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Localization of the Binding Site for Streptococcal Protein G on Human Serum Albumin. Identification of a 5.5-Kilodalton Protein G Binding Albumin Fragment†

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ABSTRACT: Protein G is a streptococcal cell wall protein with separate and repetitively arranged binding domains for immunoglobulin G (IgG) and human serum albumin (HSA). In this work, the binding of protein G to HSA was studied. The results suggest that a single binding site is present on HSA: the apparent size of the HSA–protein G complex (230 kDa) corresponded to two or three HSA molecules bound to one protein G molecule, and Ouchterlony immunodiffusion did not yield any precipitate between protein G and HSA. HSA was cleaved by pepsin and CNBr into several fragments which were identified by SDS–PAGE and N-terminal amino acid sequencing, and the binding of protein G to the fragments was studied in Western blot experiments. The results indicated that the binding area was located in disulfide loops 6–8, involving both the second (loop 6) and the third (loops 7 and 8) domain of HSA. One of the protein G binding pepsin fragments, with an apparent molecular mass of 5.5 kDa, located in loops 7 and 8, was isolated and found to completely inhibit the binding between protein G and the intact HSA, again suggesting a single protein G binding site on serum albumin. Reducing the disulfide bonds of HSA, and subsequent alkylation of the half-cystine residues, significantly decreased the affinity for protein G. Protein G bound to albumin from baboon, cat, guinea pig, hamster, hen, horse, man, mouse, and rat, but not to albumin from cow, dog, goat, pig, rabbit, sheep, snake, or turkey.

Protein G is an immunoglobulin G (IgG)[1] binding protein expressed by group C and G streptococci (Björck & Åkerström, 1990). The protein was originally enzymatically solubilized from the streptococcal cell wall (Reis et al., 1984; Björck & Kronvall, 1984), but the protein G gene has also been expressed in *Escherichia coli* (Guss et al., 1986; Fahnestock et al., 1986; Björck et al., 1967). Comparative studies (Åkerström et al., 1985; Guss et al., 1986; Åkerström & Björck, 1986) showed that the IgG Fc binding properties of protein G are similar to those of protein A, the IgG-binding protein of *Staphylococcus aureus* (Forsgren & Sjöquist, 1966; Langone, 1982), although protein G has a wider range of IgG-binding activity among mammalian species. The physicochemical properties of proteins A (Langone, 1982) and G (Åkerström & Björck, 1986) indicate that they are both fibrous proteins and the binding site on IgG Fc is identical or very similar for the two proteins (Stone et al., 1989). However, the IgG-binding domains of proteins A and G showed no sequence homology (Uhlén et al., 1984; Guss et al., 1986; Fahnestock et al., 1986). Apart from IgG, protein G also shows affinity for human serum albumin (HSA) (Björck et al., 1987). On the protein G molecule, HSA binding was found to be separate from IgG binding and located to repeated domains in the N-terminal half of the streptococcal protein, whereas the IgG-binding domains reside in the C-terminal half (Åkerström et al., 1987; Sjöbring et al., 1988). This unique organization of protein-binding domains will allow the protein G expressing *Streptococcus* to cover itself with an outer layer of albumin and an inner layer of IgG, which should influence the host–parasite relationship during infections with these bacteria.

In the present work, we have investigated the binding between protein G and HSA in order to localize the binding site on the HSA molecule. Albumin has been described as a cigar-shaped protein, consisting of three spherical domains. The domains are approximately equal in size, and each contains six disulfide bonds except domain 1 which contains five. Together these form disulfide loops 1–9 [for a review, see Peters (1985)]. On the basis of the exon arrangement of the gene, each domain is also divided into two subdomains (Minghetti et al., 1986). The three-dimensional structure of HSA has been determined, and confirms the model with three domains and six subdomains (Carter et al., 1989). By the position and size of the loops, the three-dimensional structure and, by amino acid sequence comparison, the three domains are homologous. In this work, HSA was fragmented by pepsin and CNBr treatment, and the binding of protein G to each fragment was analyzed. The results suggest that protein G binds to a single site located in the second and third domains. The mapping of the binding to this region is described, and

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[1] Abbreviations: HSA, human serum albumin; SPRIA, solid-phase radioimmunoassay; Ig, immunoglobulin; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; kDa, kilodalton(s).

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the possible functional consequences of the interaction are discussed.

Materials and Methods

Proteins and Other Chemicals. HSA was obtained from Kabi (Stockholm, Sweden), mouse albumin from Sigma Chemical Co. (St. Louis, MO), and bovine albumin (fraction V) from Boehringer Mannheim (Mannheim, West Germany). Rat serum albumin was isolated as described earlier (Åkerström & Landin, 1985). Human IgG and ovalbumin were purchased from Sigma Chemical Co. Baboon, hamster, snake (S-3507), and turkey sera were also from Sigma Chemical Co., and horse serum was from Flow Laboratories Ltd. (Irvine, Scotland). Cat, cow, dog, goat, guinea pig, hen, human, mouse, pig, rabbit, rat, and sheep sera were obtained by bleeding the animals and removing the clots by centrifugation. Porcine pepsin (P6887), L-cystine (C8755), and sodium caprylate (C3901) were all bought from Sigma Chemical Co. We also used CNBr purchased from Merck, Darmstadt, FRG. A 35-kDa protein G fragment was prepared by papain digestion of group G streptococci as reported (Björck & Kronvall, 1984), and a 65-kDa protein G was purified from lysates of *Escherichia coli* expressing protein G (Björck et al., 1987).

Labeling of Proteins. Protein G (35 and 65 kDa) and human IgG were radiolabeled with ^{125}I (Svensk Radiofarmaka AB, Bromma, Sweden) using the chloramin T method (Greenwood, et al., 1963).

Solid-Phase Radioimmunoassay (SPRIA). Microtiter plates (Falcon 3912, Becton Dickinson, Oxnard, CA) were coated with HSA (0.25 μg/well) in 50 μL of PBS (10 mM phosphate + 0.12 M NaCl + 3 mM KCl, pH 7.4) and incubated for 2 h. The wells were then washed 3 times with 0.9% NaCl + 0.05% Tween 20. After that, 50 μL of radioiodinated 35-kDa protein G (1×10^6 cpm mL^{-1}) in a buffer containing 0.05% Tween 20 (v/v), with pH ranging from 4 to 9, was added to the wells and incubated at 4, 20, or 37 °C for 0.5, 1, 3, 6, and 18 h. Ultimately, the plate was washed and dried, and the wells were cut out for counting of radioactivity. Cross-blocking studies were done by SPRIA, coating the wells for 2 h with human, rat, or mouse albumin (0.1 μg in 50 μL of PBS), and then incubating for 4 h at 20 °C with a mixture of ^{125}I-labeled 35-kDa protein G (2×10^6 cpm mL^{-1}) and increasing concentrations (200 mg/L to 1600 mg/L in PBS + 0.05% Tween 20) of one of the two other albumins. Washing was done as described above. Inhibition of HSA binding to protein G by HSA peptide was also performed by SPRIA. The wells of a microtiter plate were coated with HSA (0.1 μg in 50 μL of PBS) overnight and washed. ^{125}I-Labeled 35-kDa protein G (4×10^6 cpm mL^{-1}) was mixed with HSA fragments at different stages of purification and preincubated for 1 h before addition to the wells, followed by incubation for 5 h. Ovalbumin was used as a negative control.

Pepsin Digestion of HSA. The half-cystinyl preparation and pepsin digestion of HSA were carried out as described (King & Spencer, 1970; King, 1973) with a few modifications; 150 mg of HSA was dissolved in 5 mL of 0.1 M Tris + 0.06 M HCl, pH 7.96. L-Cystine, 3.5 mg, was dissolved in 75 μL of 1 M NaOH and then diluted to 7 mL with the same Tris buffer. The HSA and L-cystine solutions were mixed and incubated for 18 h at room temperature, rotating end-over-end. A third of the HSA solution (4 mL) was concentrated to about 250 μL in a Centricon (Amicon, Danvers, MA). Then 1.6 mL of 0.1 M ammonium formate buffer, pH 3.7, + 3.2 mM octanoic acid, 50 μL of 1 M HCl, and finally 100 μL of pepsin solution (1 mg/mL) were added. After 1 min in a sonicator bath at room temperature, the digestion was started by raising the temperature to 37 °C and terminated after 1, 5, 10, 15, or 30 min by adding 13% (v/v) 2 M Tris and 2 parts of H_2O.

CNBr Cleavage of HSA. To a 100-μL solution of 0.1 M HCl containing 1 mg of HSA was added 9.6 μL of a solution of CNBr (50 g/L). The reaction was carried out at room temperature for 6 or 24 h and terminated by diluting 10 times with H_2O and freeze-drying. The fragments were redissolved in 500 μL of PBS. The cleavage was performed in 0.1 M HCl, as described above, or in 70% formic acid.

Electrophoresis. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS–PAGE) was done according to Laemmli (1970). The acrylamide concentration (*T*) was 10, 12, or 14% (w/v) and the cross-linking (*C*) 3.0, 3.7, or 4.5%, respectively. Rainbow-colored protein markers in the molecular weight range of 2350–46 000 or 14 000–200 000 (Amersham, Buckinghamshire, England) were used as molecular mass standards.

Western Blots. Proteins separated by SDS–PAGE were also transferred to poly(vinylidene difluoride) membranes (Immobilon Transfer, Millipore, Bedford, MA) or nitrocellulose membranes (Bio-Rad, Richmond, CA) electrophoretically (Towbin et al., 1979). The membranes were stained with 0.1% Coomassie Blue R-250. For blotting purposes, the membranes were blocked for 3 h in 1% gelatin (Bio-Rad) in PBS, pH 7.4. Poly(vinylidene difluoride) membranes with SDS–PAGE-separated HSA fragments were incubated overnight at 4 °C with either 35- or 65-kDa protein G, at a concentration of 10 mg/L in PBS + 0.5% gelatin + 0.25% Tween 20. The membranes were then washed with the PBS/gelatin/Tween buffer at 4 °C and finally incubated with ^{125}I-labeled human IgG (5×10^5 cpm mL^{-1}), diluted in the same buffer for 3 h at room temperature. Sera from different species were transferred to nitrocellulose membranes following SDS–PAGE. These membranes were blocked and then incubated with ^{125}I-labeled protein G (35 kDa, 5×10^5 cpm mL^{-1}) diluted in PBS/gelatin/Tween, containing human IgG (1 g/L). All membranes were finally washed at 4 °C with PBS/gelatin/Tween + 1 M NaCl + 1 mM EDTA before air-drying and autoradiography at −70 °C. Peptides to be analyzed for amino acid sequence were electroblotted to poly(vinylidene difluoride) membranes, stained, and cut out according to Matsudaira (1987) except that we used 25 mM Tris + 192 mM glycine + 20% methanol, pH 8.4, as the transferring buffer.

Slot Blots. Proteins were applied directly to nitrocellulose membranes using the slot blot apparatus Minifold II (Schleicher & Schuell, Dassel, West Germany). After being blocked, the nitrocellulose membranes were incubated with ^{125}I-35 kDa-protein G (5×10^5 counts min^{-1} mL^{-1}) diluted in PBS/gelatin/Tween for three hours. Blocking, washing and autoradiography was done as described for Western blots.

Reduction and Alkylation. Albumin from different species (10 g/L) in 0.25 M Tris-HCl, pH 8.5, + 1 mM EDTA + 6 M guanidine hydrochloride was reduced with 0.125 M dithioerythritol (Sigma Chemical Co.) at room temperature for 2 h. Alkylation was accomplished by adding iodoacetamide to 0.25 M (Sigma Chemical Co.) and incubating at room temperature in the absence of light for 30 min. Excess reagents were removed by dialysis against several volumes of 2 mM NH_4HCO_3.

Gel Chromatography. Separation of pepsin fragments was performed using a column (2.1 × 110 cm) of Sephadex G-100 (Pharmacia LKB Biotechnology, Bromma, Sweden) equilibrated in 20 mM Tris-HCl, pH 8.0, + 0.15 M NaCl + 0.02% NaN_3. The elution rate was 4.7 mL/h, and 2.5-mL fractions

Table I: Binding Reaction between Protein G and Albumin from Different Sources

albumin	binding to protein G[a]	albumin	binding to protein G[a]
baboon	+[b]	human	+
cat	+	mouse	+
cow	–	pig	–
dog	–	rabbit	–
goat	–	rat	+
guinea pig	(+)	sheep	–
hamster	+	snake	–
hen	(+)	turkey	–
horse	(+)		

[a] The binding of the 35-kDa protein G was measured by SDS–PAGE and blotting of sera from different sources, probing with the ^{125}I-labeled protein G. [b] +, strong binding; (+), weak binding; –, no binding.

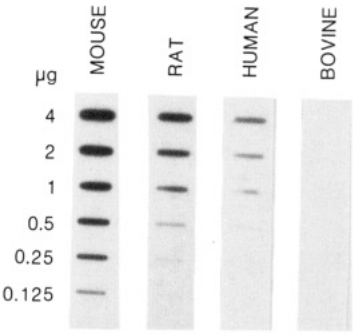

FIGURE 1: Binding of albumins from different species to radiolabeled protein G. Various amounts of mouse, rat, human, and bovine albumin were applied to nitrocellulose filters in a slot blotting apparatus. The filters were incubated with ^{125}I-labeled 35-kDa protein G (5×10^6 cpm mL^{-1}) for 3 h and autoradiographed for 3 days.

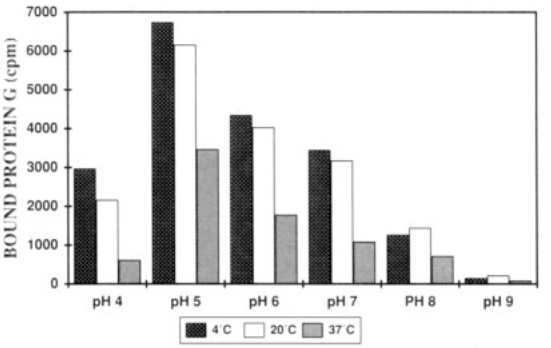

FIGURE 2: Protein G binding to HSA at different pHs and temperatures. The wells of a microtiter plate were coated with HSA (5 mg/L) in PBS, washed, and incubated with ^{125}I-labeled 35-kDa protein G (1×10^6 cpm mL^{-1}) diluted in buffers with different pH, which all contained 0.05% Tween 20. After incubation at 4, 20, or 37 °C for 2 h, the wells were washed, cut, and counted for radioactivity.

were collected. In order to determine the size of a complex between 65-kDa protein G and HSA, ^{125}I-labeled 65-kDa protein G and HSA were mixed, incubated for 1 h, and applied to gel chromatography on a Sepharose 6B-CL (Pharmacia LKB Biotechnology) column. The column had been calibrated with Blue Dextran (Pharmacia LKB Biotechnology), human IgG, myoglobin (Sigma Chemical Co.), and dinitrophenyl-alanine (Sigma Chemical Co.) and was eluted in 20 mM Tris-HCl, pH 7.2, + 0.15 M NaCl + 0.2% NaN_3. The flow rate and fraction volume per hour were approximately 1% of the column volume. Both types of chromatography were carried out at 4 °C.

Other Methods. Attempts to precipitate protein G with HSA or rat serum albumin were done by double diffusion in agarose as outlined by Ouchterlony (1958). The amino-terminal amino acid sequence of HSA fragments was determined on a Applied Biosystems 470A gas–liquid–solid-phase sequenator as described (Grubb et al., 1986). Alignment of sequences was performed using Beckman Microgenie software (Queen & Korn, 1984). Analysis of the amino acid composition of HSA fragments was done, after hydrolysis for 24 h in 6 M HCl in vacuo of poly(vinylidene difluoride) membranes, on an LKB Plus Alpha amino acid analyzer.

Results

Binding of Protein G to Serum Albumin from Different Species. Sera from several different species were separated by SDS–PAGE, electroblotted to nitrocellulose membranes, and incubated with ^{125}I-labeled 35-kDa protein G. To block the binding of protein G to the IgG in the sera, an excess of human IgG was included in the probing solution. Baboon, cat, hamster, man, mouse, and rat albumin were clearly positive with respect to protein G binding (Table I), and a weaker, but clearly positive, binding was seen with guinea pig, hen, and horse serum albumin. Human, bovine, rat, and mouse serum albumin have been sequenced (Brown, 1975; Behrens et al., 1975; Meloun et al., 1975; Sargent et al., 1981; Minghetti et al., 1985). These proteins, either native or reduced and alkylated, were applied to nitrocellulose membranes and probed with radiolabeled protein G. Mouse and rat albumin showed the strongest binding, human albumin was slightly weaker, and bovine albumin was negative (Figure 1). The binding to protein G by human, rat, and mouse serum albumin was almost completely abolished by reduction and alkylation (not shown). Each of the three albumins completely inhibited the binding of radiolabeled protein G to microtiter wells coated with the other two homologues (not shown), which suggests that human, rat, and mouse albumin bind protein G with similar affinities. In an attempt to find regions where the bovine albumin sequence was different from the other three albumin homologues, all four sequences were aligned and compared. However, no definite regions of that kind were seen.

Characterization of the Binding between Protein G and Serum Albumin. Figure 2 shows the temperature and pH dependence of the binding between protein G and HSA, analyzed by SPRIA. Like protein G binding to IgG (Åkerström & Björck, 1986), optimal binding was obtained at acidic pH. The binding was lower at higher temperatures. An HSA-coated microtiter plate was incubated with radiolabeled 35-kDa protein G for 0.5, 1, 3, 6, or 18 h at room temperature and at pH 6.5. The results demonstrated that the binding was almost completed after 6 h (not shown).

The 65-kDa protein G and HSA or rat serum albumin did not form precipitates when tested by Ouchterlony immunodiffusion. Protein G has two or three binding sites for HSA (Åkerström et al., 1987; Nygren et al., 1990; Sjöbring et al., 1991). The results from the Ouchterlony test are compatible with multiple binding sites on protein G and one binding site on HSA. Also, gel chromatography with ^{125}I-labeled 65-kDa protein G and HSA gave a complex of apparent molecular weight 230 000, indicating only one binding site on HSA.

Localization of Protein G Binding on HSA. The free SH group in HSA at amino acid position 34 was blocked by half-cystinylation, and the molecule was subsequently pepsin-digested as described under Materials and Methods. Incubation with the enzyme for 30 min gave an optimal fragmentation pattern on SDS–PAGE with at least nine major peptides, ranging from 43 kDa to approximately 4 kDa (Figure 3, section I). The peptide mixture was analyzed by Western blotting, incubating first with nonlabeled 35- or 65-kDa protein

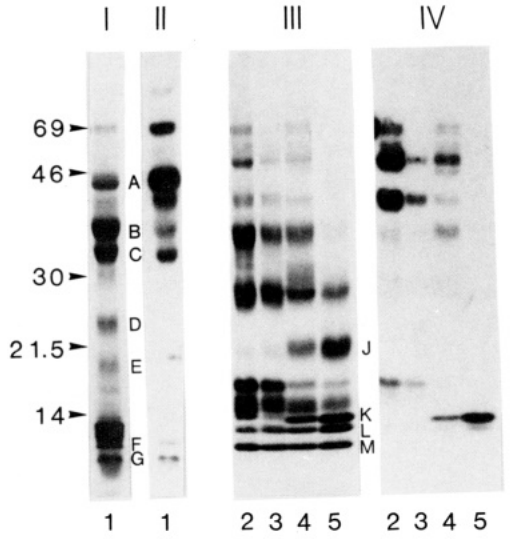

FIGURE 3: Binding of protein G to HSA cleaved by pepsin or CNBr. HSA was digested with pepsin (HSA:pepsin ratio, 500:1) at 37 °C for 30 min (lane 1) and with CNBr at room temperature either in 0.1 M HCl for 6 (lane 2) or 24 h (lane 3) or in 70% formic acid for 6 (lane 4) or 24 h (lane 5). The samples were separated by SDS–PAGE (T = 12%, C = 3.7%) under reducing conditions and were either stained (panels I and III) or transferred to poly(vinylidene difluoride) membranes (panels II and IV). The membranes were incubated with 35-kDa protein G (10 mg/L), washed, and probed with ^{125}I-labeled human IgG. Autoradiography was done at −70 °C. Markers of molecular mass are shown in kilodaltons. Peptic fragments (A–G) and CNBr fragments (J–M), which also are illustrated in Figure 4, are marked.

G followed by ^{125}I-IgG. All peptides were transferred to the membranes to a similar degree, since no difference in staining intensity of the pepsin digest could be seen between the gels and the poly(vinylidene difluoride) membranes. Both types of protein G bound similarly to the peptides, and the result from 35-kDa protein G binding is illustrated in Figure 3, section II, lane 1. Higher resolution of the smallest peptides could be achieved with SDS–PAGE on a gel with a higher acrylamide concentration (T = 14%, not shown). Thus, six HSA peptides, 43, 40, 38, 33, 5.5, and 3.5 kDa, showed affinity for protein G. Among these, the 38-kDa peptide showed a weaker binding than the other five peptides. The remaining three peptides, 23, 19, and 16 kDa, did not bind.

HSA was also cleaved by the CNBr reaction. Two different solutions, 0.1 M HCl and 70% HCOOH, and two different incubation times, 6 and 24 h, were used. Cleavages were analyzed similarly to the pepsin digestion, by SDS–PAGE and Western blotting. A higher concentration of low molecular weight peptides appeared when 70% HCOOH and a longer incubation time were used. This is shown in Figure 3, section III. Section IV shows that several protein G binding as well as nonbinding fragments were obtained by CNBr cleavage.

N-Terminal amino acid sequence and total amino acid composition analyses were performed on selected peptides separated by SDS–PAGE and transferred to poly(vinylidene difluoride) membranes. High-resolution separation was obtained by SDS–PAGE (T = 14%), and in all cases, only one sequence was seen (Table II). The positions of the peptides could thus be mapped in the HSA molecule. The positions of the CNBr fragments were corroborated by amino acid composition analysis. The C-terminal amino acids of the peptides were not determined, but the size of the peptides was estimated by SDS–PAGE, thus giving the approximate positions of the C-terminal ends. These agreed well with the positions of methionine in the intact HSA molecule. The deduced C-terminal of some of the peptic fragments, however,

Table II: Automated Sequential Degradation of Albumin Fragments[a]

	cycle no.					
fragment	1	2	3	4	5	6
A	Asp	Ala	His	Lys	Ser	Glu
	83[b]	131	?[c]	131	29	48
B	Asp	Ala	–[d]	Lys	Ser	Glu
	130	100		290	10	110
C	Phe	Ala	Lys	Thr	–	Val
	220	260	370	180		180
D	Val	Arg	Pro	–	Val	Asp
	110	?	59		35	23
E	Phe	Ala	Lys	Thr	–	Val
	25	36	22	9		13
F	Val	Glu	Val	Ser	Arg	Asn
	61	36	51	44	13	23
G	Val	Val	Leu	Asn	Gln	Leu
	160	100	100	40	50	80
J	–	Thr	Ala	Phe	His	–
		39	53	45	?	
K	Phe	Leu	Tyr	Glu	Tyr	–
	270	250	270	160	240	
L	Pro	–	Ala	Glu	–	–
	120		120	50		
M	Asp	Ala	His	Lys	Ser	–
	120	150	13	100	58	

[a] The albumin fragments were separated by SDS–PAGE and transferred to poly(vinylidene difluoride) membranes. [b] Repetitive yield of each degradation cycle in picomoles. [c] Not determined. [d] Not identified.

Table III: Size and Position of Albumin Fragments

		amino acid position[b]	
fragment	size[a]	pepsin fragment	CNBr fragment
A	43 000	1–380	
B	38 000	1–	
C	33 000	49–	
D	23 000	116–308	
E	19 000	49–	
F	5 500	424–	
G	3 500	455–	
J	21 000		124–298
K	13 000		330–446
L	12 000		447–548
M	11 000		1–87

[a] The size of the fragments (in daltons) was determined by SDS–PAGE. [b] The N-terminal positions of the fragments were determined by amino acid sequence analysis. The approximate positions of the C-terminal ends were calculated from the length of the peptides, estimated by SDS–PAGE. The deduced C-terminus of a pepsin fragment is then given in the table if it agreed with previous reports. The deduced C-termini of all CNBr fragments agreed with methionine positions and are thus given.

did not agree with previous reports, and could not be exactly located. Table III summarizes the size and location of the HSA fragments obtained by pepsin and CNBr cleavage.

Figure 4 shows a peptide map of both the peptic (A–G) and CNBr (J–M) fragments, binding or not binding to protein G. Two peptic fragments are excluded, the 40- and 16-kDa peptide bands, which gave rise to faint bands containing too small amounts to allow sequencing. Four of the CNBr fragments were sequenced and mapped. Of the fragments that are marked as protein G binding, fragments B and L bound weaker than the others, suggesting that they represent structures of minor importance for the binding. Fragments K and L, which have similar sizes, show strikingly different binding strength to protein G. Fragment K (loops 6 and 7) binds strongly and fragment L (loops 8 and 9) weakly. The results suggest that protein G binds to both the second and third domains. More specifically, the binding seems to be directed to disulfide loops 6, 7, and 8. No fragment was totally

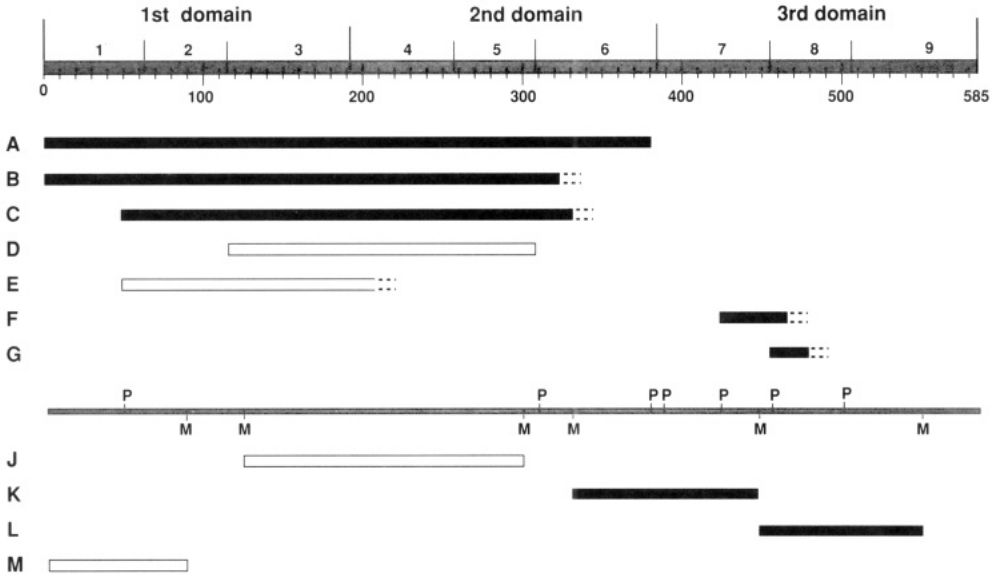

FIGURE 4: Map of HSA and its pepsin and CNBr fragments. Peptides were identified and located in the HSA map by amino-terminal amino acid sequence analysis. The upper, shaded bar represents the whole amino acid sequence of HSA with the three domains and nine double-disulfide loops marked with Arabic numerals. The lower shaded line shows reported pepsin cleavage sites (P) and CNBr cleavage sites (M). A–G illustrate HSA peptides derived from pepsin digestion and J–M peptides from CNBr fragmentation. Peptide F was also isolated by Sephadex G-100 gel chromatography of the pepsin digest (see Figure 6, lane 4). Black bars represent peptides binding to protein G and white bars nonbinding peptides. The C-termini of all peptides are unknown, and if they did not correspond to previously published sites of fragmentation, they have been marked by dashes. The length of each peptide was calculated from the apparent molecular weight, determined by SDS–PAGE.

contained within loop 9, and therefore it is not possible to determine whether this loop is included in the binding site. However, the weak binding of fragment L, the only peptide that contains structures from loop 9, suggests that at least the N-terminal 60% of loop 9 does not participate in the binding.

Isolation of Protein G Binding Peptic Fragments. The relatively large binding area suggested by the results shown in Figure 4 raised the possibility that the binding took place at two or more nonrelated binding sites interacting with multiple sites in the protein G molecule. This hypothesis could be tested by separating one of the small protein G binding fragments, F or G, from all other protein G binding fragments and determining whether such a fragment could inhibit the protein G binding to whole HSA completely, or only partially. Thus, the mixture of fragments from a 12-min pepsin digestion of HSA was applied to a Sephadex G-100 column. Longer pepsin incubation times gave higher amounts of fragments F and G, but also more of other low molecular weight peptides. The incubation time used in this digestion (12 min) thus seemed optimal for this purpose. The UV light absorbance of the eluted fractions was measured as an estimate of the total protein content (Figure 5), the distribution of the fragments in the fractions was analyzed by SDS–PAGE, and their protein G binding activity was determined by Western blotting (Figure 6). Thus, the main peak contained a mixture of undigested HSA and larger protein G binding and nonbinding fragments. The smaller peptides could be seen later in the eluate, and the two small protein G binding fragments F and G (5.5 and 3.5 kDa, respectively) were isolated from each other as well as from all other protein G binding fragments. The F- and G-containing fractions were pooled separately and found by SDS–PAGE, Western blotting, and amino acid sequence analysis to be contaminated with only a few other peptides showing no affinity for protein G.

The isolated fragment F was then chosen for inhibition studies, since the yield of this fragment, 1.2 mg from 70 mg of HSA, was higher than the yield of fragment G. Fragment F was concentrated by dialysis, lyophilization, and reconstitution in PBS. The peptide was then tested in a SPRIA, and

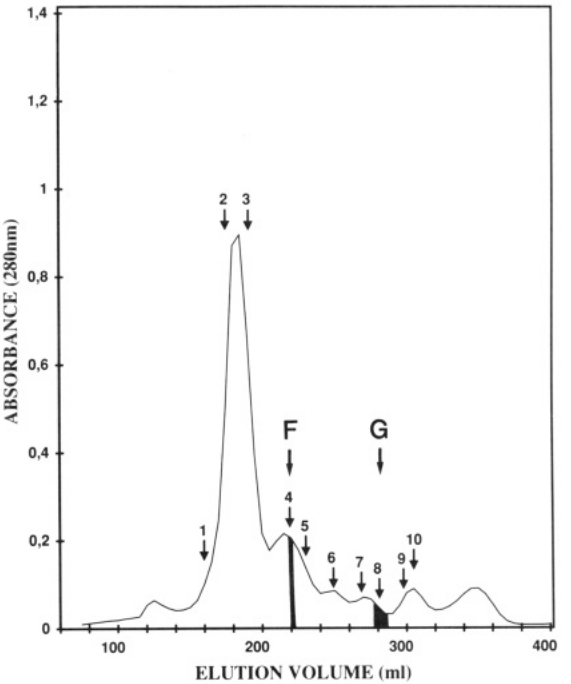

FIGURE 5: Gel chromatography of pepsin-digested HSA. Seventy miligrams of pepsin-digested HSA was applied to a Sephadex G-100 gel filtration column (0.38 L), with 20 mM Tris-HCl, pH 8.0, + 0.15 M NaCl + 0.02% NaN_3 as eluent. The UV absorbance of the fractions was measured as shown in the figure. The fractions were also separated by SDS–PAGE, and selected fractions (marked by small arrows) are shown in Figure 6. The fractions containing isolated fragments F or G were pooled and correspond to the shaded areas.

the binding of radiolabeled protein G to HSA-coated microtiter plate wells could be completely inhibited by the 5.5-kDa fragment (Figure 7). Ovalbumin, which was tested as a control, showed no inhibition.

Discussion

In this work, we have shown that the binding site for protein G on HSA was located to the second and third domains,

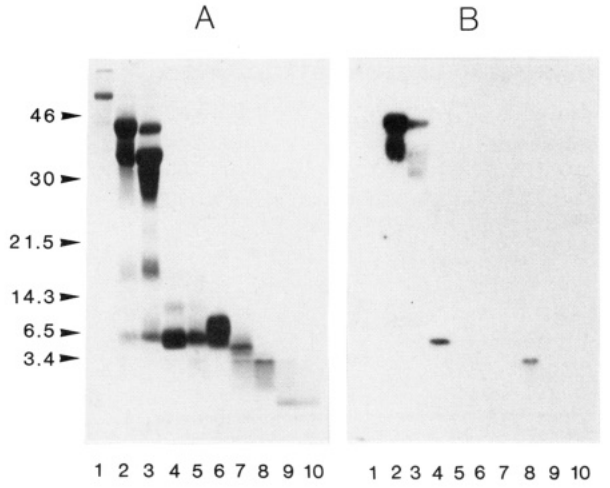

FIGURE 6: Binding of protein G to HSA fragments separated by gel chromatography. Selected fractions from Sephadex G-100 gel chromatography were separated by SDS–PAGE ($T = 14\%$, $C = 4.5\%$) under reducing conditions. In panel A, the protein bands were stained directly, and in panel B, the bands were transferred to poly(vinylidene difluoride) membranes, incubated with 35-kDa protein G, and finally probed with ^{125}I-labeled human IgG as described in Figure 3. The numbers of the lanes correspond to the selected fractions, marked by arrows in Figure 5. Markers of molecular masses are shown in kilodaltons.

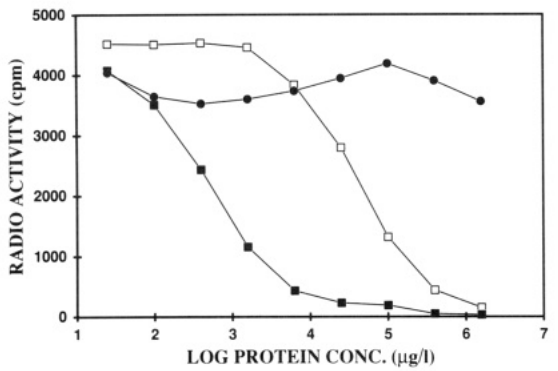

FIGURE 7: Inhibition of protein G binding to HSA by purified fragment F. Aliquots of HSA (50 μL, 2 mg/L) in PBS were coated to the wells of a microtiter plate overnight. ^{125}I-Labeled 35-kDa protein G (4×10^6 cpm mL^{-1}) was mixed with a pepsin digest of HSA (■), the purified 5.5-kDa HSA fragment (□), or ovalbumin (●) (0.1–3200 mg/L), all diluted in PBS + 0.05% Tween, and preincubated for 1 h before addition to the washed wells. After 5 h, the plates were washed, cut, and counted in a γ-counter.

including loops 6–8, and that most likely only a single binding site exists. HSA was fragmented, and the binding of protein G to separated fragments with known positions in the intact protein was studied. For this purpose, the HSA molecule was cleaved with both pepsin and CNBr. Pepsin has been used by several investigators for the fragmentation of HSA, and the results of the pepsin cleavage agreed to some extent with previous publications (Heaney-Kieras & King, 1977; Giesow & Beaven, 1977; Ledden et al., 1980; Ledden & Feldhoff, 1983). These investigators have reported that limited pepsin cleavage, similar to the conditions used here, yielded breaks in HSA at positions 48, 308, 380, 387, 423, 455, and 500 (marked by "P" in Figure 4). This agrees with the N-terminal position of all fragments in this study except for fragment D, which thus defines a novel pepsin cleavage site. The pattern of the binding and nonbinding fragments showed that protein G binds to the C-terminal half of HSA, which is compatible with the results of Widebäck-Hansson (1987), who suggested that radiolabeled HSA was bound to whole group G streptococci through its C-terminal region.

Furthermore, the results suggest that HSA contains one binding site for protein G, formed by loops 6–8. This is primarily based on four observations. (1) No precipitation was formed between protein G and HSA on Ouchterlony immunodiffusion experiments. Since protein G has two or three HSA binding sites (Åkerström et al., 1987; Nygren et al., 1990; Sjöbring et al., 1991), the lack of precipitation is compatible with one binding site on HSA. (2) The size of the ^{125}I-protein G–HSA complex was approximately 230 000 daltons, which is consistent with a complex of two or three HSA molecules and one protein G molecule, thus strongly arguing against two binding sites on the HSA molecule. (3) Fragment L, which contains part of loop 9, bound protein G very weakly as compared to the other binding fragments. This suggests that loop 9 does not participate in the binding. Loops 6–8, the remaining binding area, show only weak homology to each other (McLachlan & Walker, 1977) and no repeated three-dimensional structural elements (Carter et al., 1989). If the binding area contain two binding sites, these would then have to be different, binding to different parts of protein G. (4) The purified fragment F with a molecular mass of 5.5 kDa could block all binding of radiolabeled protein G to HSA, again indicating a single binding site for protein G on HSA.

Loops 6–8 contain the two most important binding sites for fatty acids, and the binding site for tryptophan as well as for thyroxine (Peters, 1985). It was reported that the binding of fatty acids to HSA was not interfering with the binding to streptococci (Widebäck-Hansson, 1987). Thus, it would be of interest to test if the binding of fatty acids, thyroxine, or tryptophan to HSA interferes with the interaction between protein G and HSA. The results of such functional tests could be a possible clue to the biological role of albumin binding to protein G.

Reduction and alkylation of the intrachain disulfide bonds of HSA almost completely eliminated the binding to protein G. This indicates that the binding of protein G to HSA is largely dependent on the three-dimensional structure of the binding area. In this context, it should be noted that both fragments F and G contain one disulfide bond, thus maintaining at least one disulfide loop of the peptide backbone intact.

For many years, it has been speculated that a function of microbial surface proteins capable of interacting with human plasma proteins would be to disguise the microorganism, thereby preventing its identification and elimination by the host. The organization of protein G with multiple binding sites for the two most abundant human extracellular proteins certainly makes bacteria expressing protein G very potent in this respect. However, given the complexity of the host–parasite relationship, one can speculate on other functional consequences of the protein G mediated protein–protein interactions. For instance, it has been shown that the physicochemical surface properties of protein G expressing bacteria are changed when host proteins, including HSA, are bound to the cells (Miörner et al., 1980). Such changes probably modulate the adherence between bacteria and host cells. Moreover, albumin is a major transport protein, and the molecule could, after binding to the bacterial surface, hypothetically deliver substances used by the bacterium. Finally, recent data have indicated that protein G may be involved in proteolytic events at the bacterial surface (Sjöbring et al., 1989), which in turn may influence essential bacterial functions such as growth (Björck et al., 1989; Björck, 1990). The molecular characterization of these protein–protein interac-

tions, e.g., the high-affinity interaction between HSA and streptococcal protein G described in this work, will hopefully provide a better understanding of the role played by microbial cell surface proteins with specific protein-binding properties.

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