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EVIDENCE FOR THE SPONTANEOUS FORMATION OF INTERSPECIES HYBRID MOLECULES OF HUMAN, RAT AND BOVINE SERUM ALBUMINS

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Utilizing electrophoretic and gel filtration techniques it was shown that a bovine C-terminal peptic fragment [residues 307-582] spontaneously forms interspecies hybrid molecules with three complementary N-terminal fragments derived from human [residues 1-308; 49-308] and rat [residues 1-308] albumins. The fragments associate with 1:1 stoichiometry to produce an albumin-like complex which has a molecular weight and electrophoretic mobility similar to intact albumin. These data demonstrate, for the first time, that albumin fragments derived from different species associate in a complementary fashion and provide direct evidence that the tertiary structure may be highly conserved.

Plasma albumins are complex proteins whose structure:function relationships are not fully understood (1, 2). Although the complete amino acid sequences of bovine, human and rat albumins have been determined, relatively little is known about the secondary and tertiary structure (3-5).

Despite the lack of X-ray crystallographic data, several models describing the 3-dimensional structure of albumin have been proposed (3, 6, 7). The most widely accepted model to date is the triple domain structure of Brown (3) which is based on sequence data and the proposed disulfide bond pairings of serum albumin. Briefly, the Brown model suggests that albumin consists of three separate domains, each having two subdomains and that both the domains and subdomains are connected by relatively short flexible polypeptide segments.

A consequence of this structure is that under controlled conditions it is possible to cleave connecting links between domains thereby liberating a small number of albumin fragments. Because plasma albumins undergo a reversible ex-

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pansion at acid pH, pepsin has been used to prepare overlapping and non-overlapping fragments (8). Some peptic fragments of bovine albumin retain specific ligand binding sites. In addition, two complementary fragments of bovine albumin [residues 1-306, 307-582] have been shown to spontaneously associate at alkaline pH to form an albumin-like complex (9). Studies of proteolytic fragments of bovine albumin have enhanced our understanding of structure:function relationships in the intact molecule.

In this paper we have investigated the ability of seven peptic fragments derived from human, rat and bovine albumins to form stable intra- and interspecies hybrid molecules.

MATERIALS AND METHODS

Chemicals and biochemicals were generally of the purest grade available and were obtained from either Sigma Chemical Company, St. Louis, MO or Fisher Scientific, Cincinnati, OH. Cibacron Blue F3GA-agarose (Affi-Gel Blue) was purchased from Bio-Rad Laboratories, Richmond, CA. Concanavalin A-Sepharose, Sephadex G-100 SF and G-150 were obtained from Pharmacia, Piscataway, NJ. DEAE-cellulose (DE-52) and CM-cellulose (CM-52) were purchased from Pierce Chemical Co., Rockford, IL. Fresh or outdated human plasma was generously donated by the American Red Cross, Louisville, KY.

Albumin Purification. Bovine serum albumin was purchased from Sigma Chemical Company, St. Louis, MO. The lone free sulfhydryl group of BSA was blocked with half-cystine according to the procedure of Reed et al. (9) in order to prevent dimerization of albumin or its peptic fragments. A human albumin monomer preparation was prepared from plasma, according to our modification of the method of Travis et al. (10). Briefly, an enriched albumin fraction was prepared by ammonium sulfate precipitation followed by chromatographic purification on Cibacron Blue F3GA-agarose and Concanavalin A-Sepharose at pH 7.4 (11). Rat serum albumin (RSA) monomer was purified by conventional techniques utilizing ammonium sulfate precipitation, ion-exchange chromatography on CM-52 and DE-52 and gel filtration. Again, the lone free sulfhydryl of both rat and human albumin was protected as described above. Albumin concentrations were determined by using $A^{1\%}_{1\ cm}$ = 6.6, 5.3 and 5.9 at 280 nm for BSA, HSA and RSA, respectively.

Human, Bovine and Rat Albumin Peptic Fragment Preparation and Purification. The preparation, purification and identification of the four peptic fragments of HSA (HSA [1-387], HSA [1-308], HSA [309-585] and HSA [49-308]) are described elsewhere (12-13). Both peptic fragments of bovine albumin (BSA [1-306] and BSA [307-582]) and the single rat albumin peptic fragment (RSA [1-308]) were prepared utilizing the procedures of King (14) and Feldhoff and Peters (8). The peptic digestion conditions for albumin species were similar; that is, a 20-min digestion of half-cystinyl albumin monomer preparations in the presence of octanoic acid at pH 3.7; 25 or 35°C. The purification schemes of the peptic fragments varied depending on the albumin species but generally involved an initial gel filtration step at pH 3.0 on Sephadex G-150 followed by either ion-exchange chromatography on CM-52 or DE-52 or affinity chromatography on Cibacron Blue F3GA-agarose (8, 13).

Cellulose Acetate Electrophoresis. Electrophoresis was performed on cellulose acetate ("Titan III"; Helena Laboratories, Beaumont, TX) at 23°C in

sodium barbital buffer (pH 8.6; μ = 0.075) for 15 min at 180 V. Following electrophoresis of individual peptides (20 mg/mL) or fragment mixtures (40 mg/mL; approximately equimolar ratios) in barbital buffer, the plates were stained with 0.5% Ponceau S in 3.5% trichloroacetic acid-3.5% sulfosalicylic acid, destained with 5% acetic acid and cleared with methanol:acetic acid (7:3).

Thin Layer Gel Filtration. Thin-layer gel filtration of albumins and peptic fragments was performed on Sephadex G-100 SF coated glass plates (180 x 160 x 0.75 mm) in a humidified chamber according to the procedure of Andrews (15). Plates were equilibrated with pH 8.6 barbital buffer (μ = 0.075) for 1 hour at 23°C with a plate angle of 12°. Samples (5 μL) containing peptides (100 μg) or fragment mixtures (200 μg) were applied to the equilibrated gels while the eluent was running through them. Gels were developed for approximately 5 hours at 23°C with the equilibration buffer. Filter paper imprints of the wet gels were heated to 110°C for 5 minutes, stained with 2% Light Green Yellowish in 8% sulfosalicylic acid and destained with 5% acetic acid. Control samples consisting of human albumin, human albumin dimer and Blue Dextran 2000 were run on each plate.

RESULTS AND DISCUSSION

In previous studies we have utilized controlled limited proteolysis with pepsin to prepare fragments of albumin which retain individual binding sites (9, 12, 13, 16). By digestion in the presence of a protecting ligand, at acid pH where the binding domains start to dissociate, fragments can be obtained which contain specific binding sites.

Because of the biomedical relevance of human albumin:ligand interactions we have concentrated our recent efforts on the purification and characterization of fragments of human albumin. Eight fragments of HSA have been prepared (12, 13). Some of these fragments are similar to peptides which have been prepared by the peptic digestion of BSA (8, 14). In previous studies two of the bovine fragments corresponding to residues 1-306 and 307-582 were shown to reassociate forming an albumin-like complex with the reconstitution of one or more binding sites (9). We have prepared the analogous peptides from HSA, however, the C-terminal human fragment [309-585] contains an internal cleavage site and two peptide chains which are held together by disulfide bonds. This is due to the presence in HSA of an additional and highly susceptible pepsin cleavage site [Leu_{423}-Val_{424}]. Recently, we have also found that residues 408-423 are missing from this fragment (13).

Because albumins have similar conformations, yet different sequences, in the present investigation we have utilized our human, bovine and rat fragments

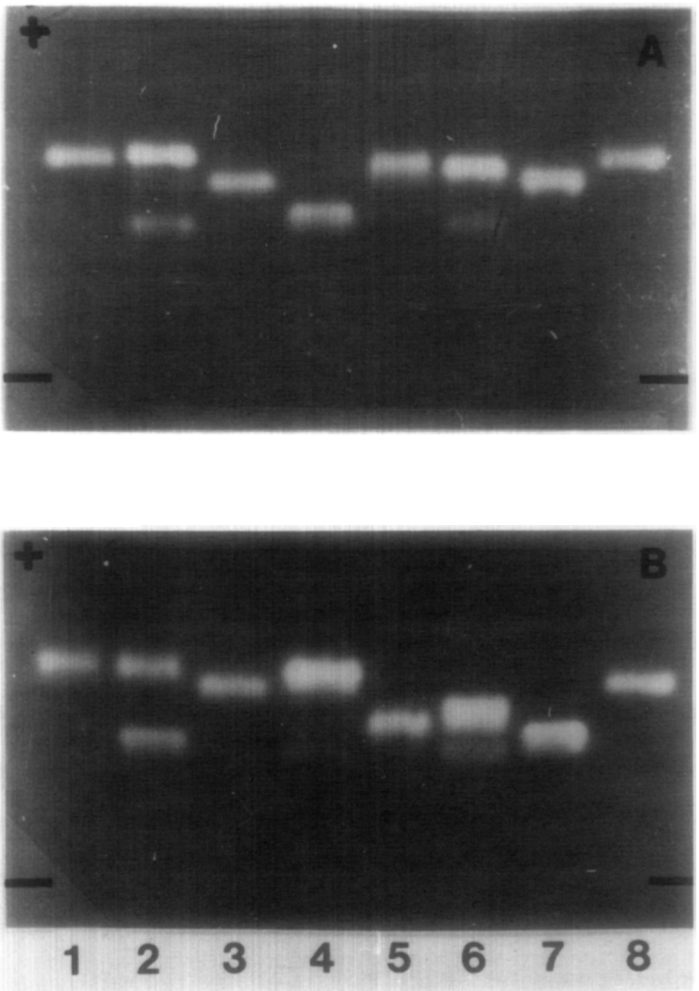

Figure 1. Cellulose Acetate Electrophoresis of Albumins and Purified Albumin Fragments. Fragment notation indicates species of origin (BSA = bovine serum albumin; HSA = human serum albumin; RSA = rat serum albumin) with amino acid residues given within brackets.

(A) Lane (1) BSA; (2) BSA [1-306] + BSA [307-582]; (3) BSA [1-306]; (4) BSA [307-582]; (5) RSA [1-308]; (6) RSA [1-308] + BSA [307-582]; (7) RSA; (8) BSA.

(B) Lane (1) HSA [1-387]; (2) HSA [1-387] + BSA [307-582]; (3) HSA [1-308]; (4) HSA [1-308] + BSA [307-582]; (5) HSA [49-308]; (6) HSA [49-308] + BSA [307-582]; (7) BSA [307-582]; (8) HSA.

Complex formation is evident in (A) lanes 2 and 6 and in (B) lanes 4 and 6. In (B) lane 2 it is clear that HSA [1-387] did not reassociate with BSA [307-582].

to ascertain whether or not the domain:domain or subdomain interactions are conserved across species lines. As shown in Fig. 1A, during cellulose acetate electrophoresis the individual BSA fragments 1-306 and 307-582 (lanes 3, 4) exhibit decreased electrophoretic mobilities relative to intact BSA (lane 1). However, when the fragments are mixed together an albumin-like complex forms

which has an electrophoretic mobility identical to BSA (lane 2). Similarly, the rat albumin (RSA) fragment 1-308 (lane 5) associates with the complementary C-terminal fragment of BSA (lane 6). The mobility of the RSA:BSA hybrid appears to be intermediate between that of intact RSA (lane 7) and BSA (lane 8). The 3 intact N-terminal HSA fragments which we have purified were also tested for complex formation as shown in Fig. 1B. Significantly HSA [1-387] does not associate with BSA [307-582] (lanes 1, 2) whereas both HSA [1-308] (lane 3) and HSA [49-308] (lane 5) do form interspecies hybrid molecules (lanes 4, 6). For reference, lanes 7 and 8 contain BSA [307-582] and intact HSA, respectively. These data suggest that residues 1-48 are not involved in complex formation, while the presence of residues 309-387 in HSA [1-387] appear to inhibit complex formation. It is noteworthy that residues 309-387 by themselves constitute a separate disulfide-bonded loop structure or subdomain (3).

Utilizing a thin layer gel filtration procedure (15), we confirmed the cellulose acetate electrophoresis results and also demonstrated that all complexes formed in a 1:1 molar ratio with no indication of aggregate formation. Electrophoretic and gel filtration experiments were undertaken with BSA [307-582] or HSA [309-585] and the complementary N-terminal fragments from the various species. Relevant examples of our thin layer gel filtration analyses are shown in Fig. 2.

Panel A shows the intraspecies complex formed by BSA [1-306] + BSA [307-585] (lane 5) has a mobility the same as intact BSA (lanes 2 or 6). Panel B shows that the analogous peptides derived from HSA do not form an albumin-like complex. As noted previously this is not unexpected since HSA [309-585] is missing residues 408-423.

As shown in panels C and D HSA [1-308] does form an interspecies hybrid complex with BSA [307-582] whereas HSA [1-387] which contains an additional 79 residues does not form a similar complex. These extra residues (309-387) may be blocking the contact site or altering the conformation of HSA [1-387] such that complex formation cannot occur. It is noteworthy that residues 1-48 of

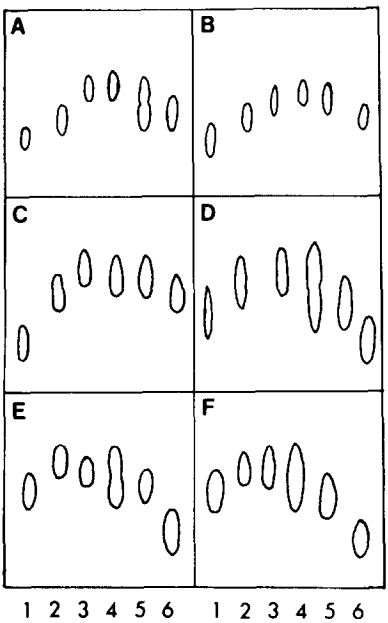

Figure 2. Thin Layer Gel Filtration on Sephadex G-100 SF of Albumins and Purified Albumin Fragments. The procedure of Andrews (15), was employed as described in the Methods section. The direction of migration was from the top to bottom of each panel. Abbreviations for albumins and peptides are defined in the legend to Fig. 1.

(A) Lane (1) Blue Dextran 2000; (2) BSA; (3) BSA [1-306]; (4) BSA [307-582]; (5) BSA [1-306] + BSA [307-582]; (6) BSA.
(B) Lane (1) Blue Dextran 2000; (2) HSA; (3) HSA [1-308]; (4) HSA [309-585]; (5) HSA [1-308] + HSA [309-585]; (6) HSA.
(C) Lane (1) Blue Dextran 2000; (2) BSA; (3) BSA [307-582]; (4) HSA [1-387]; (5) BSA [307-582] + HSA [1-387]; (6) HSA.
(D) Lane (1) BSA; (2) BSA [307-582]; (3) HSA [1-308]; (4) BSA [307-582] + HSA [1-308]; (5) HSA; (6) HSA dimer.
(E) Lane (1) BSA; (2) BSA [307-582]; (3) HSA [49-308]; (4) BSA [307-582] + HSA [1-308]; (5) HSA; (6) HSA dimer.
(F) Lane (1) BSA; (2) BSA [307-582]; (3) RSA [1-308]; (4) BSA [307-582] + RSA [1-308]; (5) RSA; (6) HSA dimer.

HSA do not appear to affect complex formation since both HSA [1-308] (panel B) and HSA [49-308] (panel E) do associate with BSA [307-582].

An additional interspecies hybrid forms between RSA [1-308] and BSA [307-582] (panel F). In all cases the stoichiometry of complex formation was 1:1. All of these data are summarized in Table 1 and suggest that a variety of interspecies hybrid molecules can be formed utilizing BSA [307-582]. However HSA [309-585] which is missing residues 408-423, did not form an albumin-like complex with any of the complementary N-terminal fragments.

The electrophoretic behavior of the bovine peptides (BSA [1-306] and BSA [307-582]) indicates that positive charges are exposed in the fragments

Table 1. Summary of Electrophoresis and Gel Filtration Experiments on the Association of Albumin Fragments.

	C-Terminal Fragments			
N-Terminal	BSA [307-582][a]		HSA [309-585]	
Fragments	CAE[b]	TLGF[c]	CAE	TLGF
BSA [1-306]	+[d]	+	-	n.d.[e]
RSA [1-308]	+	+	-	n.d.
HSA [1-308]	+	+	-	-
HSA [49-308]	+	+	-	n.d.
HSA [1-387]	-	-	-	n.d.

[a] Fragment notation is defined in the legend of Fig. 1.
[b] Cellulose acetate electrophoresis.
[c] Thin layer gel filtration.
[d] The demonstration of 1:1 complex formation is indicated by (+) and the lack of complex formation in both procedures by (-).
[e] Not determined.

which are masked in both the complex and the intact molecule (Fig. 1, panel A, lanes 1-4). This suggests that some of the bonds which maintain domain:domain interactions are likely to involve salt bridges or intrachain hydrogen-bonding between amino acids which have ionizable side chains. Our hypothesis is further supported by titration data which show an increase in the number and accessibility of ionic groups when the albumin molecule undergoes reversible acid expansion (6).

Our demonstration of the formation of stable interspecies albumin-like molecules suggests that the contact points between domains have been conserved in a manner which maintains the overall conformation. These fragments and complexes should be useful as probes to define the involvement of specific amino acid residues at the contact surfaces between domains or subdomains and for other investigations of structure:function relationships of plasma albumins.

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