Multiple effects of haemin binding on protease susceptibility of bovine serum albumin and a novel isolation procedure for its large fragment

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The effects of ligand binding on the proteolytic susceptibility of BSA were investigated. The rate for proteolytic digestion with either trypsin or chymotrypsin decreased in the presence of bilirubin and fatty acids, suggesting that overall albumin conformation is stabilized by these ligands. In contrast, haemin showed multiple effects on a proteolytic digestion pattern: the rate for the degradation of intact albumin greatly increased, but a large 45 kDa fragment accumulated during proteolytic digestion in the presence of this ligand. This unique fragmentation pattern allowed us to isolate the 45 kDa fragment at a high yield (about 30 % on a molar basis) by one-step purification. Sequence analyses indicated that this fragment lies between residues Thr^{190} and Ala^{583}, which constitutes domains II and III of the albumin molecule. Far-u.v. c.d. spectra strongly suggested that the secondary structure in the intact albumin is almost retained in the 45 kDa fragment. The isolated 45 kDa fragment showed haemin-binding ability, as evaluated by spectroscopic titration; upon re-digestion of the 45 kDa fragment, haemin showed strong protective effects. These results were consistent with the idea that haemin binding to BSA induces an increased protease susceptibility in the loop region between domains I and II, but in the overall conformation of domains II and III, a protease-resistant property.

INTRODUCTION

Serum albumin is the most abundant plasma protein. It consists of a single chain with a molecular mass of about 66 kDa organized into nine loops stabilized by 17 disulphide bridges (Peters, 1985). Recent X-ray crystallographic data demonstrate that the human serum albumin molecule is composed of three domains, I, II and III, each of which can be further divided into two subdomains, A and B (He and Carter, 1992). This complex multidomain structure allows the albumin molecule great flexibility and conformational adaptability, as seen by its ability to rapidly convert into a number of isomeric forms and to bind a number of ligands, including fatty acids, bilirubin, amino acids, hormones, drugs and metal ions (Peters, 1985). Interestingly, the conformational transition may also be involved in ligand transport to the target cells (Reed and Burrington, 1989).

Because of the large size and complex structure, the use of isolated fragments having native function is important for the detailed analyses of ligand-induced local conformational change in serum albumin. For this reason, extensive studies have been done to isolate functional fragments (Peters, 1985). The fragmentation procedure, however, must include minimal chemical damage, so that highly cross-linked disulphide bonds essential for the native conformation (Lee and Hirose, 1992) remain intact. This has made it difficult to obtain a variety of albumin fragments with intact function: only limited numbers of procedures have been widely accepted, such as proteolytic fragmentation procedures for the two half-molecules (King, 1973; Feldhoff and Peters, 1975; Geisow and Beaven, 1977).

The proteolytic digestion approach has been used as a useful probe for monitoring ligand-induced conformational changes in a large protein (Ueno and Harrington, 1984). The approach is based on an assumption that if the conformational state of a ligand-bound protein is significantly different from the time-average conformation in the absence of a ligand, then their products of proteolysis could also be different. Ligand binding usually confers a more resistant conformation upon a globular protein. Thus if a ligand induces a protease-resistant conformation in its binding domain of serum albumin, a variety of functional fragments will be obtained by proteolysis in the presence of various ligands. Typical successful examples of this procedure are the cases of the isolation of the two half-molecules and domain III by proteolysis of fatty acid-bound albumin (King, 1973; Peters and Feldhoff, 1975).

In the present study, we have searched for conditions in which a large fragment of BSA accumulates during proteolysis in the presence of a ligand. Among several tested ligands, haemin showed unique effects on the proteolytic profile of BSA: the digestion rate of the intact molecule was greatly accelerated, but a fragment with a molecular mass of 45 kDa, that was assigned to the sequence Thr^{190}–Ala^{583}, accumulated. The haemin-induced modulation in the protease susceptibility allowed us to isolate a large fragment corresponding to domains II and III in a high yield by one-step purification.

EXPERIMENTAL

Materials

Fatty acid-free BSA (type A-7511) was obtained from Sigma Chemical Company. In the present study, we used a so-called 'mercaptalbumin' fraction that was essentially purified as described by Noel and Hunter (1972) except that the DEAE-cellulose column was replaced by an f.p.l.c. column (HiRoad XK-26, Pharmacia-LKB Co.). The concentration of BSA was determined using $A^{1\%}_{1\,cm}$ 6.67 at 279 nm (Sun and Maximos, 1989). Haemin chloride was a crystalline product from Sigma Chemical Company. It was dissolved at 8.0 mM in 0.01 M NaOH, rapidly diluted with 10 mM Tris/HCl buffer, pH 8.0, to a required concentration, and used in this form within 1 h. The concentration was determined spectrophotometrically using a molar absorption coefficient of 58400 $M^{-1} \cdot cm^{-1}$ at 385 nm (Beaven et al., 1974). Chymotrypsin A (EC 3.4.21.1) (type II), trypsin (EC

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3.4.21.4) (diphenylcarbamyl chloride-treated, type XI), bilirubin, and oleic acid were purchased from Sigma Chemical Company. Carboxypeptidase Y (EC 3.4.16.1) was purchased from Oriental Yeast. DEAE-cellulose (DEAE-cellulofine, A-500) was obtained from Seikagaku Kogyo. Other chemicals were guaranteed analytical grade from Nacalai Tesque.

Time course of proteolytic digestion

BSA or its fragment was digested at 0.5 mg/ml in 50 mM Tris/HCl buffer, pH 8.0, at 37 °C for various time periods with chymotrypsin or trypsin at a protein to enzyme ratio of 60:1 (w/w). To monitor the proteolytic progress, part of the mixture corresponding to 2.6 mg of original BSA was withdrawn and analysed by SDS/PAGE according to the standard method of Laemmli (1970) using a 12.5% polyacrylamide gel. The gel was stained with Coomassie Blue and band intensities were determined with a densitometer (Shimadzu, CS-9000).

Determination of protease activities with synthetic substrates

Assays for proteolytic activities using synthetic substrates were essentially carried out as described by Walsh and Wilcox (1970). In brief, *N*-benzoyl-L-tyrosine for chymotrypsin or *p*-toluenesulphonyl-L-arginine methyl ester for trypsin were incubated at 1.0 mM with a protease in 10 mM Tris/HCl buffer, pH 8.0, at 37 °C in the absence or presence of 7.5, 15, or 30 mM haemin. Time-dependent increases in absorption at 247 nm for trypsin and 256 nm for chymotrypsin were recorded for 5 min, and activities were calculated from the slope.

Purification of 45 kDa fragment

BSA was digested at 0.5 mg/ml with 8.3 μg/ml of chymotrypsin at 37 °C for 30 min in 50 mM Tris/HCl buffer, pH 8.0, in the presence of 30 μM haemin (ligand to protein molar ratio of 4:1). Proteolysis was terminated by the addition of 0.01 vol. of 50 mM phenylmethanesulphonyl fluoride, and the pH of the mixture was adjusted to 3.0 by the addition of 0.1 M HCl. Haemin was removed from the mixture by treatment with 1 vol. of water-saturated n-butanol, then the pH of the mixture was raised up to 8.0 with 0.1 M Tris base. The chymotrypsin digests were loaded on to a DEAE-cellulose column (1.0 cm × 7.0 cm) equilibrated with 10 mM Tris/HCl buffer, pH 8.0, and eluted with an 80 ml linear gradient (10–500 mM) of the same buffer. The temperature was kept at 4 °C during the isolation procedure. Eluted protein fragments were analysed by SDS/PAGE in the same way as described above. Peak fractions containing a 45 kDa fragment were collected, dialysed against distilled water, concentrated with a Centricon-10 ultrafilter (Amicon), and used for further analyses. The absorption coefficient of the 45 kDa fragment was determined as described by Gill and von Hippel (1989).

N- and C-terminal sequence analyses

The N-terminal amino acid sequence of the 45 kDa fragment was determined with a protein sequenator (Applied Biosystems model 477A/120A). The C-terminal amino acid sequence was determined by sequential digestion with carboxypeptidase Y. The 45 kDa fragment, or intact BSA as a control, was denatured by incubation in 20 mM *N*-ethylenemorphyrine buffer, pH 7.0, containing 8 M urea at 30 °C for 10 min. The sample was diluted with 1 vol. of the same buffer and incubated at 30 °C for various times with carboxypeptidase Y at a protein to enzyme molar ratio of 50:1. Aliquots of the digestion sample were withdrawn and mixed with 0.1 vol. of 6 M HCl, and then diluted with 2.47 vol. of 0.2 M sodium citrate buffer, pH 2.2. Released amino acids were quantified with an amino acid analyser (Hitachi amino acid analyser, Type-835).

Measurement of c.d. spectra

The far-u.v. c.d. spectra of intact BSA and the 45 kDa fragment were measured at 0.1 mg/ml, using a cuvette of 0.1-cm light path, in 50 mM Tris/HCl buffer, pH 8.0, at room temperature with a spectropolarimeter (Jasco J-720). The data were expressed as mean residue ellipticity (degree·cm^2·dmol^{-1}), using the mean residue weights of 113.9 for the intact molecule, and 113.3 for the 45 kDa fragment.

Spectrophotometric titration with haemin

The solution containing 6.6 mM of intact BSA or of the 45 kDa fragment was titrated with haemin in 50 mM Tris/HCl buffer, pH 8.0. The reference mixture containing the same buffer was titrated in the same way, and differences in absorbance at 410 nm were recorded at 37 °C with a u.v.–visible recording spectrophotometer (Shimadzu, UV-260) as described by Beaven et al. (1974).

RESULTS

Effects of ligands on proteolytic digestion rates

BSA was digested at 0.5 mg/ml with trypsin or chymotrypsin in the presence or absence of a ligand, and the time course of digestion was monitored by SDS/PAGE. A previous report demonstrates that the K_m value (Michaelis constant) of the enzyme is 11.0 mg/ml for serum albumin (Markus et al., 1967), indicating that the proteolysis should proceed via pseudo-first-order kinetics at an employed substrate concentration as low as 0.5 mg/ml. Thus the rate for proteolysis was determined from the slope in the semi-logarithmic plots of the amount of intact albumin versus digestion time.

As summarized in Table 1, the rate for proteolytic digestion greatly decreased in the presence of bilirubin or oleic acid at a

Table 1 Effects of ligands on the rates for proteolytic digestion of serum albumin

BSA was digested with chymotrypsin or trypsin for various times in the presence or absence of a ligand and electrophoresed on a polyacrylamide gel as described in the text. The band intensities of intact BSA were determined with a densitometer. Pseudo-first-order rate constants for the proteolytic digestion were determined from the slope of the semi-logarithmic plots of intact BSA versus digestion time. Relative rate constants were calculated as the rate in the absence of a ligand taken as 1.0. The data are the averages for four determinations (± S.E.M.). N.D. represents 'data not determined'.

Ligand	Molar ratio (ligand:albumin)	Relative rate constant	
		Chymotrypsin	Trypsin
Bilirubin	1.0	0.11 ± 0.04	0.34 ± 0.04
Oleic acid	1.0	0.66 ± 0.08	0.68 ± 0.05
Linoleic acid	1.0	0.40 ± 0.03	0.68 ± 0.05
	2.0	0.11 ± 0.01	N.D.
	4.0	0.02 ± 0.01	N.D.
Haemin	1.0	1.88 ± 0.30	2.90 ± 0.50
	2.0	N.D.	6.12 ± 1.18
	4.0	N.D.	12.06 ± 0.42

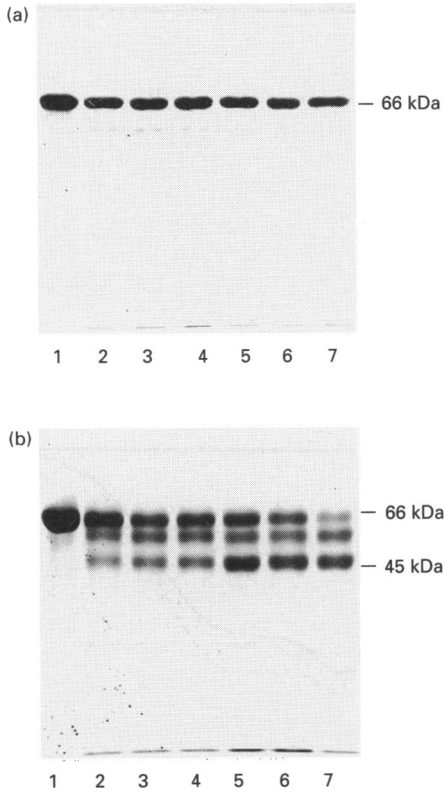

Figure 1 Effects of haemin on the fragmentation pattern of BSA

BSA was digested with chymotrypsin in the absence (**a**) or presence (**b**) of haemin at a ligand to albumin molar ratio of 4:1 for 0 min (lane 1), 5 min (lane 2), 10 min (lane 3), 15 min (lane 4), 30 min (lane 5), 45 min (lane 6), or 60 min (lane 7). The samples corresponding to 2.6 μg of original BSA were analysed by SDS/PAGE as described in the text.

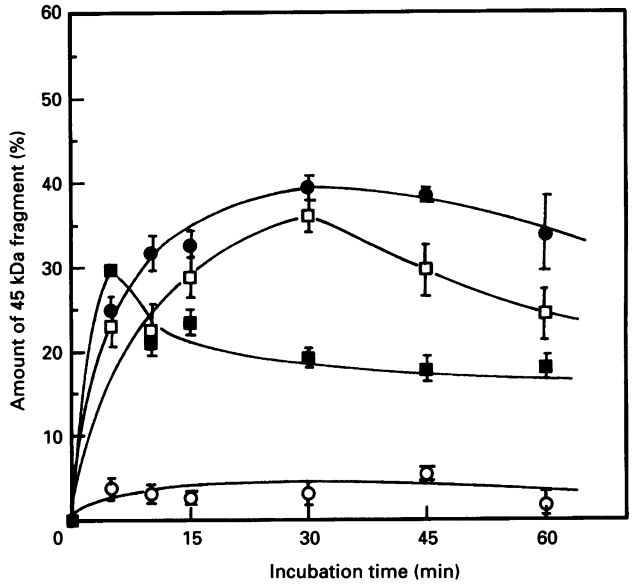

Figure 2 Quantitative analyses for the accumulation of a 45 kDa fragment

BSA was digested with chymotrypsin for various times in the absence (○), or the presence of haemin at a ligand to protein ratio of 1:1 (■), 2:1 (□), or 4:1 (●). The samples, corresponding to 2.6 μg of original BSA, were analysed by SDS/PAGE and the band intensities for the 45 kDa fragment were determined with a densitometer. The ordinate represents the percentage of the band intensity of the 45 kDa fragment compared with the intensity of intact BSA at incubation time zero. Data are the averages of duplicate determinations.

ligand to albumin molar ratio of 1:1, and the extent of chymotrypsin inhibition was increased with increasing molar ratio of linoleic acid. In contrast, haemin increased the rate for the proteolytic digestion with increasing molar ratios of ligand to albumin. We confirmed that the increased digestion should not be accounted for by an activation of protease activity, since the proteolytic activities for the synthetic substrates of *N*-benzoyl-L-tyrosine for chymotrypsin and *p*-toluenesulphonyl-L-arginine methyl ester for trypsin were not affected by the same concentrations of haemin (results not shown). In addition, no BSA proteolysis by haemin was detected in the absence of an added protease (results not shown). Thus we concluded that the increased proteolysis rate by haemin can be accounted for by an alteration in the protease susceptibility of BSA.

Accumulation of the 45 kDa fragment

The preceding proteolysis data were determined as degradation rates of intact albumin. Figure 1, however, demonstrates that a 45 kDa fragment accumulated during chymotrypsin digestion in the presence of haemin. We quantitatively analysed the extent of accumulation of the 45 kDa fragment at various molar ratios of haemin to BSA. Figure 2 shows that the accumulation of the fragment was much greater in the presence of haemin than in the absence of the ligand. At a haemin to BSA molar ratio of 4:1 and 30 min digestion, the amount of fragment was almost 40 % of the original intact form. Essentially the same accumulation of the 45 kDa fragment in the presence of haemin was also observed for trypsin digestion (data not shown). Thus the results in Figure 2, along with those in Table 1, strongly suggest that haemin has at least two types of effects on the protease susceptibility of BSA, thereby facilitating the degradation of the intact form but protecting the 45 kDa fragment that is an early degradation product of the intact form.

Purification of the 45 kDa fragment

To assign the N-terminal amino acid sequence of the 45 kDa fragment, BSA was digested with trypsin or chymotrypsin in the presence of haemin (ligand to albumin molar ratio 4:1), electrophoresed on a polyacrylamide gel and then electroblotted on to a poly(vinylidene difluoride) membrane. The band corresponding to the 45 kDa fragment was excised and subjected to N-terminal sequence analysis. Data showed that the tryptic 45 kDa fragment consisted of two N-terminal amino acid sequences: one is Gln-Arg-Leu-Arg- and the other, Leu-Arg-Xaa-Ala-. In contrast, chymotrypsin generated a single N-terminal sequence: Thr-Ser-Ser-Ala-Arg-. From these results, the N-terminal amino acid of the chymotryptic fragment can be assigned according to the established primary sequence of BSA (Brown, 1975; Reed et al., 1980; Hirayama et al., 1990) as Thr^{190}, and those of the tryptic fragments, as Gln^{195} and Leu^{197}, strongly suggesting that the loop region between the first and second domains was made more susceptible by haemin to either trypsin or chymotrypsin.

To isolate a 45 kDa fragment having a single N-terminal sequence, we employed proteolytic conditions using chymotrypsin. BSA (17 mg) was digested at 0.5 mg/ml with chymotrypsin in the presence of haemin at a ligand to albumin ratio of

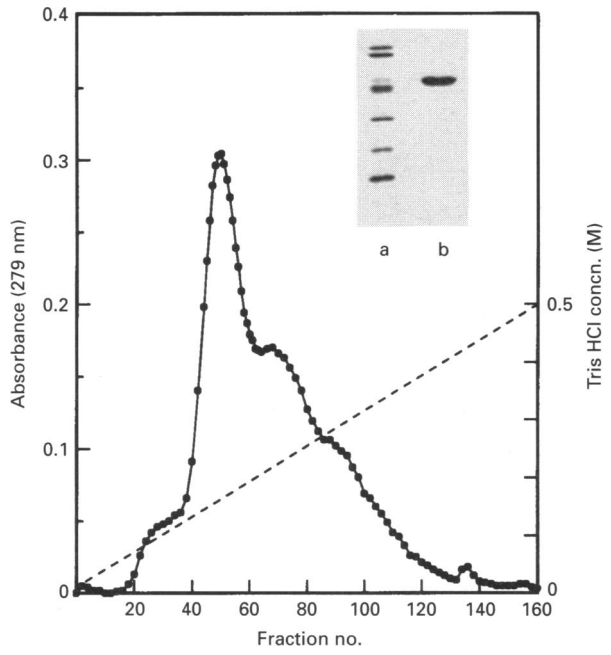

Figure 3 Elution profile of chymotrypsin digests from a DEAE-cellulose column

BSA (17 mg) was digested at 37 °C for 30 min with chymotrypsin in the presence of haemin (ligand to protein molar ratio 4:1). The sample was loaded on to a DEAE-cellulose column and eluted with a linear gradient (———) of Tris/HCl buffer, pH 8.0 (10–500 mM) as described in the text. The absorbance (●) of each fraction (0.5 ml) was determined at 279 nm, and peak fractions (fractions 40 to 58) that corresponded to the 45 kDa fragment were collected, dialysed against distilled water, and concentrated with a Centricon-10 (Amicon) to about 1.7 mg/ml. In the inset, this purified 45 kDa fragment in lane b and a mixture consisting of standard proteins (fructose-6-phosphatase, 85.2 kDa; BSA, 66.2 kDa; aldolase, 39.2 kDa; triosephosphate isomerase, 26.6 kDa; soybean trypsin inhibitor, 21.5 kDa; lysozyme, 14.4 kDa) in lane a were analysed by SDS/PAGE as described in the text.

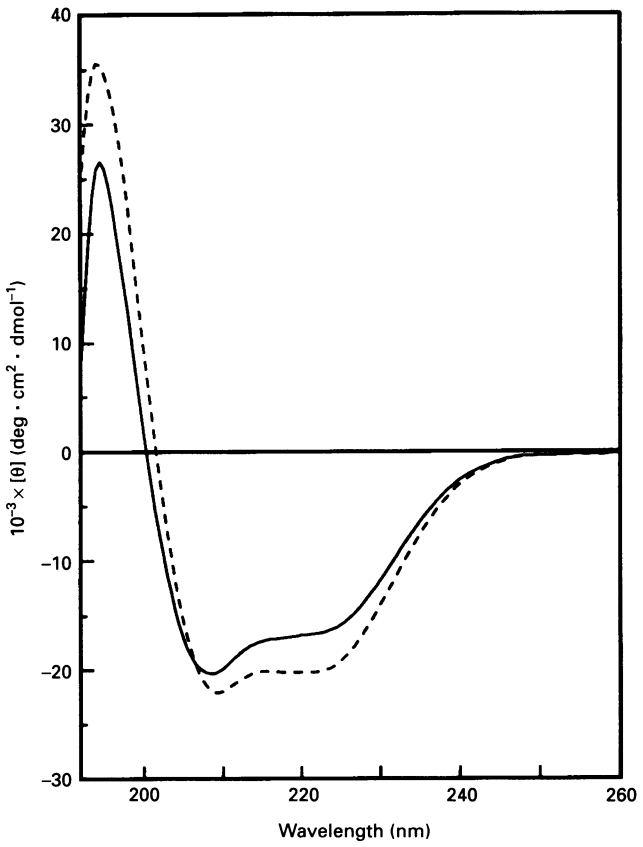

Figure 4 Far-u.v. c.d. spectra

The far-u.v. c.d. spectra of intact BSA (broken line) and the 45 kDa fragment (solid line) were measured as described in the text. The data, the averages of duplicate determinations, were expressed as mean residue ellipticity (degree · cm^2 · dmol^{-1}).

4:1 for 30 min. The digest was applied to an ion-exchange column and eluted as shown in Figure 3. SDS/PAGE analyses for the fractions revealed that the major peak contained the 45 kDa fragment. Peak fractions were collected, dialysed against distilled water, and concentrated. On SDS/PAGE, the sample showed a single band (Figure 3, inset). From 17 mg of original BSA (mercaptalbumin), 3.43 mg of the purified fragment was obtained. Thus the recovery was 29.6% on a molar basis.

The value for $A^{1\%}_{1\,cm}$ at 280 nm of the 45 kDa fragment determined by the method of Gill and von Hippel (1989) was 4.98. The concentration of the 45 kDa fragment was determined spectrophotometrically on the basis of this value.

N- and C-terminal sequence analyses of the isolated fragment

To assign more exactly the primary structure, we determined the N- and C-terminal sequences of the isolated chymotrypsin fragment. The N-terminal amino acid sequence, determined with a protein sequenator, was again Thr-Ser-Ser-Ala-Arg-, indicating that the N-terminal amino acid of the fragment corresponds to Thr^{190} in intact BSA.

We determined the C-terminal amino acid sequence of the 45 kDa chymotrypsin fragment in such a way that the time course for the digestion with carboxypeptidase Y is compared between the fragment and intact BSA. The time course for the amino acid release from intact albumin was consistent with the established C-terminal sequence (-Ser-Thr-Gln-Thr-Ala-Leu-Ala): alanine and leucine were, respectively, the first and second amino acids released. The release of threonine plus glutamine was followed, and then a slow release of serine was observed. This profile was essentially the same as the time course of amino acid release from the 45 kDa fragment. Thus, we concluded that the C-terminal amino acid sequence of the chymotryptic 45 kDa fragment is the same as that of intact BSA.

From the N- and C-terminal sequence data, the 45 kDa fragment was assigned to the sequence of Thr^{190} to Ala^{583}, which corresponds to domains II and III of BSA.

C.d. spectra

To obtain conformational information about the purified 45 kDa fragment, we compared the far-u.v. c.d. spectra of this fragment with that of intact BSA. As shown in Figure 4, the spectrum of intact BSA showed two minima at 209 nm and 222 nm. This profile was essentially the same as previous data for intact BSA (Sun and Maximos, 1989). The c.d. spectrum of the 45 kDa fragment was very similar to that of intact BSA, although the absolute intensity of the fragment was slightly decreased compared with that of intact BSA (Figure 4). Previous X-ray crystallographic analyses have demonstrated that serum albumin comprises three homologous structural domains, I, II and III

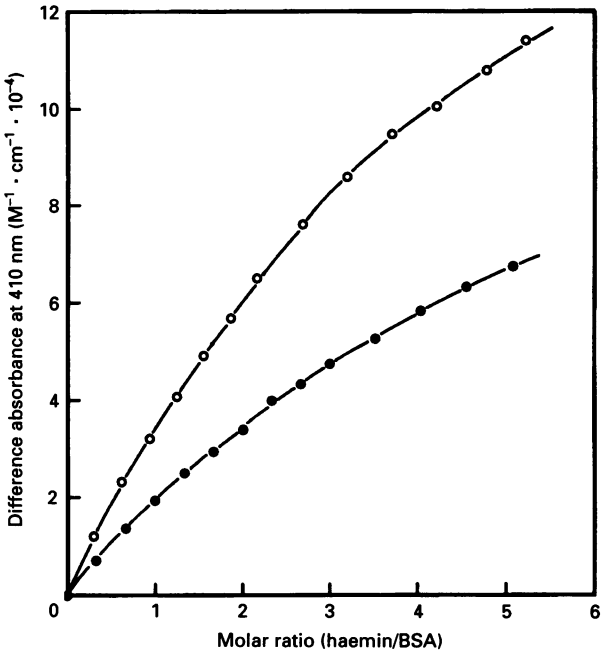

Figure 5 Spectroscopic titration with haemin

Intact BSA (○) or the isolated 45 kDa fragment (●) was titrated with haemin, and difference molar absorption at 410 nm was plotted versus the ligand to protein molar ratios. Data are the averages of duplicate determinations.

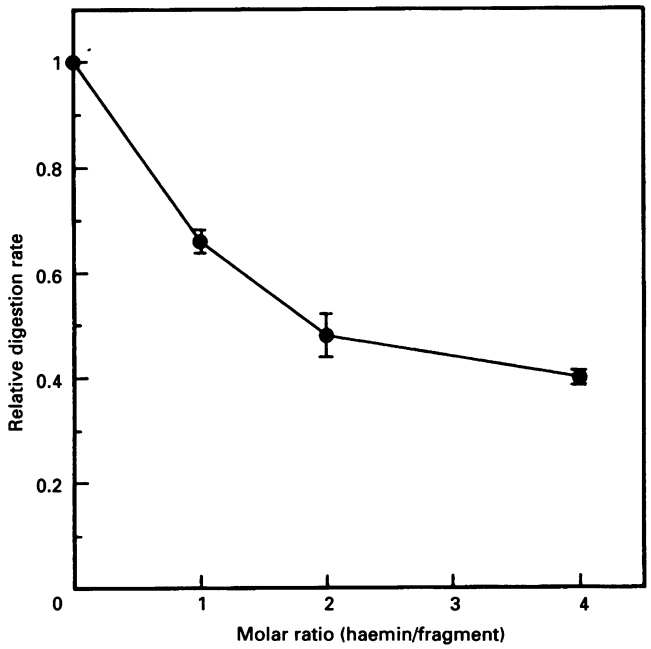

Figure 6 Protection by haemin of the 45 kDa fragment from proteolytic digestion

The isolated 45 kDa fragment was digested at 37 °C for various times with chymotrypsin in the presence of haemin at different molar ratios to albumin, and analysed by SDS/PAGE as described in the text. The band intensities for the 45 kDa fragment were determined with a densitometer and the rates for digestion were calculated by the semi-logarithmic plots of the band intensities versus incubation time as described in Table 1. Relative rates to a control (the rate in the absence of the ligand) are shown in the ordinate. Data are the averages of duplicate determinations.

(He and Carter, 1992). It is therefore very likely that the fragmentation by chymotrypsin induces no drastic alteration in the conformation of domains II and III of BSA.

Spectrophotometric titration with haemin

The difference u.v. spectrum in the Soret region showed a maximum at 410 nm with either the 45 kDa fragment or intact BSA (results not shown). Thus spectroscopic titration with haemin was carried out at 410 nm. As shown in Figure 5, in the titration of intact albumin the difference absorbance gradually increased with increasing molar ratios of ligand to protein, but there was no defined end-point. This titration profile was very similar to the previous one for intact human serum albumin, in which there is one strong binding site and several weaker binding sites for haemin (Beaven et al., 1974). The titration profile of the 45 kDa fragment was similar to that of intact albumin in terms of the absence of any sharp end-point, but the difference absorption intensity was much lower in the fragment than in the intact protein. These results suggested that only weak binding sites are included in the 45 kDa fragment.

Effects of haemin on the digestion rate of the isolated 45 kDa fragment

To investigate the effects of haemin on the proteolytic susceptibility of domains II and III, the isolated 45 kDa fragment was digested with chymotrypsin in the presence of haemin at various molar ratios of ligand to protein. Figure 6 shows that the rate for digestion of the 45 kDa fragment was markedly decreased with increasing haemin to albumin molar ratios, indicating that haemin induces some protease-resistant conformation in the 45 kDa fragment.

DISCUSSION

In an early study, haemin transfer from globin to albumin was found to be species specific, and did not occur with many animal albumins other than primate proteins (Liang, 1957). On the basis of this observation, most studies of haemin bindings have been done with human serum albumin. In the present study, however, we have found that haemin has multiple effects on the proteolytic digestion of BSA: in the presence of this ligand, the rate of degradation of intact BSA with either trypsin or chymotrypsin increased; but a large 45 kDa fragment consisting of domains II and III (Thr^{190}–Ala^{583}) accumulated (Table 1 and Figures 1 and 2). These effects should not be accounted for by changes in enzymic activity but by conformational changes in the substrate molecule due to binding of the ligand, since either trypsin or chymotrypsin activity, when assayed using a synthetic substrate, was not affected by haemin. More directly, the spectroscopic titration data (Figure 5) demonstrate that haemin binding does occur in BSA. Binding of haemin to BSA has also been shown in previous spectroscopic studies by Marden et al. (1989).

The present study provides some information about the mode of haemin binding in intact BSA. Previous studies have shown that human serum albumin has one high-affinity binding site and additional low-affinity sites for haemin (Beaven et al., 1974). The acceleration of the degradation rate of intact albumin can be observed at a haemin to protein molar ratio of 1:1 (Table 1); the N-terminal amino acid of the large fragment generated by proteolysis is Thr^{190} with chymotrypsin, or Gln^{195} and Leu^{197} with trypsin. A previous report has shown that haemin binds to a cyanogen bromide fragment, M (residues 124–298), with a higher affinity than to fragment N (1–123) or C (299–585) of

human serum albumin (Hrkal et al., 1978). These results strongly suggest that the primary high-affinity binding occurs in the proximity of the loop between the first and the second domain, thereby inducing some accessible conformation against a protease. The spectroscopic titration profile indicates the occurrence of haemin binding in the isolated large fragment with a lower affinity than in the intact form (Figure 5); but the haemin binding protects the fragment from proteolysis (Figure 6). Thus it is very likely that haemin binds to secondary weak binding sites in domain(s) II and/or III, thereby protecting the fragment from proteolysis.

This unique fragmentation profile allowed us to isolate the 45 kDa fragment in a high yield by one-step purification. Previous studies have shown that a similar large fragment consisting of domains II and III can be produced by limited trypsinization of bovine (King and Spencer, 1970; Peters and Feldhoff, 1975) and human (Bos et al., 1988) serum albumin in the absence of a ligand. Trypsin, however, generates heterogeneous N-terminal amino acids, such as Leu, Thr, Val and Tyr (Peters and Feldhoff, 1975), as also observed in trypsinization in the presence of haemin in the present study (Gln^{195} and Leu^{197}). In contrast, chymotrypsin digestion in the presence of haemin, as employed in the present study, gives a single N-terminal amino acid and reproducibly high yield (about 30% on a molar basis) of the fragment.

In conclusion, isolation procedures for albumin fragments having the native conformation and function are very important for studies of pH-dependent multiple conformational transitions and of the localization of ligand-binding sites in serum albumin. Although mercaptalbumin was used for the proteolytic fragmentation in the present paper, we have observed that the same chymotryptic 45 kDa fragment can be obtained from non-fractionated fatty acid-free BSA at a high yield (W.-S. Shin and M. Hirose, unpublished work). Partial chymotrypsinization of BSA in the presence of haemin, therefore, appears to be a useful procedure for the isolation of a large fragment consisting of domains II and III.

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