Preparation and characterization of fragments from the N-terminal end of bovine serum albumin under native conditions

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The domain I of BSA, containing residues 1–183 of the protein sequence, was isolated by CNBr treatment. It was further reductively cleaved into two subfragments, N₁ and N₂, in 8 M urea; the subfragments were regenerated in GSH and GSSG. The fragment N and subfragments N₁ and N₂ were found to be homogeneous with respect to size and charge. Results for amino acid composition, N-terminal amino acid sequence, thiol groups and *M_r* suggested that the fragments N₁ and N₂ contain residues

88–183 and 1–87 of the intact BSA respectively. Optical studies, intrinsic-viscosity measurements, gel-filtration data and derived hydrodynamic parameters, taken together with the results on proteolytic digestion, showed that fragment N, as well as its subfragments N₁ and N₂, exist in compact and globular conformation and that the conformation of N₂ fragment is more compact than that of the N₁ fragment.

INTRODUCTION

On the basis of internal sequence similarity and the unique position of 17 disulphide bridges in the BSA sequence, its polypeptide chain is organized into a series of nine loops which are further grouped into three structural domains: I, II and III (Brown, 1977; Peters, 1985). BSA and human serum albumin (HSA) show 80% sequence similarity (Peters, 1985) and are expected to have similar structural organization. Recently the crystal structure of HSA has been published (He and Carter, 1992) and the results showed that each domain of the heart-shaped molecule contains ten helices and it is further subdivided into two subdomains, A and B. The principal binding sites for bilirubin and palmitate are located in domains II and III respectively; domain I offers sites for binding of thiol compounds, mercurials and metal ions (Peters, 1985; Pedersen, 1987; He and Carter, 1992).

Studies on specific fragmentation of BSA were carried out in the last two decades primarily to locate the binding site of specific ligands and to gain an understanding of the structural organization of the fragments/domains and eventually of the native protein itself. One of the critical requirements in such studies is that the fragments should be obtained in the native form. One such BSA fragment, containing residues 1–183 and thus constituting domain I of the protein, can be isolated by specific cleavage of the protein by CNBr (King and Spencer, 1970; Benjamin and Weigle, 1971; Goossens et al., 1973). This fragment would contain a nicked peptide bond (Met⁸⁷–Ala⁸⁸) and, therefore, it can be further fragmented into two subfragments, one containing residues 1–87 (fragment N₂) and other residues 88–183 (fragment N₁) (King and Spencer, 1970). However, the two subfragments could be obtained only in denatured and reduced forms and therefore their properties could not be compared with those of native domain I (BSA). We have, therefore, attempted first to isolate these subfragments in native form and subsequently to investigate their physicochemical properties as well as structural flexibility under native conditions.

MATERIALS AND METHODS

Materials

Proteins and enzymes used in these studies were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). All types of Sephadex, and Blue Dextran 2000, were obtained from Pharmacia Fine Chemicals (Uppsala, Sweden). CNBr was the product of Sisco Research Laboratories, Bombay, India. Other chemicals were of analytical grade.

Gel filtration

Analytical gel chromatography of proteins on a Sephadex G-200 column (61 cm × 2.38 cm) was performed as described previously (Ansari and Salahuddin, 1973). The column was equilibrated with 60 mM sodium phosphate buffer, pH 7.0, at 25 ± 0.5 °C. The marker proteins used were cytochrome *c*, ribonuclease A, myoglobin, trypsinogen, chymotrypsinogen A, ovalbumin and BSA (Saeed, 1986).

Electrophoresis, viscosity measurements and other analytical procedures

SDS/PAGE was performed as described previously (Ip and Saeed, 1990). Viscosity measurements of the protein solutions were made as described by Khan and Salahuddin (1984). The protein concentration of BSA fragments was determined by the method of Lowry et al. (1951), with BSA as standard (Saeed and Ip, 1989a). The N-terminal amino acid was identified by the method of Gray (1967). The thiol content of the protein was estimated by the method of Ellman (1959). The amino acid composition of the protein was determined (Saeed, 1986) on an LKB Biochrom automatic amino acid analyser (model 4101) by using the procedure recommended by the manufacturer. Antibodies to the N-fragment of BSA were raised as described by Saeed and Ip (1989b).

Abbreviation used: HSA, human serum albumin.

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Proteolytic digestion

Proteolytic digestion of BSA and its fragments was carried out at 30 °C in 0.1 M sodium phosphate buffer, pH 8.0, as described by Ansari et al. (1975).

RESULTS

Isolation and purification of fragments in native form

BSA monomer was isolated by gel filtration of commercial samples of the protein. The CNBr fragments, N, N₁ and N₂, were prepared as described by King and Spencer (1970) (see Figure 1). The fragments were regenerated by the method of Johanson et al. (1981), using a regenerating mixture comprising 0.1 M Tris/HCl buffer, pH 8.0, containing 0.01 M EDTA, 3×10^{-4} M GSH and

Figure 1 Chromatography of reduced CNBr fragment on Sephadex G-75 column

About 125 mg of the reduced fragment N was applied on a Sephadex G-75 column (2.96 cm × 123 cm) equilibrated with 0.1 M acetic acid. The protein, collected in 2.5 ml and 5.0 ml fractions at a flow rate of 10 ml/h, was assayed by the method of Lowry et al. (1951). A, incompletely cleaved N-fragment; B, reduced N₁ fragment; C, reduced N₂ fragment.

Figure 2 Re-chromatography of the regenerated fragments N₁ and N₂ on a Sephadex G-75 column

The experimental conditions were the same as those described in the legend to Figure 1. The inset shows SDS/PAGE of N₁ and N₂ fragments.

3×10^{-5} M GSSG. The two regenerated fragments, N₁ and N₂, obtained from the fragment N are seen to be homogeneous with respect to size (see Figure 2). N-terminal amino acid residues of N, N₁ and N₂ were identified as aspartic acid, alanine and aspartic acid respectively. The number of thiol groups in regenerated N₁ and N₂ fragments was found to be 1 and 2 respectively.

Physicochemical properties

Hydrodynamic properties of N, N₁ and N₂ fragments of BSA were studied by gel filtration on an analytical Sephadex G-200 column. The values of V_o , V_i , V_e , K_a and their derived functions were computed using standard procedure (Ansari and Salahuddin, 1973). The M_r values of the fragments N, N₁ and N₂ thus computed were 23 500, 12 200 and 10 300 respectively, which are close to those (21 494 for N fragment, 11 580 for N₁ fragment and 9914 for N₂ fragment) deduced from the amino acid sequence (Peters, 1985). The Stokes radii determined for the three fragments N, N₁ and N₂ would be respectively 2.37, 1.66 and 1.49 nm. The values of diffusion coefficient and frictional ratio (in parantheses) were thus computed to be 3.2×10^{-7} (1.19), 13.2×10^{-7} (1.07) and 14.7×10^{-7} cm²/s (1.01) respectively for the fragments N, N₁ and N₂. Thus the fragments appear to have similar hydrodynamic volume. The partial specific volumes (Cohn and Edsall, 1965) of the fragments were calculated from their amino acid composition and were 0.727, 0.722 and 0.710 ml/g respectively for the N, N₁ and N₂ fragments.

Optical properties, such as u.v.-absorption and fluorescence spectra, urea-induced difference spectra and solvent-perturbation difference spectra of N and regenerated fragments N₁ and N₂ were also studied (Saeed, 1986). The spectral properties of the three fragments were found to be consistent with their aromatic-amino-acid contents. Fragments N and N₁ both contain tyrosine and tryptophan, whereas the N₂ fragment was devoid of tryptophan (Saeed, 1986). Accordingly, maxima in fluorescence emission spectra of N and N₁ occurred at 340 nm, whereas the maximum in the fluorescence spectra of N₂ was observed near 310 nm. The degree of exposure of aromatic amino acids (tryptophan and/or tyrosine) as measured by perturbation with 20% ethylene glycol, dimethyl sulphoxide and glycerol was nearly the same in the two regenerated fragments. Further, urea-induced difference spectra indicated extensive disruption of structure in the fragments caused by 8 M urea. A precipitin reaction against anti-(N fragment) antiserum by an immunodiffusion test revealed that N, N₁ and N₂ fragments show complete immunological cross-reactivity under native conditions. Intrinsic-viscosity values obtained under different conditions are shown in Table 1.

Proteolytic digestion

The conformational flexibility of the fragments N, N₁ and N₂ of BSA was studied by determining the rate of their digestion by trypsin and chymotrypsin. The amino group formed due to the proteolytic digestion of the peptide bond in protein was allowed to react with ninhydrin to give rise to a deep blue colour absorbing maximally at 570 nm. The change in A_{570} was taken to be a measure of protein digestion (Ansari et al., 1975). Results are shown in the form of a plot of ΔA_{570} as a function of time. The values of the initial slopes ($d\Delta A_{570}/dt$) of the curves (see Figure 3) were determined to be 2.0×10^{-3} , 2.2×10^{-3} , 0.9×10^{-3} and 0.68×10^{-3} for the tryptic digestion of BSA, N, N₁ and N₂ fragments respectively. Since tryptic digestion will depend upon the number of scissile peptide bonds, the slope must be corrected for the number of such bonds in BSA and its fragments. The

Table 1 Intrinsic viscosities $[\eta]$ of the fragments of BSA

Conditions	$[\eta]$ (ml/g)		
	N fragment	N ₁ fragment	N ₂ fragment
Native state	3.9	3.6	3.2
8 M urea	5.5	5.4	5.2
8 M urea plus 0.1 M 2-mercaptoethanol	10.5	10.2	9.0

Figure 3 Extent of proteolytic digestion of BSA and its fragments

(a) Tryptic and (b) chymotryptic digestion of BSA and its fragments (N, N₁ and N₂) in 0.1 M sodium phosphate buffer, pH 8.0, at 30 °C using trypsin or chymotrypsin at a substrate molar ratio of 1:2 and at an enzyme concentration of 0.5 mg/ml. Aliquots (0.5 ml) were taken from the reaction mixture at different time intervals and mixed with 0.5 ml of 1 M sodium acetate buffer, pH 5.1, pre-cooled in an ice bath to stop the reaction. Freshly prepared ninhydrin reagent (0.5 ml) was added to the tube and mixture was heated in a boiling-water bath for 15 min and cooled after diluting with 50% ethanol; the precipitate was removed by filtration and the A_{570} recorded.

Table 2 Rate constant for the proteolytic digestion of BSA and its fragments (N, N₁ and N₂)

Digestion	Slope (dA_{570}/dt)	No. of scissile peptide bonds	Effective rate constant, K_e (A unit/min)
Tryptic digestion			
(i) BSA	2.0×10^{-3}	72	2.8×10^{-5}
(ii) N fragment	2.2×10^{-3}	21	10.6×10^{-5}
(iii) N ₁ fragment	0.9×10^{-3}	12	7.6×10^{-5}
(iv) N ₂ fragment	0.68×10^{-3}	9	6.6×10^{-5}
Chymotryptic digestion			
(i) BSA	3.1×10^{-3}	92	3.4×10^{-5}
(ii) N fragment	2.4×10^{-3}	38	6.3×10^{-5}
(iii) N ₁ fragment	1.0×10^{-3}	20	5.1×10^{-5}
(iv) N ₂ fragment	0.6×10^{-3}	18	3.9×10^{-5}

slopes were therefore divided by the number of scissile peptide bonds to find out the effective rate constant, K_e . The values for K_e of BSA and its fragments are listed in Table 2. Likewise the values of K_e were determined for chymotryptic digestion of BSA

and its fragments (see Figure 3). The values of K_e thus obtained are listed in Table 2.

DISCUSSION

BSA fragments N, N₁ and N₂ isolated in the present study were essentially homogeneous as shown by our gel-filtration and electrophoretic results. Previously, N₁ and N₂ were obtained in reduced and denatured forms (King and Spencer, 1970; Benjamin and Weigle, 1971). These fragments in regenerated form were prepared in this study for the first time. The contention that the fragments N₁ and N₂ were in the native form finds support from our results on intrinsic viscosities, gel filtration and immunological cross-reactivity. It is instructive to recall that the intrinsic viscosities of native and globular proteins lie in the range of 3.0–4.0 ml/g (Tanford, 1968), and the intrinsic viscosity of the three fragments were found to be in the range 3.2–3.9 ml/g. Further, the frictional ratios of the three fragments were also found to be consistent with a compact and globular conformation. Results on difference spectra and the viscosity of urea-denatured fragments suggested extensive disruption of the structure of fragments in concentrated urea.

The fragment N contains 183 amino acids from the N-terminal end of BSA and therefore it would consist of nearly all the residues of domain I of BSA (residues 1–190) (Peters, 1985). Assuming that BSA and HSA possess a similar conformation, domain I would be further subdivided into two subdomains, A and B, which share a common structural motif (He and Carter, 1992). The fragment N₂ (residues 1–87), comprising the complete loop 1 and part of loop 2 (Peters, 1985), would form most of the subdomain A, whereas the N₁ fragment, comprising residues 88–183, will have subdomain B along with the connecting loop consisting of residues 106–119 (He and Carter, 1992). Since the structural fold of both the domains A and B are the same, one should expect similar overall hydrodynamic volume and, indeed, the two fragments in the native state possess similar intrinsic viscosities (see Table 1) and other hydrodynamic parameters such as diffusion coefficient and frictional ratio. That the regenerated fragments N₂ and N₁ contain sizable elements of structure was indicated by the urea-induced difference spectral results, as well as by the viscosity of the two fragments in 8 M urea (see Table 1). The three fragments, N, N₁ and N₂, were immunologically indistinguishable and had expected optical properties. The fact that the rates of tryptic and chymotryptic digestion were found to be of the same order of magnitude for the two fragments (see Table 2) suggested that the fragments possess similar conformational flexibility under native conditions.

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