**Cyanogen Bromide Fragments of Human Serum Albumin*\(^1\)**

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**SUMMARY**

CNBr cleaves nonreduced human serum albumin into three large fragments, A, B, and C, which account for the total amino acid composition of albumin. Reduction and carboxamidomethylation of the free —SH groups produce four subfragments from A, identified according to their NH\(_2\)-terminal amino acid residues as A-ProI (32 residues), A-AsxI (38 residues), A-ProII (109 residues), and A-Phe (92 residues). A small residue which contains no homoserine is unaccounted for in this sum of the subfragments in A. A-AsxI contains the COOH-terminal leucine of albumin. Reduction and carboxamidomethylation of fragment B produce two further subfragments, B-Ala (36 residues) and B-AspII (89 residues). These subfractions account for all amino acid residues in B. B-AspII contains the NH\(_2\)-terminal Asp and free —SH group of albumin. Fragment C has only one peptide chain (164 residues) with an NH\(_2\)-terminal Cys residue. Heterogeneity in disulfide linkages is evident in some preparations.

King and Spencer (3) earlier reported success in initial fragmentation of bovine serum albumin by reaction of CNBr with nonreduced albumin and separation of the cleavage products into two major fragments by passage over Sephadex G-100. We have found it advantageous also to initially react CNBr with nonreduced HSA. By use of Sephadex G-100 and carboxymethylcellulose chromatography, three (rather than two) major fragments were resolved from the CNBr-treated, nonreduced HSA preparation. By reduction and blockage of the freed —SH groups in the major fragments and various resin column techniques, seven CNBr peptides of whole albumin have been isolated. Since the isolation procedures must be closely followed for good resolutions, they are described in detail.

**EXPERIMENTAL PROCEDURE**

A number of sources of HSA were investigated for the cleavage studies. Albumin obtained from fresh plasma by modification of the methods of Kendall (4) and Janatova, Fuller, and Hunter (5) was by far the best preparation for the cleavage studies. All steps in these procedures were carried out at room temperature. A crude preparation of albumin was first obtained by removing from 125 ml of plasma the protein soluble in 45% saturated (NH\(_4\))\(_2\)SO\(_4\), pH 6.5, but precipitated by 75% saturated (NH\(_4\))\(_2\)SO\(_4\), pH 4.5. After centrifugation the precipitate was removed, placed on a pad of filter paper for approximately 30 min in order to remove excess moisture, scraped off, and solubilized by addition of a small amount of water and by adjusting the pH to 5.5 (total volume ~40 ml). The solution was passed through Sephadex G-100 (Pharmacia) in 0.05 M NaCl, column size 5 x 220 cm, flow rate 40 to 60 ml per hour. The albumin fraction appeared in the elution volume of 1000 to 1400 ml. It followed a turbid region of high lipid content and was readily visible through the yellow color of protein-bound serum bilirubin. The albumin tubes were pooled; the pH of the solution was raised to 7.5 by addition of 1 N NaOH; 25 mg of iodoacetamide were added; the solution was allowed to stand for 1 hour, deionized through a column (2 x 40 cm) of mixed resins at a flow rate of ~15 ml per hour (6), placed on a column (5 x 7 cm) of DEAE-cellulose (Whatman DE 52), and eluted at a flow rate of 300 to 400 ml per hour with a linear gradient of 0.02 M PO\(_4\)(Na\(^+\)), pH 7.0 (3 liters), against 0.02 M PO\(_4\)(Na\(^+\)), 0.1 M NaCl, pH 7.0 (3 liters). The albumin fraction eluted between 500 and 5000 ml. The cut showing no impurities by rellulose acetate electrophoresis in pH 8.6 barbital buffer (characteristically in the elution position of 700 to 3500 ml) was isolated, adjusted to pH 5.5 by addition of 1 N HCl, freeze-dried, dissolved in the minimum amount of water to obtain solubility, deaeted in 50 ml...
batches at a flow rate ~15 ml per min through a column (4 × 50 cm) of Sephadex G-25 (coarse) in water, freeze-dried, and stored (yield ~3 g). Several lots of Mann chromatographically isolated albumin, an albumin Fraction V preparation from Nutritional Biochemicals, and crystalline mercurialalbumin (Pentex) were also used in initial cleavage studies.

In a few studies the —SII group on albumin was not blocked by reaction with iodoacetamide prior to CNBr treatment. However, no differences in fragment resolutions were recognized in these untreated preparations.

CNBr was purified by sublimation.

Formic acid, technical grade (97%+), was purified by fractional crystallization according to the general technique described by Whitaker (7). In this technique, a 3-kg bottle of formic acid was slowly magnetically stirred at -20°. After 16 to 24 hours the bottle was removed to a 4° environment and inverted, and the unfrozen solution was allowed to drain for several hours. The solid formic acid was thawed and the entire freezing, thawing, and draining steps were repeated two more times. The melted solution was then placed at 6-10° without stirring. The material which did not solidify in several days was further drained (avoiding supercooling). A 50% yield of solid formic acid with a sharp melting point was obtained.

GuHCl (7 M) was prepared from twice crystallized guanidinium carbonate (250 g of the latter material were crystallized from 500 ml of water and 500 ml of methanol). The GuHCl solution was prepared shortly before use by neutralization of the guanidinium carbonate in the approximate proportions of 9 ml of concentrated HCl to 10 g of guanidinium carbonate.

The concentrated urea solutions (~8 M) were prepared by passage of a 10 M urea solution over a deionizing column (6). The urea solutions were stored frozen at -20° until ready for use.

The buffer solutions for gradients at pH 2.0 and 2.7 were prepared from 1.0 M phosphate stock solutions. Buffers solutions at pH 9.5 and 10.0 were prepared from 0.5 M borate and phosphate stock solutions, respectively. The cation was always Na+ and the pH listed was that of the concentrated stock solution.

CNBr Reaction—One gram of albumin isolated from fresh plasma was dissolved in 4 ml of H2O and 16 ml of formic acid were added. One gram of CNBr was added and the reaction was allowed to proceed for 20 to 24 hours at 4°. All further experiments were conducted at room temperature. The solution was passed through a column (4 × 40 cm) of Sephadex G-25 (coarse) in 1% propionic acid, the protein was collected, and the concentrated urea solution was stored frozen at -20° until ready for use.

The monomer fraction of CNBr, nonreduced HSA fragments (1 g of albumin) from a column (5 × 220 cm) of Sephadex G-100 with 5% propionic acid, 0.1 M NaCl, 25°, flow rate 40 to 60 ml per hour.

The aggregate fraction is at the position of the exclusion volume of the column. The monomer fraction has the approximate amino acid composition of whole albumin. The components in the I and II fractions were further resolved by carboxymethyl cellulose chromatography (see Figs. 2 and 3).

Reduction and Separation of Fragments—After the freeze-drying, the A concentrate residue was dissolved in ~5 ml of 7 M GuHCl and transferred to a reaction container, the gas phase was changed to N2, 200 mg of dithiothreitol dissolved in 2 ml of 7 M GuHCl were added, the pH was adjusted to 10.0 by addition of 6 N NaOH, and the reaction was held under N2 at room temperature for 3 hours. The pH was adjusted to 8.5 by addition of 6 N HCl and 0.8 g of iodoacetamide dissolved in 3 ml of 7 M GuHCl solution was added. The reaction chamber was covered with aluminum foil and the mixture was held at pH 8.5 under N2 for 1 ½ hours. The pH was reduced to 2 to 2.5 by addition of 1 N HCl and the solution was immediately passed through a Sephadex G-25 column (2 × 90 cm) in 0.01 M PO4, pH

Fig. 1. Elution of CNBr, nonreduced HSA fragments (1 g of albumin) from a column (5 × 220 cm) of Sephadex G-100 with 5% propionic acid, 0.1 M NaCl, 25°, flow rate 40 to 60 ml per hour. The aggregate fraction is at the position of the exclusion volume of the column. The monomer fraction has the approximate amino acid composition of whole albumin. The components in the I and II fractions were further resolved by carboxymethyl cellulose chromatography (see Figs. 2 and 3).

Fig. 2. Fractionation of zone I (from Fig. 1) on a column (4 × 5 cm) of carboxymethyl cellulose, 0.075 to 0.3 m NaCl gradient in 0.01 m PO4, pH 2.7, 25°, flow rate 300 to 400 ml per hour.

Fig. 3. Fractionation of zone II (from Fig. 1) on a column (4 × 5 cm) of carboxymethyl cellulose, 0.05 to 0.5 m NaCl gradient in 0.01 m PO4, pH 2.7, 25°, flow rate 300 to 400 ml per hour. The starting gradient contained 0.05 M NaCl in lieu of 0.075 M NaCl. The concentration of NaCl was increased in the approximate proportions of 9 ml of concentrated HCl to 10 g of guanidinium carbonate.
A-ProII, and A-Phe zones from this elution were adjusted to pH 4 to 4.5 by addition of 1 M NaCl (1 liter). pH 2.7, 25°C, column flow rate 300 to 450 ml per hour. The peptide fraction was collected; the pH was raised to 3.1 by addition of 1 M NaOH, and the solution was desalted at a flow rate of 10 ml per min. The peptide fraction was collected, diluted 1:1 with water, placed on a DEAE-cellulose column (Whatman DE-52), and eluted according to the conditions described in Fig. 5. The B-Ala fraction from the column was adjusted to pH 4.5 by addition of 1 M NaOH, freeze-dried, dissolved in 8 M urea, and desalted in the same manner as the fragments from the A reduction. The B-AspII fraction was adjusted to pH 4.5 by addition of 1 M NaOH and freeze-dried, 15 ml of water were added to the residue, the pH was raised to 8 by addition of 1 M NaOH to obtain a clear solution, and the solution was deacetylated at a flow rate of 10 ml per min over a column (2 x 90 cm) of Sephadex G-25 (coarse) in water and the freeze-dried. Alternately, the reduced B fragment was fractionated under conditions identical with those described except that a gradient at pH 10.0, consisting of 0.005 M NaCl against 0.02 M NaCl, 0.2 M NaCl was used.

Fragment C was reduced and passed through Sephadex G-25 at pH 2.0 in the same manner as A. The reduced C fragment was then placed on a carboxymethylcellulose column and eluted according to the conditions given in Fig. 6. The C-Cys fraction obtained from this elution was adjusted to pH 4 to 4.5 by addition of 1 M NaOH, freeze-dried, dissolved in 8 M urea, and desalted over Sephadex G-25 in the same manner as the fragments from A reduction.

**NH₂-terminal Groups**—They were determined by reaction with 1-dimethylaminonaphthalene-5-sulfonylchloride (dansylation). To be as certain as possible that no groups were missed by improper dansylation conditions, dansylation of all fragments was carried out by two methods; one sample in the presence and the other in the absence of urea solution, as described in the procedures of Gros and Labrousse (8). The small fragments tended to be too soluble in urea solutions and did not precipitate on addition of trichloroacetic acid, resulting in their loss. On the other hand, the large fragments tended not to react well in the absence of urea. Reacting the same fragment under both conditions reduced chances of missing the end groups. The identification of the dansylated amino acids was performed by high voltage electrophoresis as described by Gray (9). Standards and samples were run at three concentrations; 1, 2, and 4 nmoles. All questionable zones on the electrophoresis papers were cut out and their identity was made positive by re-electrophoresis in a second buffer. The possible presence of an end group unreactive with dansyl-chloride (e.g. the pyrrolidonecarboxyl group) could not be ruled out, although no fragments were isolated without identifiable end groups.

**Amino Acid Analyses**—Amino acid analyses were conducted with a Beckman 120B or 120C analyzer. For hydrolyzed samples the recovery of serine was taken as 0.95 and threonine as 0.98. Homoserine lactone values were added to the homoserine values. A value of 0.65 leucine eq was taken for the lactone peak (10). The lactone value was always small in
RESULTS AND DISCUSSION

Under the conditions described, the cleavage of methionine by CNBr seemed by all evidence to be complete. No methionine, methionine sulfoxide, or methionine sulfone was found in amino acid analysis of unfraccionated CNBr-treated albumin, or, for that matter, in the analysis of any CNBr fragments.

In developing systems for separation of the fragments, the initial screening for fragment purity was conducted by determining electrophoresis of the dapsylated products as described, there was usually no difficulty in visualizing zones at 1-nmole application. Applications of the dansylated products as described, there was usually no difficulty in visualizing zones at 1-nmole application. Applications

| Amino acid | Analyses | B | B at integer | Analyses | Best integer | Analyses | Best integer | Total (A, B, C) | HSA | HSA
|-----------|----------|---|-------------|----------|--------------|----------|--------------|----------------|-----|-----
| Lys.      | 32.6     | 31.4 | 32            | 9.1      | 9.3           | 9        | 16.4         | 15.3           | 16  | 57  | 53.0 | 54.0 | 54 |
| His.      | 5.8      | 5.9  | 6             | 4.9      | 5.0           | 5        | 4.0          | 4.8            | 4   | 15  | 14.8 | 14.1 | 14 |
| Arg.      | 9.5      | 9.9  | 10            | 4.8      | 4.8           | 5        | 8.1          | 8.5            | 8   | 23  | 21.1 | 23.3 | 21 |
| Asp.      | 25.4     | 26.6 | 26            | 16.2     | 14.7          | 15       | 12.3         | 13.2           | 13  | 54  | 53.0 | 53.0 | 53 |
| Thr.      | 15.8     | 15.0 | 15            | 6.0      | 6.1           | 6        | 4.7          | 5.0            | 5   | 26  | 27.1 | 26.4 | 27 |
| Ser.      | 12.1     | 13.1 | 13            | 3.6      | 3.1           | 3        | 7.6          | 8.1            | 8   | 24  | 22.4 | 23.5 | 23 |
| Glu.      | 44.0     | 41.0 | 42            | 21.2     | 19.6          | 20       | 22.6         | 25.0           | 24  | 86  | 83.7 | 82.0 | 81 |
| Pro.      | 10.6     | 12.1 | 11            | 4.9      | 5.4           | 5        | 4.5          | 4.8            | 5   | 21  | 21.4 | 21.6 | 22 |
| Gly.      | 6.0      | 6.0  | 6             | 3.0      | 3.0           | 3        | 3.0          | 3.0            | 3   | 12  | 11.4 | 12.5 | 12 |
| Ala.      | 31.5     | 29.0 | 31            | 9.8      | 11.0          | 11       | 19.5         | 23.2           | 21  | 63  | 62.0 | 60.0 | 61 |
| Val.      | 25.7     | 25.2 | 25            | 9.6      | 10.2          | 10       | 4.9          | 5.6            | 5   | 40  | 37.2 | 39.1 | 38 |
| Cys*      | 15.5     | 14.7 | 15            | 7.2      | 7.9           | 8        | 9.8          | 11.1           | 10  | 33  | 36.1 | 34.3 | 35 |
| Met.      | 0        | 0    | 0             | 0        | 0             | 0        | 0            | 0              | 0   | 0   | 5.3  | 6.2  | 6  |
| Ile.      | 2.5      | 2.9  | 3             | 0.9      | 1.0           | 1        | 3.6          | 3.9            | 4   | 8   | 8.4  | 8.9  | 9  |
| Leu.      | 33.2     | 31.5 | 32            | 12.2     | 12.4          | 12       | 18.3         | 16.3           | 17  | 61  | 60.4 | 61.0 | 61 |
| Tyr.      | 8.2      | 8.1  | 8             | 1.9      | 2.2           | 2        | 4.7          | 5.5            | 5   | 15  | 16.3 | 16.4 | 16 |
| Phe.      | 15.2     | 14.3 | 15            | 6.2      | 6.8           | 6        | 8.8          | 10.0           | 9   | 30  | 29.4 | 30.4 | 30 |
| His*      | 2.6      | 2.2  | 3             | 1.4      | 1.8           | 2        | 0.8          | 0.8            | 1   | 6   | 0    | 0    | 0  |
| Trp.      | 0        | 0    | 0             | 0        | 0             | 0        | 0.8          | 0              | 1   | 1   | 1    | 1    | 1  |
| Total     | 293      | 123  |               | 159      | 575           |          | 570          | 576            |     |     |      |      |    |

*Best composite integers, Schultz, Heimburger, and Frank (13), Spahr and Edsall (14), and Theodore Peters, Jr. (the later a private communication).

†Includes cysteic acid when present.

‡Homoserine.
in A: the A-ProI, A-AsxI, A-ProII, and A-Phe fragments. The 
sum of the amino acid residues in the four subfragments is some-
what less than that obtained from the analysis of A. Within 
the variability of the analyses, amino acids not totally accounted 
for are Arg, Asp, Glu, Gly, Ala, and Leu. This shortage suggests 
the loss of a small fragment after the reduction step. The lost 
fragment would contain no homoserine since the latter has been 
accounted for in the other fragments isolated. The matter is 
being further explored by the use of other techniques.

Traces of glycine (up to 0.4 residue) were sometimes present 
in the A-ProII fragment. A second reaction of this fragment with 
either CNBr or performic acid, followed by rechromatogra-
phy on carboxymethylcellulose, did not change the level of 
glycine (or other amino acids) in the fragment recovered. We 
suspect that this trace level of glycine is related to the missing 
amino acid residues in A, described above, one of which is glycine.

The A-ProII fragment in polyamide 6DS electrophoresis some-
times showed a weak but easily distinguishable band trailing 
the major band. There were indications that the presence of 
this second band depended on the presence of glycine in the 
A-ProII analysis.

Table III gives the amino acid analyses of fragments B-Ala 
and B-AspII, which were obtained on reduction of Fraction B. 
The sum of the amino acids in the two subfragments agrees 
well with the amino acid analysis of the whole B fragment. Also 
compared in this table are the amino acid analyses of C-Cys and 
the unreduced C fragment. The good agreement between these 
latter two amino acid analyses is evidence that reduction has 
ot further cleaved the C fragment.

With albumins from fresh plasma, Fragments A, B, and C 
were obtained in ~75% yield. The yields of the smaller frag-
ments ranged for 20% to 50%.

After coverage of the free —SH group in fresh plasma albumin 
with a 14C-carboxamidomethyl group, the amount of radioactivity 
in the separate fragments showed that the —SH group is totally 
in the B-AspII fragment. The B-AspII fragment is also from 
the NH2-terminal end of whole albumin. The solution contain-
ing this fragment turns blue on addition of Cu++, a reaction 
which, as has been shown by Shearer et al. (17), occurs only 
in the presence of the NH2-terminal end of albumin. The other 
fragment with NH2-terminal A-AsxI contains no COOH-terminal 
homoserine and is clearly identified as a fragment at the COOH-
terminal end of albumin.

The B fraction elution profile from the carboxymethylcellulose 
column sometimes showed distortions suggesting the presence 
of more than one component. We suspect that this was due 
to the attachment of different radicals to the —SH group in this 
fragment: carboxamidomethyl (which we add), cysteine, gluta-
mine, or possible other adducts (18) might well be present 
in mixtures at this site. Partial formation of a disulfide with 
cysteine was confirmed by the elution of free dansyl-cysteine 
after coverage of the free —SH group in fresh plasma albumin.
Hydrolysis was conducted in 5.7 N redistilled HCl in sealed evacuated tubes for 22 hours at 110°.

| Amino acid | B-Ala fragment | B-AsxI fragment | Cys (Cm) fragment | Cys | C* |
|------------|----------------|------------------|------------------|-----|----|
| Lys        | 1.9 ± 0.03b    | 6.8 ± 0.04b      | 16.7             | 17  | 16 |
| His        | 0.9 ± 0.03     | 3.7 ± 0.07       | 4.4              | 4   | 4  |
| Arg        | 2.8 ± 0.07     | 2.1 ± 0.11       | 8.3              | 8   | 8  |
| Cys (Cm)   | 2.6 ± 0.13     | 3.8 ± 0.18       | 11.0             | 11  | 0  |
| Asp        | 6.9 ± 0.10     | 9.2 ± 0.14       | 13.2             | 13  | 13 |
| Thr        | 0              | 3.4 ± 0.23       | 5.0              | 5   | 5  |
| Ser        | 0              | 3.0 ± 0.09       | 7.4              | 7   | 8  |
| Glu        | 6.1 ± 0.10     | 13.1 ± 0.33      | 22.6             | 23  | 23 |
| Pro        | 4.0 ± 0.09     | 1.5 ± 0.10       | 4.8              | 5   | 5  |
| Gly        | 0              | 3.0 ± 0.06       | 3.0              | 3   | 3  |
| Ala        | 2.0 ± 0.03     | 8.8 ± 0.14       | 23.2             | 23  | 21 |
| Val        | 3.0 ± 0.02     | 6.0 ± 0.08       | 4.7              | 5   | 5  |
| CysSH      | 0              | 0                | 0                | 0   | 0  |
| Met        | 0              | 0                | 0                | 0   | 0  |
| Ile        | 0              | 0.9 ± 0.04       | 3.6              | 4   | 4  |
| Leu        | 3.0 ± 0.04     | 9.2 ± 0.17       | 18.0             | 18  | 17 |
| Tyr        | 0              | 1.8 ± 0.00       | 5.4              | 5   | 5  |
| Phe        | 1.0 ± 0.01     | 5.6 ± 0.10       | 10.1             | 10  | 9  |
| HSer*      | 0.8 ± 0.04     | 0.7 ± 0.01       | 0.8              | 1   | 1  |
| Try        | 0              | 0                | 0.6              | 1   | 1  |
| Total*     | 30             | 89               | 122              | 164 | 159 |

* Table I values.
* Means of five analyses on five different preparations with standard errors.
* S-Carboxymethylcysteine.
* Homoserine.
* Total of best integer values.

The most troublesome separations were those required for the purification of Fragments A-Pro11 and A-Phe. When evaluated by SDE polyamide electrophoresis these fragments were obtained pure when albumin isolated from fresh plasma was used for the cleavage studies, and then only by following the precise steps indicated. It was found to be very important that the pH of A fragment solution be adjusted to 4.0 to 4.5 before the exposure of the dried A residue to air for any length of time. For this reason the A fragment was reduced immediately after it was taken off the lyophillizer.

The commercial albumin preparations after CNBr reaction gave elution profiles which were frequently different from those of fresh plasma albumin. Thus, after CNBr cleavage of the Nutritional Biochemicals preparation, a prominent peak was consistently found between Peaks I and II (Fig. 1). Upon purification of the fragment in this peak, it was found by end group and amino acid analyses to have the composition of Fragment B. The position of elution of this peak suggested that it was dimerized B fragment, presumably with the attachment via the —SH groups. With most commercial albumins a small peak was found between the A-AsxI and A-Pro11 zones in the elution profile of the reduced A fragment. This zone was identified as the B-Ala fragment by amino acid analysis and end group analysis. In albumin from fresh plasma this zone was found only in fraction B. With the CNBr-treated crystalline mercaptalbumin preparation, traces of A-Pro11 and A-AsxI fragments were found in the elution of the reduced C fragment from the carboxymethylcellulose column. Again, this mixing of small fragments among the major fragments was never found in the albumin preparations isolated from fresh plasma. The implication here is that analoguous disulfide interchange has occurred in albumin preparations from various commercial sources. Disulfide interchange has been implicated as the primary cause of at least a part of the microheterogeneous behavior of albumin (19). Little is gained by preliminary purification of the commercial albumins. Almost all of the aggregates in such preparations appear as an aggregate in the elution of the CNBr fragments from Sephadex G-100. In preparations in which the aggregate content was high, however, the B fragment was reduced in yield more so than the C fragment. This is consistent with the B fragment, which contains a free—SH group, being more involved in aggregate formation than the C fragment.

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