved in NO conjugation (17).

Effects of intrinsic functional properties of HSA other than its ligand binding ability on its role as a drug carrier are poorly understood. There is little doubt that carrier proteins can strongly influence the magnitude of the therapeutic response to a conjugated drug. This influence is thought to be especially significant in the case of peptide drugs and drugs conjugated to a carrier protein via a peptide linker. For example, Albuferon is reportedly approximately 20 times less potent than IFN-α on a molar basis (5). Thus, detailed investigation of the influence of HSA on drugs conjugated to it is important for understanding drug targeting, modification of drug pharmacokinetics, stability of drug-albumin conjugates and, most importantly, design of more sophisticated drug delivery carrier systems. In the present study, we used *Pichia pastoris* to separately express the three recombinant domains of HSA, in order to determine the location of its active sites and clarify the mechanisms underlying its functions such as ligand binding, antioxidant activity and enzyme-like activities.

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ABBREVIATIONS: DHT, 5α-dihydrotestosterone; DNSA, dansyl-L-asparagine; DNSS, dansylsarcosine; DRD, dihydrorhodamine 123; HSA, human serum albumin; rHSA, recombinant HSA; *n*-butyl *p*-AB, *n*-butyl *p*-aminobenzoate; RD, rhodamine 123; RSA, rabbit serum albumin.

MATERIALS AND METHODS

Materials

Racemic ketoprofen was a gift from the Kissei Pharmaceutical Co. (Matsumoto, Japan). The following were obtained as pure substances from the manufacturer: warfarin (Sigma, St. Louis, MO, USA), dansyl-L-asparagine (DNSA) (Sigma), dansylsarcosine (DNSS) (Sigma), dihydrorhodamine 123 (DRD) (Sigma), *n*-butyl *p*-aminobenzoate (*n*-butyl *p*-AB) (Wako, Osaka, Japan), *p*-nitrophenyl acetate (Nacalai Tesque, Kyoto, Japan), 5α-dihydrotestosterone (DHT) (Merck, Darmstadt, Germany), and tritiated (1,2) dihydrotestosterone (40 Ci/mmol) (PerkinElmer Life Sciences, Inc., Boston, MA, USA). The scintillation cocktail Hionic-fluor was purchased from Packard Co. (Meriden, CT, USA). Restriction enzymes, calf intestinal alkaline phosphatase and TaKaRa EX *Taq* DNA polymerase were obtained from Takara Shuzo Co. Ltd (Kyoto, Japan). A DNA sequence kit was obtained from Applied Biosystems (Foster City, CA, USA). The *Pichia* Expression kit was purchased from Invitrogen Corp. (Carlsbad, CA, USA). Other chemicals used were obtained from commercial suppliers.

Synthesis and Purification of rHSA and Domains

The protocol used to express and purify domains I, II, and III of HSA was a modification of a previously published protocol (14,18). The segments coding for rHSA and the individual HSA domains were amplified by the polymerase chain reaction using a cDNA clone of HSA as the template. The domains contained the following amino acids: domain I, 1-197; domain II, 187-385; domain III, 381-585. The 5′-sense primers encoded an *Xho* I restriction site. The 3′-antisense primers were designed to incorporate an *Eco* RI site, and to add a stop codon to terminate the translation. The sequences of the 5′-primers were as follows: 5′-GGACTAGTCTCGAGAAAAGAGATGCACACAAGAGTG-3′ (rHSA and domain I), 5′-GGACTAGTCTCGAGAAAAGAGATGAAGGGAAGGC-3′, (domain II), and 5′-GGACTAGTCTCGAGAAAAGAGTGGAAGAGCCTCAG-3′ (domain III). The sequences of the 3′-primers were as follows: 5′-CGCGAATTCTTATCTCTGTTTGGCAGACGAAGCC-3′ (domain I), 5′-CGCGAATTCTTACTGAGGCTCTTCCACAAGAGG-3′ (domain II), and 5′-CGCGAATTCTTATAAGCCTAAGGCAGC-3′ (rHSA and domain III). The amplified gene segments were ligated into the pPIC9 vector, which contains an α-factor secretion signal sequence. The resulting vector was introduced into the yeast species *Pichia pastoris* (strain GS115). The secreted rHSA and individual domains were isolated from the growth medium by a combination of precipitation with 60% (w/v) $(NH_4)_2SO_4$ and purification on a Blue Sepharose CL-6B column (Amersham Pharmacia Co., Uppsala, Sweden) and a TSKgel SuperQ-TOYOPEARL650 column (Tosoh Co., Tokyo, Japan). Isolated protein was defatted using the charcoal procedure described by Chen (19), deionized, freeze dried and then stored at –20°C until used. The resulting rHSA and individual domains (treated with dithiothreitol) exhibited a single band on SDS-PAGE. N-terminal amino acid sequences of the proteins were determined using a Perkin-Elmer ABI 477A protein sequencer.

Fluorescence and CD Spectra Measurements

Intrinsic fluorescence spectra were obtained using a Jasco FP-777 spectrofluorometer (Tokyo, Japan) equipped with thermostatically controlled 1-cm quartz cells and 5-nm excitation and emission bandwidths. rHSA and individual domains were excited at 295 nm, and the spectra were corrected for buffer baseline fluorescence. CD spectra were obtained using a JASCO J-720 spectropolarimeter (JASCO, Tokyo, Japan) at 25°C. Far- and near-UV CD spectra were recorded at protein concentrations of 5.0 μM and 15 μM, respectively, in 67 mM phosphate buffer (pH 7.4).

Ultrafiltration

The interaction of site I ligands (warfarin, DNSA, *n*-butyl *p*-AB) and site II ligands (ketoprofen, DNSS) with rHSA and individual domains in 67 mM phosphate buffer (pH 7.4) was examined using ultrafiltration at 25°C. An amicon MPS-1 micropartition system with a YMT ultrafiltration membrane was used. First, 1-ml samples were ultrafiltered at 2000 rpm for 40 min at 25°C. The concentration of the free drug was determined by HPLC using a system consisting of a Hitachi L-6200 Pump and an F-1050 Fluorescence Spectrophotometer or L-4000 UV Detector. LiChrosorb RP-18 (Cica Merck, Tokyo, Japan) was used as the stationary phase. The mobile phases were as follows: 30 mM phosphate buffer (pH 7.7)–acetonitrile (57.5:42.5, v/v) for warfarin, DNSA and DNSS; deionized water-acetonitrile (30:70, v/v) for *n*-butyl *p*-AB; 0.2 M acetate buffer (pH 4.5)-acetonitrile (60:40, v/v) for ketoprofen. Ketoprofen was detected at 257 nm using a UV monitor. Warfarin, DNSA, DNSS and *n*-butyl *p*-AB were detected using a fluorescence monitor. The excitation/emission wavelengths were as follows: warfarin, 300 nm/400 nm; DNSA, and DNSS, 330/550 nm; *n*-butyl *p*-AB, 290 nm/350 nm. Because we detected slight adsorption of *n*-butyl *p*-AB to the membrane and apparatus, the ultrafiltration system was siliconized by treatment with Sigmacote (Sigma, St. Louis, MO, USA) to prevent adsorption. The data were corrected using a calibration curve obtained from the concentrations of ultrafiltered ligand in the absence of protein. The adsorption of other ligands to the membrane and apparatus was negligible. The bound ligand concentration (C_b) was calculated using the following equation:

$$C_b = \text{total ligand concentration (before ultrafiltration)} - (C_t) - \text{ligand concentration in filtrated fraction } (C_f) \quad (1)$$

The bound percentage was calculated using the following equation:

$$\frac{C_b}{C_f + C_b} \times 100 \quad (2)$$

Determination of Esterase-like Activity

The reaction of *p*-nitrophenyl acetate with rHSA and individual domains was followed spectrophotometrically at 400 nm (JASCO Ubest-35 UV-VIS spectrophotometer) by monitoring the appearance of *p*-nitrophenol. The reaction mixtures contained 5 μM *p*-nitrophenyl acetate and 20 μM protein in 67 mM phosphate buffer (pH 7.4). Reactions were followed at 25°C. Pseudo-first-order rate constant analysis

was performed, as described elsewhere (11,12), and the apparent hydrolysis rate constants (k_{obs}) were calculated.

Enolase-like Activity Assay

The final volume of the reaction mixture used in the measurement of enolase activity of rHSA and individual domains was 250 μl and contained the following: 67 mM phosphate buffer (pH 7.4) or carbonate buffer (pH 9.2), 0.4 μCi [1,2-3H] DHT (about 300,000 CPM), 20 μM radioinert DHT, and enzyme preparation. After incubation at 37°C for 90 min, 1 ml of a dextran-coated charcoal suspension (1% charcoal and 0.1% dextran [av. mol. wt. 35,000–45,000] in 67 mM phosphate buffer [pH 7.4] or carbonate buffer [pH 9.2]) was added, and the suspension was then allowed to stand for 10 min at room temperature. The suspension was then centrifuged for 5 min at 10,000 rpm. A 0.4-ml aliquot of the resulting supernatant was added to 4 ml of scintillation cocktail, and the radioactivity was determined using a LSC-5100 liquid scintillation counter (Aloka, Tokyo, Japan). Total enolase activity was subtracted from activity in the absence of protein (background). The steroid was removed by adsorption on charcoal. The counted radioactivity was due to tritiated water released from DHT, as evidenced by the following: 1) radioactivity was not measurable in the absence of protein; 2) evaporation of the reaction product (after treatment with charcoal suspension) to dryness resulted in the loss of radioactivity (13).

Reactive Oxygen Species (ROS) Quenching Capacity

H_2O_2 oxidizes DRD to rhodamine 123 (RD), which fluoresces at 536 nm when excited at 500 nm. We prepared 2-ml samples of rHSA or individual domains (7.5 μM) and DRD (5 μM) in 67 mM phosphate buffer (pH 7.4), and at predetermined times, H_2O_2 (25 mM) was added. The progress of the reactions was followed spectrophotometrically by measurement of the RD formed at 25°C by fluorescence. Controls were performed without additives. The quenching of the control was 0%.

In Vivo Experiments

Animals

Male ddY mice (6 weeks old, 25–35 g) were purchased from the Kyudou Co (Kumamoto, Japan). The animals were maintained under conventional housing conditions. The experiments were performed in accordance with the Principles of Laboratory Animal Care adopted and promulgated by the United States National Institutes of Health.

Biodistribution Experiment

rHSA and individual domains were radiolabeled with ^{111}In using DTPA anhydride, as described elsewhere (20). Each radiolabeled derivative was purified by gel-filtration chromatography using a Sephadex G-25 column with elution by a 0.1 M acetate buffer (pH 6.0). The absorbency of the eluants was measured at 280 nm, and the protein-containing fractions were pooled. The resulting solution was concentrated and washed with 0.9% NaCl by ultrafiltration. The specific activity of each derivation was approximately 37 MBq/mg protein. The mice received a 0.1 mg/kg dose of ^{111}In-rHSA conjugate in saline by injection into the tail vein. At 1, 3, 5, 10, and 30 min after injection of the ^{111}In-rHSA conjugate, blood was collected from the vena cava with the animal under ether anesthesia, and plasma was obtained by centrifugation. At each of these time points, an animal was sacrificed for excision of liver and kidney. These organs were then rinsed with saline, weighed, and examined for radioactivity. ^{111}In radioactivity was counted using a well-type NaI scintillation counter (ARC-2000; Aloka, Tokyo, Japan).

Pharmacokinetic Analysis

The tissue distribution patterns of the ^{111}In-rHSA derivatives were evaluated according to organ uptake clearance using a previously reported method (21). In the early period after injection, the efflux of ^{111}In radioactivity from the organs was assumed to be negligible, because the degradation products of ^{111}In-labeled ligands, prepared using DTPA anhydride, do not easily pass through biologic membranes (21). This assumption was supported by the fact that no ^{111}In was detected in the urine. Based on this assumption, organ uptake clearance was calculated by dividing the amount of radioactivity in an organ at 30 min by the area under the plasma concentration-time curve (AUC) at the same time point. The AUC was calculated by fitting an equation to the plasma concentrations of the derivatives using MULTI, a nonlinear least-squares program (22). Tissue distribution patterns were evaluated using tissue uptake clearance data according to the integration plot analysis. Tissue accumulation at time t was proportional to the $AUC_{0\text{-}t}$. By dividing the tissue accumulation at time t (X_t) and the $AUC_{0\text{-}t}$ by the plasma concentration (C_t), CL tissue was obtained from the slope of the plot of X_t/C_t vs. $AUC_{0\text{-}t}/C_t$. A previous report (23) has shown that ^{111}In is not suitable for evaluating the dynamic phase of a protein with a long *in vivo* half life. Therefore, we estimated the total, liver and kidney clearance within a period of 30 min.

Statistical Analysis

All data are presented as mean ± SD. Statistical analysis of differences was performed by one-way ANOVA followed by a modified Fisher's least squares difference method.

RESULTS

Structural Aspects of the Recombinant HSA Domains

Previous experiments in our laboratory demonstrated that rHSA expressed with a yeast expression system and then purified exhibited structural and functional properties that were very similar to those of HSA. The recombinant domains expressed in the present study exhibited structural properties identical to those reported by Dockal *et al.* (data not shown). Individual domains expressed in the present study were similar in length to those reported by Dockal *et al.* (14), except that domain II had 2 additional residues at the N-terminal in the present study (187-385). N-terminal amino acid sequences of the proteins expressed in the present study were consistent with the corresponding portions of native HSA. The three recombinant domains of HSA exhibited secondary and tertiary structures comparable to those of rHSA. The sum of the observed ellipticities of the three domains shows the same

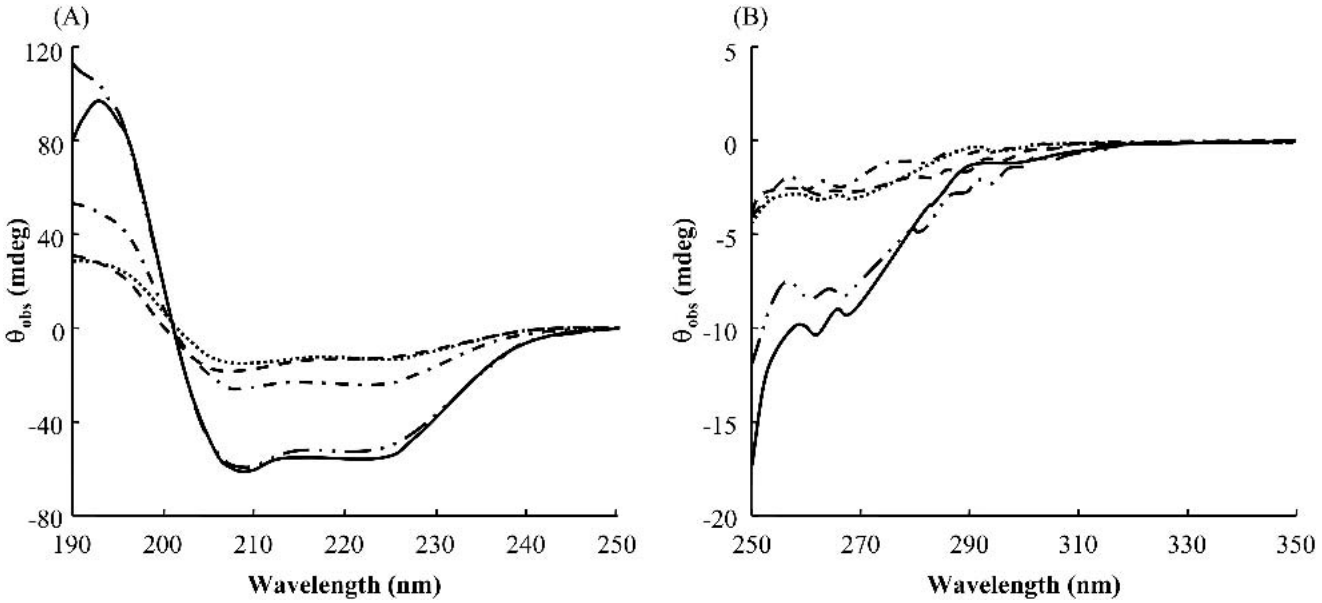

Fig. 1. Observed ellipticities of far-UV (A) and near-UV (B) CD spectra for rHSA (——), domain I (·····), domain II (**----**), domain III (– · – ·) and calculated signal of the three domains combined (— ·· —). Far- and near-UV CD spectra were recorded at protein concentrations of 5 μM and 15 μM, respectively, in 67 mM phosphate buffer (pH 7.4 and 25°C).

minima and shape as rHSA, especially at wavelengths above 265 nm in the near-UV CD spectra, suggesting that their conformation is similar to their native structure as constituents of rHSA (Fig. 1).

Ligand Binding Ability of the Recombinant HSA Domains

Although there have been reports of the ligand binding ability of each HSA domain, there have been no reports of the quantitative binding and ligand binding to subsites of site I. In this study, the percentage of ligand (2.5 μM) bound to rHSA and individual domains (5 μM) was determined by ultrafiltration. Table I shows the binding percentage of warfarin, DNSA and *n*-butyl *p*-AB, which are markers of subsite Ia, Ib, and Ic, respectively (24). Negligible amounts of warfarin and DNSA bound to each single domain. However, the binding percentage of *n*-butyl *p*-AB to rHSA was 50%, and a significant amount of *n*-butyl *p*-AB bound with domain II.

Table I. Binding of Warfarin, DNSA, and *n*-Butyl *p*-AB to rHSA and Individual Domains

	Percentage bound (%)		
HSA	Warfarin (Ia)	DNSA (Ib)	*n*-butyl *p*-AB (Ic)
rHSA	50.93 ± 1.94	42.36 ± 2.74	14.38 ± 5.72
Domain I	6.48 ± 0.67[a]	3.29 ± 2.96[a]	1.27 ± 0.37[a,c]
Domain II	7.46 ± 4.26[a]	2.46 ± 1.69[a]	9.39 ± 1.79[b]
Domain III	5.18 ± 2.00[a]	3.36 ± 2.94[a]	3.00 ± 1.06[b,d]

The sample solutions contained 2.5 μM warfarin, DNSA or *n*-butyl p-AB and 5 μM rHSA or individual domains in 67 mM phosphate buffer (pH 7.4 and 25°C). All values are mean ± SD (n = 3 to 5).
[a] $p < 0.01$ vs. rHSA.
[b] $p < 0.05$ vs. rHSA.
[c] $p < 0.01$ vs. domain II.
[d] $p < 0.05$ vs. domain II.

The intrinsic fluorescence spectra originating from Trp-214 shows that the relative fluorescence intensity of domain II was low and its λ_{max} was blue-shifted, compared with rHSA (Fig. 2). Table II shows the percentage of bound ketoprofen and DNSS, which are site II markers of HSA. Ketoprofen and DNSS bound to domain III with greater affinity than the site I markers to domain II, but ketoprofen and DNSS bound with greater affinity to rHSA than to domain III.

Esterase-like Activities of the Recombinant HSA Domains

The catalytic activity of rHSA and individual domains with respect to *p*-nitrophenyl acetate is shown in Table III. Esterase-like activity was detected in domain III, where site II is located (18), but this activity was about 45% of that of rHSA. Activity was also detected in domain I, although it was much lower than that of rHSA or domain III. However, no activity was detected in domain II.

Enolase-like Activities of the Recombinant HSA Domains

We used a radioisotope tracer to measure the enolase-like activity (conversion of the 3-keto form of DHT to the 3-enol form) of the HSA domains (Table IV). The enolase-like activity of HSA is influenced by pH and the type of buffer used. The activity increases with increasing pH, but at very high pH, especially over pH 11.0, enolization is a self-catalyzed reaction for compounds containing a keto group. The behavior of DHT in the present study was the same as reported previously (13). Accordingly, we examined enolase-like activity under two conditions: in a carbonate buffer at pH 9.2, conditions under which the highest activity has been observed and the self-catalyzed reaction is negligible; and in a phosphate buffer at pH 7.4, which are more typical experimental conditions. All HSA domains showed only low activity in the phosphate buffer (pH 7.4). The activity of rHSA was higher in the carbonate buffer (pH 9.2) than in the phosphate

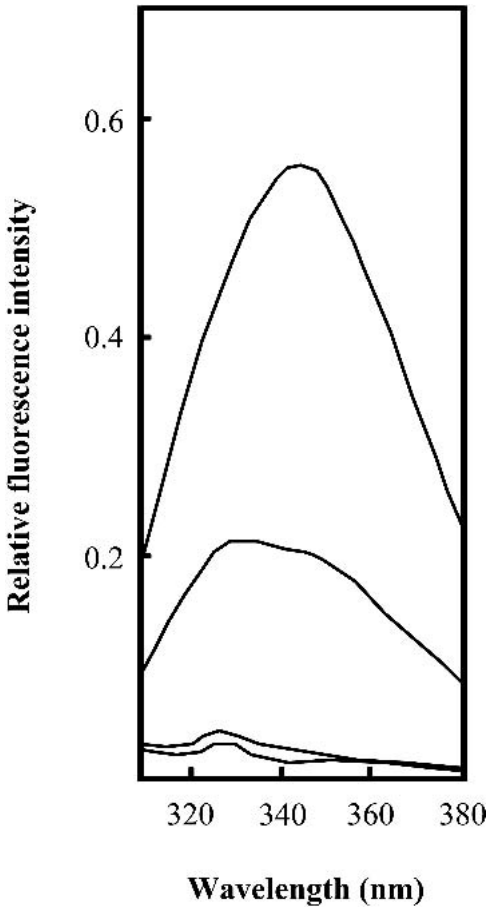

Fig. 2. Intrinsic fluorescence spectra of rHSA and individual domains. The protein concentration was 6 μM in 67 mM phosphate buffer (pH 7.4 and 25°C). From top to bottom, spectra for rHSA, domain II, domain III, and domain I.

buffer (pH 7.4), and a similar difference in activity was observed for domain II. However, this pattern of activity was not observed for the other two domains.

In order to confirm the involvement of domain II in the enolase-like activity of HSA, the enolase-like activity of rHSA (5 μM) for DHT (2 μM, 0.4 μCi) was measured in the presence of warfarin or ketoprofen (20 μM). No inhibition was observed in the presence of ketoprofen at pH 7.4 or 9.4. In contrast, in the presence of warfarin, a decrease in activity of about 35% was observed at pH 7.4, whereas no significant inhibition was observed at pH 9.4 (data not shown). These results suggest that the active site in site I is functional at pH 7.4, and that a new site different from site I is formed in domain II at pH 9.4, a process which is thought to be influenced by fragmentation.

Table II. Binding of Ketoprofen and DNSS to rHSA and Individual Domains

HSA	Percentage bound (%)	
	Ketoprofen	DNSS
rHSA	80.17 ± 6.73	62.43 ± 5.38
Domain I	6.07 ± 3.95[a,b]	4.21 ± 2.90[a,b]
Domain II	4.58 ± 1.61[a,b]	7.02 ± 2.87[a,b]
Domain III	64.00 ± 5.83[a]	38.92 ± 7.78[a]

The sample solutions contained 2.5 μM ketoprofen or DNSS and 5 μM rHSA or individual domains in 67 mM phosphate buffer (pH 7.4 and 25°C). All values are mean ± SD (n = 3 to 5).
[a] $p < 0.01$ vs. rHSA.
[b] $p < 0.01$ vs. domain III.

Table III. Hydrolysis Rate Constants (k_{obs}) for *p*-Nitrophenyl Acetate

HSA	k_{obs} ($s \times 10^{-3}$)
rHSA	7.13 ± 0.90
Domain I	1.73 ± 0.65[a]
Domain II	ND
Domain III	3.18 ± 0.83[a,b]

The reaction mixtures contained 5 μM *p*-nitrophenyl acetate and 20 μM rHSA or individual domains in 67 mM phosphate buffer (pH 7.4 and 25°C). All values are mean ± SD (n = 5 to 7). ND, not detectable.
[a] $p < 0.01$ vs. rHSA.
[b] $p < 0.01$ vs. domain I.

Antioxidant Activity of the Recombinant HSA Domains

We examined the ability of each domain to inhibit the oxidation of DRD by H_2O_2. The RD generated by oxidation was used as an indicator of antioxidant activity (25). Figure 3 shows the capacity of rHSA and individual domains to quench DRD oxidation by H_2O_2. Domains II and III showed a weak inhibitory effect compared with rHSA, but the quenching capacity of domain I was comparable to that of rHSA.

In Vivo Studies

To evaluate whether fragmentation of domains affects the biologic fate of rHSA, we measured uptake clearance of rHSA and the corresponding domain proteins in mice (Table V). There was about a 50-fold difference in total clearance between rHSA and individual domains. However, there was no significant difference in total clearance among the domains. The three domains were mainly distributed in the kidney. However, domain III was found at slightly higher levels than other domains in the liver.

Table IV. Enolase-like Activity of rHSA and Individual Domains in Two Different Buffer Solutions

HSA	Phosphate buffer pH 7.4 (CPM)	Carbonate buffer pH 9.2 (CPM)
rHSA	5073 ± 1422	11650 ± 1374
Domain I	1467 ± 205[a]	1804 ± 36[a,b]
Domain II	727 ± 80[a]	5851 ± 1320[a]
Domain III	1110 ± 261[a]	1897 ± 171[a,b]

5 μM rHSA or individual domains was incubated with DHT (20 μM, 0.4 μCi) for 90 minutes and enzymatic activity was measured at 37°C. All values are the mean ± SD (n = 3).
[a] $p < 0.01$ vs. rHSA.
[b] $p < 0.01$ vs. domain II.

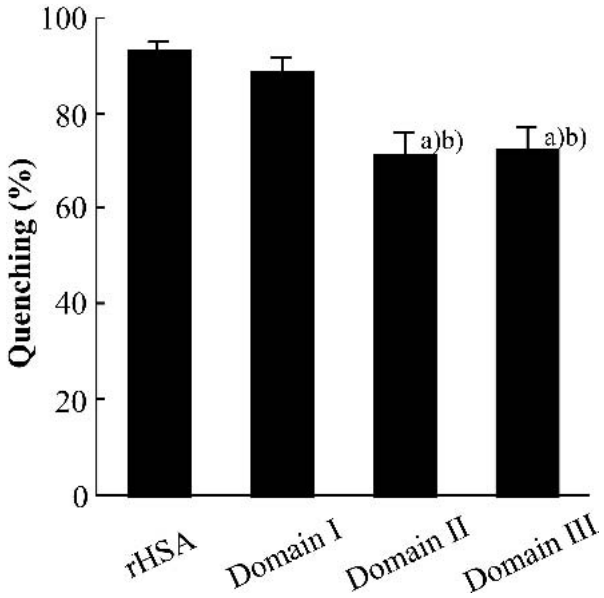

Fig. 3. Quenching of H_2O_2 oxidation of DRD by rHSA and individual domains. The sample solutions contained 7.5 μM rHSA or individual domains in 67 mM sodium phosphate buffer (pH 7.4 and 25°C), 5 μM DRD, and 25 mM H_2O_2. Each bar represents the mean ± SD (n = 3). a) $p < 0.01$ vs. rHSA; b) $p < 0.01$ vs. domain I.

DISCUSSION

The effectiveness of recombinant protein pharmaceuticals is heavily dependent on the intrinsic pharmacokinetics of the natural protein. For example, the efficacy of a drug is affected by its serum half-life. A variety of strategies have been proposed to create long-acting forms of drugs. One approach involves modification of drug formulation so that the product is slowly released from the injection site. This sustained release form requires fewer injections. However, the active agent is not changed, so elimination of the drug remains unchanged and the drug dose must be increased to cover the longer dosing interval. Another strategy is to exploit plasma protein binding; for example, noncovalent association with albumin extends the half-life of short-lived proteins. The drug is covalently linked with a component that is known to bind with high affinity to a plasma protein. Recombinant fusion of the albumin-binding domain of streptococcal protein G (which selectively binds to albumin with high affinity) to human complement receptor type 1 has been shown to increase the half-life of this receptor 3-fold to 5 h in rats (26). In another study, when insulin was acylated with myristic acids, its effects were prolonged due to its spontaneous association with albumin (27). Another approach is to modify the drug itself, so that the active drug is cleared more slowly from the systemic circulation. Because the kidney generally filters out molecules smaller than 60 kDa, efforts to reduce clearance have focused on increasing the molecular size of drugs through protein fusion, glycosylation or addition of polyethylene glycol polymers (*i.e.* PEG). Another approach is conjugation of the drug to a carrier protein to form a prodrug (5,28,29).

Table V. Clearance of ^{111}In-Labeled rHSA and Individual Domains

HSA	Clearance (μl/min)		
	Total	Liver	Kidney
rHSA	3.65 ± 0.78	0.69 ± 0.21	0.28 ± 0.10
Domain I	178.35 ± 9.72[a]	5.12 ± 0.98[b,c]	147.33 ± 10.57[a]
Domain II	181.37 ± 12.12[a]	5.20 ± 2.52[c]	173.38 ± 12.35[a,d]
Domain III	184.80 ± 15.21[a]	19.09 ± 4.16[a]	171.14 ± 5.63[a,d]

All values are mean ± SD (n = 3).
[a] $p < 0.01$ vs. rHSA.
[b] $p < 0.05$ vs. rHSA.
[c] $p < 0.01$ vs. domain III.
[d] $p < 0.05$ vs. domain I.

One of the challenges for the successful commercialization of therapeutic proteins is to maintain the safety and efficacy of the protein during its manufacturing, storage and administration. To achieve this, the purified form of the protein drug is usually formulated with carefully selected excipients. The formulation of a peptide drug preparation would require the inclusion of an antioxidant to maintain the potency of the drug as well as a lyoprotectant. A protein carrier that possesses considerable antioxidant activity could eliminate or reduce use of antioxidants in the formulation. A protein drug carrier with low or no enzymatic activity would promote the stability of a peptide drug or the peptide linker during storage, and may eliminate the need for refrigeration during storage. Low ligand binding capacity of a protein carrier may prevent accumulation of endogenous or exogenous substances which may modify the drug properties during the preparation stage or upon administration into the general circulation. Because proteins have complex molecular structures that can influence the protein stability, the development of stable formulations of protein pharmaceuticals requires an intimate knowledge of the protein structure as well as its chemical and physical properties. In particular, an understanding of the mechanisms by which a protein may degrade is crucial for designing and testing drug formulations. The major pathways of protein degradation include denaturation, aggregation, oxidation and interfacial damage.

Ligand Binding Properties

HSA is the most abundant plasma protein, and is involved in a variety of physiologic functions including maintenance of colloid osmotic pressure. One of its prominent physiologic functions is ligand transport. HSA has at least two distinct binding sites for several physiologically important compounds and a large number of hydrophobic drugs. These two major binding regions, site I and II, are located within specialized cavities in subdomain IIA and IIIA, respectively (1). Although mapping of the locations of each drug binding site had been attempted in qualitative ligand binding studies involving induced circular dichroism using recombinant domains by Dockal *et al.,* quantitative data has not been obtained.

In the current study, all domains showed a significant decrease in the percentage of ligand bound for all site I probes, compared with rHSA, except for the binding of *n*-butyl *p*-AB, a site Ic ligand that binds to domain II. It appears highly likely that the observed marked loss of the ligand binding ability of domain II, which contains site I, is due to the absence of the neighboring domain, which indicates the importance of interdomain interactions for maintaining the

structural stability of site I, particularly at subsites Ia and Ib. The importance of interdomain interactions for the integrity of site I was also indicated by the marked decrease in the intrinsic fluorescence spectra of Trp-214, the only Trp residue of HSA that is located in domain II, reflecting major changes in the environment and dynamics of the residue (Fig. 2). A corresponding decrease in the percentage of warfarin (a site I ligand that has been shown to interact with Trp-214) bound by domain II was also observed.

Domain III, which contains site II, retained most of its ability to bind site II ligands (Table II). The preservation of the binding ability of site II may be due to its structural independence, as indicated by the crystal structure of HSA. The entrance of the binding pocket of site II in domain III is in direct contact with the bulk solvent. In contrast, domain I is in contact with the entrance of the site I binding pocket in domain II (1). However, interactions between domain III and domain II also contribute to the binding of ligands to site II, as indicated by the fact that the bound percentage of site II ligand was lower than that of rHSA.

On the other hand, domain I showed negligible binding to all the ligands used in the binding studies. Kjeldsen *et al.* (30) reported that domain III, but not domain I, binds myristic acid. However, insulin acylated with myristic acid bound to domain III but bound more weakly to domain I.

Enzymatic Activity

Domain III of HSA has been reported to possess esterase-like activity. Arg-410 and Tyr-411 have been identified as the key amino acid residues responsible for the esterase-like activity of domain III. Recently, Watanabe *et al.* (18) reported that the enzymatic activity of albumin is much more dependent on the presence of Tyr-411 than on Arg-410. In the present study, the esterase-like activity of domain III was only about 40% that of the intact rHSA (Table III). This suggests that interdomain interaction is crucial for complete esterase-like activity.

On the other hand, esterase-like activity was also detected in domain I, although it was much weaker than that of domain III. However, the enzymatic site on domain I has not been identified. This esterase-like activity of domain I may be additive to the lower activity found in domain III, as indicated by the much higher total activity observed for intact HSA. However, the formation of a new active site in domain I upon fragmentation cannot be ruled out.

No enzymatic activity was detected for domain II, suggesting the absence of an active site in this domain. This finding is consistent with the fact that domain II has not been reported to possess esterase-like activity in studies of intact HSA.

The enolase-like activity of HSA for DHT has been reported to decrease with polymerization of HSA in malignant breast tumors, compared with benign breast tumors (13). All domains showed a decrease in enolase-like activity at a pH of 7.4 in phosphate buffer, with domain II showing the lowest value, compared with rHSA. In contrast, all domains and rHSA showed an increase in activity at a pH of 9.2 in carbonate buffer. Among the domains, domain II showed the largest increase, suggesting that an enolase active site is generated in domain II upon pH alteration (Table IV).

Antioxidant Properties

HSA is a mixture of mercaptalbumin (which has one free sulfhydryl group at Cys-34) and non-mercaptalbumin (which has a mixed disulfide with cysteine and glutathione at Cys-34 in serum). The fraction of human mercaptalbumin has been shown to be markedly decreased in various diseases including diabetes mellitus, and in some physiologic conditions such as aging, compared with healthy young male subjects (31). In a previous study, we used several different methods to oxidize HSA, and found that Cys-34 and Met residues are major participants in oxidative stress (10).

The antioxidant activity of domain I was found to be slightly lower than that of rHSA. Domain II was found to have antioxidant activity comparable to that of domain III but lower than that of domain I. Each domain contains two Met residues that are thought to contribute to antioxidant activity. The substantial activity of the domains may be due to a more open environment, resulting in a more exposed location of the two methionine residues, allowing them to function as antioxidants more easily. Cys-34 seems to contribute additively to the higher antioxidant activity of domain I, because domain I contains Cys-34 with a free thiol group (Fig. 4).

In the formulation of biotechnological products, albumin has been commonly used as an anti-adhesion agent and lyoprotectant. Other excipients such as nonionic surfactants have also been widely used in the development of protein pharmaceuticals. However, the low level of residual peroxides in surfactants may affect the stability of oxidation-sensitive proteins. In this respect, albumin may also function as an antioxidant, minimizing the potential undesired effects of other excipients.

Pharmacokinetics of Individual Domains

Characterization of the pharmacokinetics and pharmacodynamics of each domain of HSA will facilitate further development of drug targeting strategies. Manipulation of the size of the carrier protein may also be used to control the clearance of the conjugated drug in a graded manner. Sheffield *et al.* (32) tested the effects of truncation on the *in vivo* clearance of rabbit serum albumin (RSA) in rabbits, using recombinant RSA mini-proteins containing the first, first and second, or third domain of the protein. They reported that

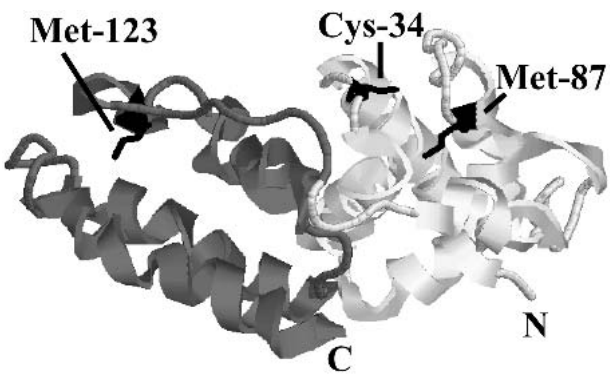

Fig. 4. Ribbon model of domain I. The subdivision of the protein into subdomains (IA and IB) indicated by color codes; N and C represent the N- and C-terminus, respectively. The figure was prepared with Molscript on the basis of the atomic coordinates 1UOR available at the Brookhaven Protein Data Bank.

half-lives did not differ among the truncated proteins, suggesting that all had been reduced below the size threshold.

In the present pharmacokinetic analysis, all three ^{111}In-radiolabeled domains exhibited very rapid elimination compared to rHSA, with a high level of accumulation in the kidney. Domain I showed a slightly lower renal clearance than the other two domains. Domain III showed about a 4-fold higher liver clearance than the other two domains (Table V). Our laboratory has recently shown that the triple-residue mutant K199A/K439A/K525A of rHSA mimics the effect of glycosylation, a reaction that alters the pharmacokinetics of proteins. This suggests that the half-lives of the recombinant domains can be manipulated by mutating certain residues such as positively charged lysine residues (33).

CONCLUSIONS

Domain I of HSA has great potential for further development as a "stand-alone" protein for potential use as a drug delivery protein carrier due to its antioxidative activity and low enzymatic activity. In addition, the presence of a free cysteine residue allows relatively easy conjugation with a drug containing a free thiol moiety. Though its short half-life may make it suitable for application as a stabilizer, polymerization may be a feasible approach for increasing the half-life of domain I protein of HSA.

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