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Isolation, characterization and effect of acidic pH on the unfolding-refolding mechanism of serum albumin domains

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Abstract. Three fragments, *viz.,* BSA-CNBr$_{1-183}$, BSA-CNBr$_{184-582}$, and BSA-T$_{377-582}$ representing domains I, II + III and III of bovine serum albumin have been isolated and purified. The physicochemical properties have been investigated and compared with their parent albumin molecule. The values of Stokes radii (nm) and intrinsic viscosities (ml/g) have been determined to be 2·36, 3·30; 3·43, 4·36; and 2·40, 3·13 for the fragments BSA-CNBr$_{1-183}$, BSA-CNBr$_{184-582}$ and BSA-T$_{377-582}$ respectively. The acid induced unfolding-refolding transitions of intact albumin and the fragment BSA-T$_{377-582}$ have been shown to occur in two steps while the fragments BSA-CNBr$_{1-183}$ and BSA-CNBr$_{184-582}$ underwent single step transitions. The formation of the acid denatured states of intact albumin, BSA-CNBr$_{1-183}$ and BSA-CNBr$_{184-582}$ was accompanied by an increase of about 86, 56 and 44% in the values of intrinsic viscosities respectively. Since all the transitions were reversible, the values of equilibrium constants, K_D, were calculated. The analysis of the dependence of K_D on pH indicated that the first transition *(N–X)* of albumin was caused due to the uptake of about 3 protons by the native albumin. ,The intermediate state, *X,* is converted to acid unfolded state, *D,* by taking up another two protons. A comparision of the results on intact albumin with that of its fragments revealed that the second transition of the fragment BSA-T$_{377-582}$ and the two single step transitions of the fragment BSA-CNBr$_{1-183}$ and BSA-CNBr$_{184-582}$ were much closer to the second transition *(X-D)* of the intact albumin. The first transition of albumin has been attributed to its domain III represented by the fragment BSA-T$_{377-582}$.

Keywords. BSA-domains; hydration; tryptic digestion; viscosity; ultraviolet absorption; acid-induced unfolding.

Introduction

The mechanism of acquisition of 3-dimensional structure by proteins has been extensively studied over the past several years (Anfinsen and Scheraga, 1975; Baldwin, 1975, 1989; Creighton, 1978; Privalov, 1982). Among others, the idea that a globular protein might fold in independent 'structural regions' or domains before forming the crucial native structure, has emerged as one of the great acheivements in the field of protein folding in recent years (Wetlaufer, 1973). This has been tested in several laboratories by studying the unfolding-refolding behaviour of small protein fragments (see Wetlaufer, 1981). However, studies with larger proteins with the possibility of having multiple nucleation centres (Wetlaufer, 1973) are still inadequate. Serum albumin is a suitable protein for this purpose because (i) it is a

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Abbreviations: BSA, bovine serum albumin; CNBr, cyanogen bromide; ATEE, N-acetyl-L-tyrosine ethyl ester; ATPA, N-acetyl-L-tryptophanamide; APEE, N-acetyl-L-phenylalanine ethyl ester; PEG-400; polyethylene glycol-400; TPCK, tosyl-L-phenylalanyl-chloromethyl ketone.

large molecule with 582 amino acid residues (Brown, 1977; Reed *et al.,* 1980), (ii) it has a 3-domain structure (Brown, 1975, 1976), (iii) several albumin fragments have been isolated and characterized (Wetlaufer, 1981), and (iv) some fragmentary data on the unfolding-refolding properties of this protein are already available (Wetlaurfer, 1981; Johanson *et al.,* 1986; Khan and Salahuddin, 1984; Khan, 1986; Khan *et al.,* 1987).

Keeping above points in mind, we have isolated and characterized 3 fragments of bovine serum albumin (BSA) corresponding to domains, I, II + III, and III of the intact albumin molecule. Two of the fragments have been prepared by cyanogen bromide (CNBr) cleavage of the albumin while the third fragment has been isolated from a hydrolyzate obtained by controlled tryptic digestion of the protein. The fragments have been named as BSA-$CNBr_{1–183}$, BSA-$CNBr_{184–582}$ and BSA-$T_{377–582}$ where 'CNBr' and 'T' indicate the mode of cleavage *i.e.*, cyanogen bromide and trypsin, and 1–183, 184–582 and 377–582 show the position of the fragments in the primary structure (Brown, 1976) of BSA respectively. The unfolding-refolding behaviour of these 3 fragments and their parent molecule under acidic conditions has been systematically investigated. We have shown that the C-terminal region of the albumin molecule is more susceptible towards acid denaturation and is important in the *N-F* transition of serum albumin (Foster, 1960; Khan, 1986). The characteristics of the acid induced transitions of the 3 albumin fragments have been reported.

Materials and methods

All the proteins including BSA (lot Nos. 10C-8080 and 100F-0249), N-acetyl-L-tyrosine ethyl ester (ATEE), N-acetyl-L-tryptophanamide (ATPA), N-acetyl-L-phenylalanine ethyl ester (APEE), dansyl chloride, dansylated amino acids, DEAE cellulose and tosyl-L-phenylalanyl-chloromethel ketone (TPCK) were obtained from Sigma Chemical Co., USA. BSA was routinely purified by Sephadex G-100 column chromatography before use. Chymotryptic activity associated with commercial trypsin was eliminated by treating the enzyme with TPCK (Carpenter, 1967). The constant boiling HCl was prepared from the concentrated analytical grade acid using the procedure of Foulk and Hollingsworth (1923). Other chemicals were of the best commercial grade available.

Measurement of pH

Since most of the experiments described in this paper required an accurate measurement of pH, an EC digital pH meter (serial 022, pH 5651) in conjunction with EC combination electrode was exclusively used for this purpose. pHs of the solutions were measured twice (before and after the experiments) and the mean of the two readings was taken as the final reading. However, there was no significant variation in the two readings in most of the measurements.

Isolation and purification of the fragments

Commercially procured BSA was routinely subjected to Sephadex G-100 column

chromatography before use. The monomeric fraction (molecular weight 68,000) of BSA thus obtained was allowed to react with L-Cys to block the lone sulfhydryl group of the protein by a method essentially due to King and Spencer (1970). The cystinylated-monomeric BSA was used for subsequent preparation of the albumin fragments. The tryptic fragment, BSA-$T_{377–582}$ representing domain III of the intact BSA molecule was isolated from TPCK-treated tryptic digests of the protein by the method of Habeeb and Atassi (1976). The two CNBr fragments, BSA-$CNBr_{1–183}$ and BSA-$CNBr_{184–582}$ were prepared by following procedures: 50 ml of a 2% (w/v) albumin solution in 60% (v/v) formic acid was taken in a 250 ml conical flask wrapped with black papers to make it opaque. An equal volume of 99% (v/v) formic acid containing 250 mg of CNBr was added into the flask and the content was left for 20 h at 25°C with a gentle stirring throughout the incubation period. The excess reagent was then removed by gel filtration on a Sephadex G-25 column (78·5 × 2·96 cm) equilibrated at pH 2·86 with 0·2 M ammonium formate buffer. The digestion mixture was finally concentrated and fractionated on a Sephadex G-75 column (135 × 2·95 cm) equilibrated with the above buffer. The process of gel filtration was repeated several times until symmetrical peaks giving homogeneous preparations of the respective fragments were obtained.

Protein estimations

Depending on the desired precision and sensitivity, the protein concentrations were determined by the methods of Lowry *et al.* (1951), Bradford (1976) or spectrophotometrically by using the values of $E^{1\%}_{1cm}$ at 278 nm as 6·52, 6·76, 5·1 and 3·29 (at 276 nm) for BSA, BSA-$CNBr_{1–183}$, BSA-$CNBr_{184–582}$ and BSA-$T_{377–582}$ respectively.

Solvent perturbation difference spectroscopy

The degree of exposure of aromatic chromophores was measured by solvent perturbation techniques, essentially as described by Herskovits and Laskowski (1962), and Herskovits (1967). The model mixtures were made by mixing ATEE, ATPA and APEE in appropriate concentrations. Other details were the same as described earlier (Baig and Salahuddin, 1978).

Gel filtration experiments

Some hydrodynamic parameters were calculated from the data obtained by gel filtration on a Sephadex G-200 column (80 × 2·74 cm) equilibrated with 0·06 M sodium phosphate buffer, pH 7, at 25±0·5°C. The elution volumes were determined gravimetrically by weighing the fractions accurately. Elution weight, W_e, thus obtained was converted to elution volume, V_e, by dividing the former with density of the eluent. The available distribution coefficient, K_{av}, (Laurent and Killander, 1964); the distribution coefficient, K_d (Ackers, 1967), the Stokes radius, r; the frictional ratio, f/f_o ;the axial ratio, a/a_o; and the apparent molecular weight, M, were calculated by the method described earlier (Ansari and Salahuddin, 1973).

Determination of intrinsic viscosity

The reduced viscosity, *R,* and the intrinsic viscosity, [η], were calculated with the help of the following equations (Tanford, 1955):

$$\eta_R = (t - t_o)\, t_0 c + (1 - \bar{V}_2\, \rho_o)/\rho_o \tag{1}$$

$$[\eta] = \underset{c \to o}{\text{limit}}\, (t - t_o) \text{ and } /t_o c + (1 - \bar{V}_2 \rho_o)/, \tag{2}$$

where is protein concentration in g/ml, p_o is the density of the solvent in g/ml, and $\bar{V}_2$ is partial specific volume of the protein in ml/g. The time of flow of the solvent t_o, and that of the protein solution, *t,* were recorded at 25°C in a Kimax G-46 (size 25) viscometer as described earlier (Ahmad and Salahuddin, 1974).

The partial specific volumes of BSA and its fragments were calculated by the method of Haschemeyer and Haschemeyer (1973), using the following expression:

$$\bar{V}_2 = \sum_i \bar{V}_i\, w_i / \sum_i w_i, \tag{3}$$

where $\bar{V}_i$ is the partial specific volume of the ith amino acid residue in the protein and w_i is the fractional molecular weight of the *i*th residue. Using the molecular weights of 68,000, 22,000, 44,000 and 22,000; the partial specific volumes were calculated to be 0·734, 0·728, 0·746 and 0·739 ml/g for BSA, BSA-$CNBr_{1–183,}$ BSA-$CNBr_{184–582}$ and BSA-$T_{377–582}$ respectively.

Tryptic digestion

Tryptic digestion of BSA and its 3 fragments was performed at 25°C by a method essentially due to Paik and Kim (1972). 21·8 mg trypsin in 5 ml of 0·1 M sodium phosphate buffer, pH 7·5, was mixed with an equal volume of the same buffer containing 21·8 mg of BSA (or its fragments). After shaking the mixture properly, 1 ml aliquots were pipetted out at different time intervals and the reaction was arrested by adding 1 ml of chilled 1 M sodium acetate buffer, pH 5·1. The extent of tryptic hydrolysis in different aliquots was followed by estimating the colour yield with ninhydrin due to the newly formed amino groups by the method of Moore and Stein (1954).

Measurement of difference spectra

The difference spectra of the proteins were recorded in 0·2 M KCl in the presence and absence of acid by employing 4-cells technique. The solutions were prepared on the basis of weight in 5 ml calibrated volumetric flasks. The two cells in the reference compartment contained protein solution in KCl (pH 6·8) and a solution of KC1-HC1 mixture, while the two cells in the sample compartment contained protein solution in KC1-HC1 mixture (pH 1·6) and a solution of KCl respectively. The protein concentration which was identical in both the compartments, was in the range 1·8–5·7 mg/ml. The difference spectra were recorded in the wavelength region 240–360 nm.

Determination of the equilibrium denaturation curve

Transition studies were performed by taking stock solutions of the protein (by weight) in calibrated flasks (5 ml). To this were added 74·6 mg of KCl crystals and varying amounts of constant boiling HCl (diluted 10 times before use) and the volume was made up to 5 ml with water. The contents were thoroughly mixed and left for about 5 h for equilibration. The solutions were then filtered through millipore filter (pore size 0·5 μm) and subsequent measurements of viscosity or absorbance were made as described above. Reversibility of the transition was tested by taking proteins already exposed to acid pH for 5 h and raising their pHs to desired level by adding known volumes of 0·05 M KOH solution followed by 5 h equilibration prior to viscosity or optical measurements.

Results

Isolation and purification of the fragments

Al the 3 fragments isolated in this study were found to be homogeneous on Sephadex G-100 gel chromatography and on polyacrylamide gel electrophoresis (see figure 1). Relative electrophoretic mobilities, R_m, of the 3 fragments and BSA were calculated in triplicate. The R_m of BSA and its fragments, BSA-$CNBr_{184–582}$ and BSA-$T_{377–582}$ were 80, 63 and 61 % of the mobility of the fragment BSA-$CNBr_{1–183}$ respectively. This clearly indicated that the R_m was essentially independent of the size of the proteins and all the fragments had probably retained the globular conformation even after the separation from their parent molecule. The substantial difference in R_m of BSA and its fragments is, however, explainable in terms of the difference in the number of negative charges on the different domains of BSA at physiological pH (see Peters, 1975).

Spectral properties

Absorption spectra of BSA and its 3 fragments were recorded in 0·06 M sodium phosphate buffer, pH 7, at 25°C. The intact protein was found to absorb maximally at 278 nm. These features were retained in the spectra of the two CNBr fragments of the albumin. However, the spectrum of the fragment, BSA-$T_{377–582}$ was significantly different from BSA in that it had its maximum at 276 nm and trough at 256 nm with some fine structure in between those two wavelengths. This is expected because the tryptic fragment is devoid of Trp and contains 4 Tyr and 9 Phe residues in a total of 206 amino acid residues present in the fragment. Obviously the spectrum is dominated by Tyr with a minor contribution from Phe which produces the fine structure in the 256–276 nm region. For the same reason, the specific extinction coefficient, $E^{1\%}_{1cm}$ of the tryptic fragment (3·29) is significantly lower than BSA (6·52) or its two CNBr fragments, BSA-$CNBr_{1–183}$ (6·76) and BSA-$CNBr_{184–582}$ (5·10), which contain relatively higher numbers of aromatic chromophores (Brown, 1975). That the tryptic fragment is devoid of Trp is also evident from its fluorescence emission maximum (313 nm). The fluorescence emission maxima of BSA and its fragments BSA-$CNBr_{1–183}$ and BSA-$CNBr_{184–582}$ were found to be at 341, 338 and 336 nm respectively.

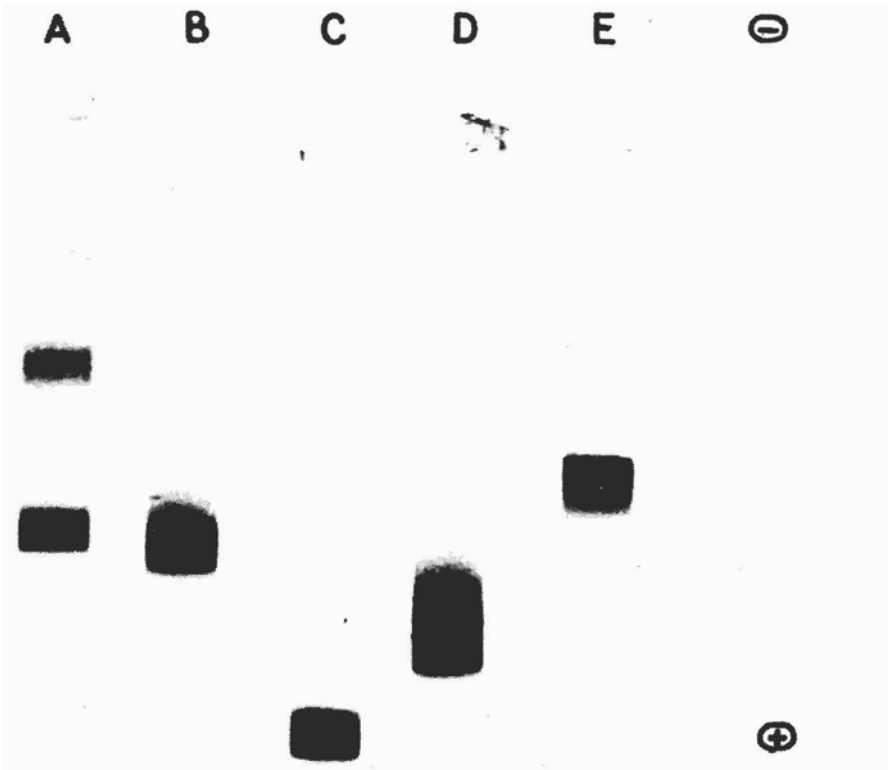

Figure 1. Polyacrylamide gel electrophoresis of **(A)** BSA, **(B)** BSA-monomer, **(C)** BSA-CN^{Br}1-183, **(D)** BSA-$CNBr_{184–582}$, and **(E)** BSA-$T_{377–582}$ in 7% gels. About 200μg of each protein was electrophoresed for about 2 h in Tris-glycine buffer, pH 8·2, ionic strength 0·02, with an anodic current of 4 mA per tube.

Solvent perturbation

Results on solvent perturbation obtained with DMSO and ethylene glycol as the perturbants suggested about 35% exposure of Tyr and about 40% exposure of Trp residues in BSA (see table 1). On the other hand, the degree of exposure of aromatic chromophores in albumin fragments was much higher as compared to the intact BSA molecule (table 1).

Like BSA, the degree of exposure of the aromatic chromophores in the 3 albumin fragments was not significantly changed on their acidification from pH 7 to pH 1·8 (see table 1) for small perturbants, DMSO and ethylene glycol. The polyethylene glycol-400 (PEG-400), which was successfully employed to show an increase in the exposure of chromophores in BSA on its acid denaturation could, however, not be used in similar experiments with the 3 albumin fragments since they were not freely soluble in 20% (v/v) PEG-400 solution.

Higher degree of exposure of aromatic chromophores in albumin fragments as compared to their parent molecule would imply that some of the chromophores are shielded from the solvent due to inter-domains interactions in the intact BSA molecule, and become exposed upon separation of the domains. An alternative explanation would be that the BSA fragments are somewhat less structured, atleast

Table 1. Solvent perturbation difference spectral results on BSA and its fragments[a].

Protein in 20% (v/v) solvent	Exposure (%)			
	Tyrosine		Tryptophan	
	pH 7·0	pH 1·8	pH 7·0	pH 1·8
BSA (DMSO)	36+1	35+1	36+2	41+2
BSA (ethylene glycol)	34+1	39+2	43+2	45+3
BSA-$CNBr_{1-183}$ (DMSO)	54+2	58+1	64+2	59+3
BSA-$CNBr_{1-183}$ (ethylene glycol)	58+3	59+4	54+2	65+2
BSA-$CNBr_{184-582}$ (DMSO)	52+2	61+3	53+2	62+1
BSA-$CNBr_{184-582}$ (ethylene glycol)	57+3	59+4	50+1	57+3
BSA-$T_{377-582}$ (DMSO)	59+2	56+2	—	—
BSA-$T_{377-582}$ (ethylene glycol)	64+3	69+3	—	—

The experiments at pH 7·0 and 1·8 were performed in 0·06 M sodium phosphate buffer and 0·15 M KCl-HCl mixture respectively. The composition of the model mixture was based on 2 Trp, 19 Tyr, 27 Phe; 1 Trp, 8 Tyr, 11 Phe; 1 Trp, 11 Tyr, 16 Phe; and 4 Tyr, 9 Phe residues per mol of BSA, BSA-$CNBr_{1-183}$, BSA-$CNBr_{184-582}$ and BSA-$T_{377-582}$ respectively (Reed *et al.,* 1980).

in the vicinity of the aromatic chromophores, than intact albumin molecule. This is, however, unlikely (except perhaps in the case of fragment BSA-$CNBr_{184-582}$) because fragments BSA-$CNBr_{1-183}$ and BSA-$CNBr_{377-582}$ exist (see below) in a compact and folded conformation. Furthermore, fragment, BSA-$T_{377-582}$ has been shown to have same percentage of the secondary structure as BSA (Reed *et al.,* 1975). As evident from table 1, with DMSO and ethylene glycol as the perturbants, the per cent exposures of Tyr and Trp in BSA are not significantly different from those found for the native proteins. However, with PEG-400 as the perturbant, the degree of exposure of Tyr and Trp increased from 20 and 22% to 39 and 44% respectively.

Thehydrodynamic properties of BSA and its fragments

The gel filtration results on Stokes radii, diffusion coefficients and the frictional ratios of the 3 albumin fragments are given in table 2. The values of these hydrodynamic parameters for the fragments BSA-$CNBr_{1-183}$ and BSA-$T_{377-582}$ suggest that the two fragments exist in a compact and globular conformation. This

Table 2. Hydrodynamic parameters of BSA and its fragments.

Parameters	BSA	BSA-$CNBr_{1-183}$	BSA-$CNBr_{184-582}$	BSA-$T_{377-582}$
Intrinsic viscosity (ml/g)	3·60	3·30	4·36	3·13
Axial ratio[a] (a/a_o)	2·64	2·30	3·44	2·04
Partial specific volume (ml/g)[b]	0·734	0·728	0·736	0·739
Hydration[b] (g water/g protein)	0·415	0·432	0·435	0·388
Stokes radius (nm)[c]	3·50[e]	2·36	3·43	2·40
Frictional ratio[d] (f/f_0)	1.30[f]	1·19	1·30	1·19
Diffusion coefficient ($cm^2/sec \times 10^7$)[d]	5·90[f]	9·32	6·39	9·16

[a]Calculated from viscosity data. [b]Calculated from amino-acid composition. [c]Determined by gel filtration. [d]Calculated from Stokes radii. [e]Taken from Tanford *et al.* (1974). [f]Taken from Peters (1975).

conclusion is strongly supported by the intrinsic viscosity data given in the same table. The values of the intrinsic viscosities of the fragments BSA-$CNBr_{1–183}$ (3·30 ml/g) and BSA-$T_{377–582}$ (3·13 ml/g) were well within the range (3–4 ml/g) expected for native proteins having compact and globular conformation (Tanford, 1968). Thus, if these two fragments are taken to be compact and globular in shape, their Stokes radii (r) can be calculated from the measured intrinsic viscosities, [η], with the help of following equation (Tanford, 1961):

$$[\eta] = 2{\cdot}5\ (N/M)\ (4/3)\ r^3\ , \qquad (4)$$

where M and N are the molecular weight of the proteins and Avogadro's Number ($6{\cdot}022 \times 10^{23}$/mol) respectively. The Stokes radii of BSA-$CNBr_{1–183}$ and BSA-$T_{377–582}$, thus calculated to be 2·26 and 2·22 nm respectively, were in agreement with those determined by gel filtration (see table 2).

Analysis of the gel filtration results on the fragment BSA-$CNBr_{184–582}$ (table 2) showed significant asymmetry and/or hydration of the fragment. This was also supported by viscosity data where intrinsic viscosity was found to be significantly higher than the expected range for the compact and globular proteins (table 2, Tanford, 1961).

Tryptic digestion

The rates of tryptic digestion of BSA and its 3 fragments, which would depend on the accessibility of scisile peptide bonds in the proteins (Klee, 1967), were studied at 25°C and the results are depicted in figure 2. The rates (ΔO.D./min) of hydrolysis were determined by measuring the slope of the initial linear phase of the curve, to be $1{\cdot}18 \times 10^{-1}$, $3{\cdot}3 \times 10^{-3}$, $3{\cdot}40 \times 10^{-3}$ and $0{\cdot}84 \times 10^{-3}$ for BSA, BSA-$CNBr_{1–183}$, BSA-$CNBr_{184–582}$ and BSA-$T_{377–582}$ respectively. The increased rate of tryptic digestion of BSA-$CNBr_{184–582}$ support the conclusion reached above that the

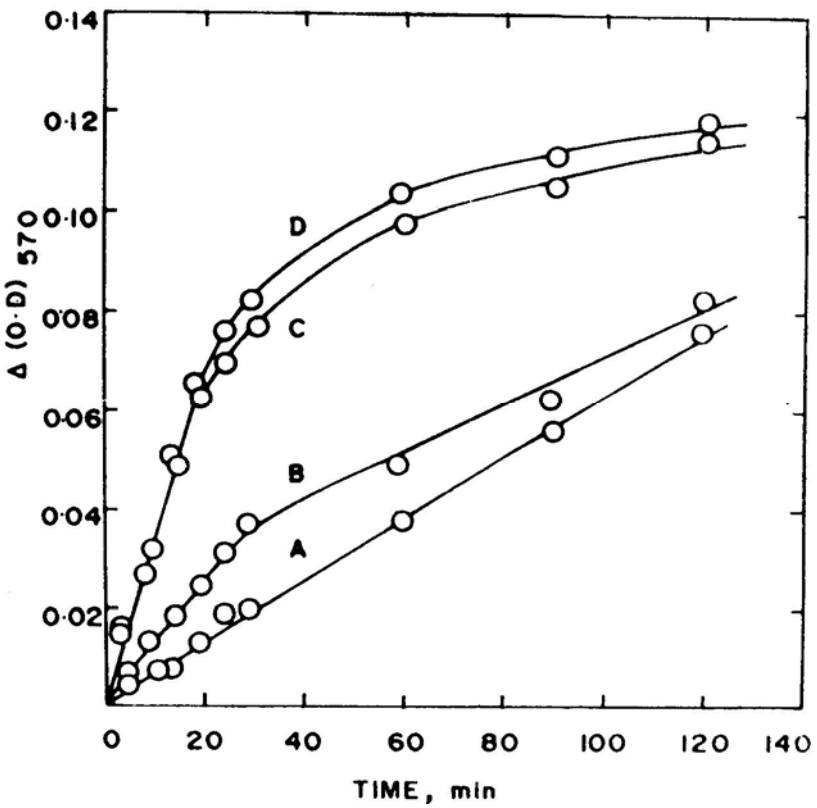

Figure 2. Rate of tryptic digestion of native fragment BSA-$T_{377–582}$ **(A)**, intact BSA **(B),** fragment BSA-$CNBr_{184–582}$ **(C)** and fragment BSA-$CNBr_{1–183}$ **(D).**

Five ml of the enzyme solution containing 21·8 mg trypsin was added to an equal volume of the protein (21·8 mg) solution in 0·1 M sodium phosphate buffer, pH 7·5. One ml aliquots were pipetted out at different time intervals and the reaction was stopped by adding 1 ml chilled 1 M sodium acetate buffer pH 5·1. The increase in the colour intensity with time due to newly formed amino groups was estimated at 570 nm by the method of Moore and Stein (1954).

fragment has a distorted structure as compared to its parent molecule. However, the enhanced rate of the hydrolysis of fragment BSA-$CNBr_{1–183}$, which has been shown above (hydrodynamic results) to exist in a compact and globular conformation, would probably indicate that among the accessible scisile peptide bonds in fragment BSA-$CNBr_{1–183}$ and BSA, the latter contained proportionally more peptide bonds refractory to tryptic attack. This indeed is the case is supported by the slowest hydrolytic rate observed for the fragment BSA-$T_{377–582}$ which constitutes domain III of the intact albumin and thus helps the latter to undergo tryptic hydrolysis at an overall slower rate as compared to the fragment BSA-$CNB_{1–183}$.

Difference absorption spectra

The difference spectra of BSA and its fragments denatured at pH 1·5 were obtained

by using native protein in 0·2 MKCl at pH 6·8 as the reference. The difference between the absorbance, Δ (O·D.), of the native and acid denatured proteins were plotted against wavelength and a representative spectrum thus obtained for fragments BSA-$CNBr_{1–183}$ is shown in figure 3. The fine structures of the difference spectrum of BSA included a pronounced trough at 287 nm, a trough at about 280 nm and 'wiggles' between 250–270 nm. These features were in general shared by the spectra of the 3 albumin fragments. However, in addition to these fine structures the spectra of BSA-$CNBr_{1–183}$ (see figure 3) and BSA-$CNBr_{184–582}$ also contained a trough at about 290 nm. Furthermore, the 'wiggles' between 250–270 nm of the spectrum of fragment BSA–$T_{377–582}$ were much more pronounced as compared to the two CNBr fragments and the intact albumin. These fine structures in the difference spectra of BSA and its 3 fragments were indicative of the exposure of aromatic chromophores in the acid denatured proteins. Since the change in absorbance was maximum at 287-288 nm, the latter were used to follow the acid denaturation of the proteins described below.

Viscosity results

Intrinsic viscosities, [η], of BSA and its fragments, BSA-$CNBr_{1–183}$ and BSA-

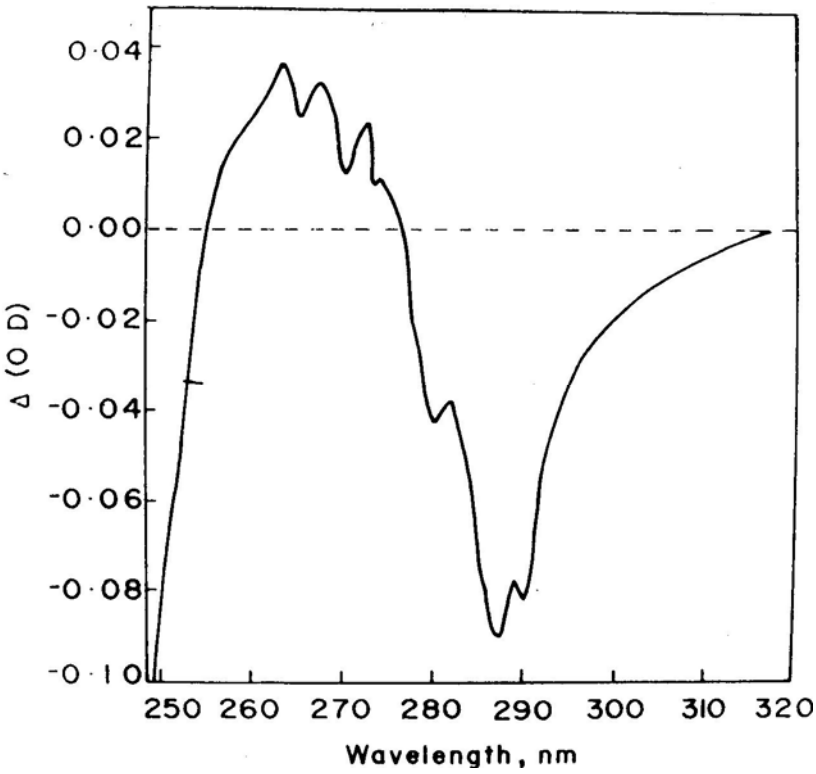

Figure 3. The acid induced difference spectrum of the fragment BSA-$CNBr_{1\_183}$.
The difference spectrum was recorded at 25°C by taking the fragment solution at pH 7 in. the reference compartment and the protein solution at pH 1·8 in the sample compartment. The protein concentration was 1·8 mg per ml.

$CNBr_{184–582}$ at pH 1·8, were found to be 6·71, 4·74 and 6·78 ml/g (see figure 4 for a representative plot for the determination of [η]) which represented an increase of 86, 56 and 44% respectively over the corresponding values of these proteins under native conditions. In view of the marked tendency of time dependent aggregation of the fragment BSA-$T_{377–582,}$ especially at higher protein concentrations, its viscosity could not be measured with any acceptable degree of precision.

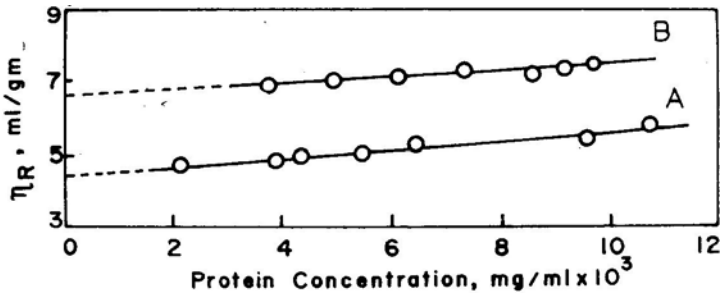

Figure 4. Reduced viscosity of BSA as a function of protein concentration in (**A**) 0·2 M KC1-HC1 mixture, pH 3·6 and (**B**) 0·2 M KC1-HC1 mixture, pH 1·8, at 25°C. The intercepts of the straight lines were computed by the method of least squares to be 4·50 and 6·71 respectively.

Acid induced transition of BSA and its fragments

The shift in equilibrium between native and acid denatured states of BSA and its 3 fragments was measured through the change in reduced viscosity and light absorption at 287 nm. Some of the important features of all these transitions are summarized in table 3. Superimposability of the transitions followed by viscosity and difference spectral measurements, was checked by plotting the fraction denatured (calculated as suggested by Tanford, 1968) measured by the two techniques against pH (see figure 5 for a representative curve obtained for the fragment BSA-$CNBr_{184–582}$).

Equilibrium data given in table 3, clearly showed that the pH induced transition of BSA and its fragment BSA–$T_{377–582,}$ occurred in two steps involving 3 distinguishable states *N, X* and *D;* the transition of the fragments BSA-$CNBr_{1–183}$ and BSA-$CNBr_{184–582}$ were essentially a single step process having only native and acid denatured conformational states. All the transitions were found to be reversible since the experimental points in the denaturation as well as the renaturation experiments lied on the same curve (Tanford, 1968) as illustrated in figure 5.

Equilibrium constant

Assuming that the one step acid induced transitions of the fragments, BSA-$CNBr_{1–183}$ and BSA-$CNBr_{184–582,}$ and both the transitions of intact albumin and BSA-$T_{377–582}$ separately follow two state mechanism, the equilibrium constants *(K_D)* for the different transitions were calculated from the equilibrium data using appropriate equation described elsewhere (Tanford, 1968). Since the dependence of K_D on pH was found to be linear, the slope and the intercepts of the straight lines

Table 3. Important features of the acid induced transitions in BSA and its fragments measured at 25°C in 0·2 M KCl.

Protein transitions and method	pH for the onset of transition[a]	pH for the middle point of transition	pH for the completion transition	$\ln K_D$/pH (slope)	$\ln K_D$ (intercept)
BSA (first transition)					
Viscosity	4·30	4·04	3·80	3·11	12·39
Difference spectra	4·45	4·25	3·95	3·68	15·69
BSA (second transition)					
Viscosity	3·40	3·15	2·75	2·56	7·99
Difference spectra	3·60	3·14	2·45	1·42	4·45
BSA-CNBr$_{1-183}$					
Viscosity	—	3·00	2·25	1·96	5·94
Difference spectra	—	3·10	2·25	1·05	3·31
BSA-CNBr$_{184-582}$					
Viscosity	—	3·20	2·50	2·21	6·97
Difference spectra	—	3·55	2·55	1·09	3·89
BSA-T$_{377-582}$ (first transition)					
Difference spectra	5·05	4·60	4·00	1·99	9·04
BSA-T$_{377-582}$ (second transition)					
Difference spectra	3·60	3·25	2·50	3·16	10·32

[a]pH for the onset of transition for the fragments BSA-CNBr$_{1-183}$ and BSA-CNBr$_{184-582}$ could not be measured with precision because the solubility of these fragments was very poor in the neighbourhood of pH 5. The dependence of equilibrium constant, K_D, on pH was analysed as described by Wyman (1964). The slopes and the intercepts of the straight lines were calculated by the method of least squares.

drawn by the method of least squares were computed and the values thus obtained are given in table 3. According to the theory of linked function, described by Wyman (1964), the dependence of K_D on pH can be described by the equation:

$$-\partial \ln K_D / \partial \mathrm{pH} = \nu H_D - \nu HN$$

where νH_D and νH_N are the number of protons bound to the acid denatured and native proteins respectively. Thus the slope of the straight line of the type given in figure 6 (representative plot given for the fragment BSA–T$_{377-582)}$ yields the difference between the number of protons bound by the denatured and the native states of the proteins whereas the intercept will give the value of log K_D which will be independent of pH.

Discussion

The transition from native to acid denatured state in intact albumin was fully reversible and occurred in atleast two well defined steps involving an inter-mediate at about pH 3·6 having about 25% higher hydrodynamic volume than the native

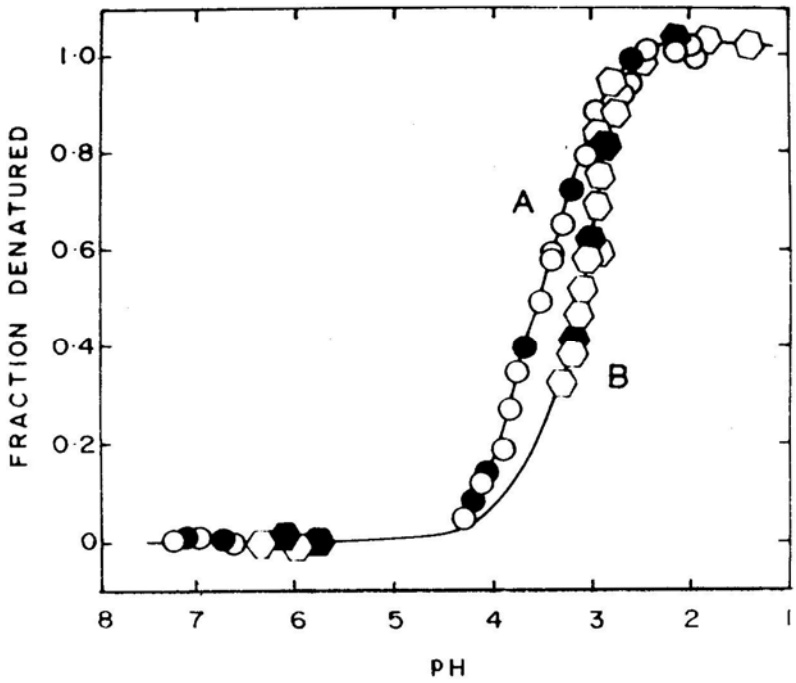

Figure 5. The acid induced transitions of fragment BSA-CNBr$_{184–582}$ as followed by difference spectral (O), and viscosity (⬡) measurements. Filled circles and hexagons represent points taken in renaturation experiments. Protein concentrations for the spectral and viscosity measurements were 3·2–2·2 mg per ml respectively.

protein. The first transition, as measured by reduced viscosity, occurred at somewhat lower pH than the transition measured by difference spectroscopy. This would imply that the exposure of aromatic chromophores precedes the overall unfolding of BSA in the pH range 4·5–3·6. Therefore, the reduced viscosity and difference spectral results (see table 3) taken together will be consistent with the following mechanism for the first transition:

$$N \rightleftharpoons X' \rightleftharpoons X \qquad (6)$$

where X' and X are distinguishable only by difference spectrpscopy. The transition $N \rightleftharpoons X$ was highly cooperative and it was found that the N state must bind about 3 protons (see table 3) in order to be converted to X state which was perhaps similar to 'F' state observed by Foster (1960) during the study on $N \rightleftharpoons F$ transition (Aoki and Foster, 1956).

Although the pH corresponding to the mid-points of the second transitions in BSA as measured by reduced viscosity and spectroscopy, were fortuitously the same, the transition measured by the two properties did not coincide with each other. The acid denatured state, D, detected by reduced viscosity was found to exist at lesser acidic pH than the corresponding state, D', monitored by difference spectroscopy. However, the difference between D and D', which are distinguishable by viscometry, is not likely to be a major one since the change in absorbance at pH values where these, two states could separately exist, were found to be very small.

Similar analysis of the data on 3 albumin fragments revealed that all the

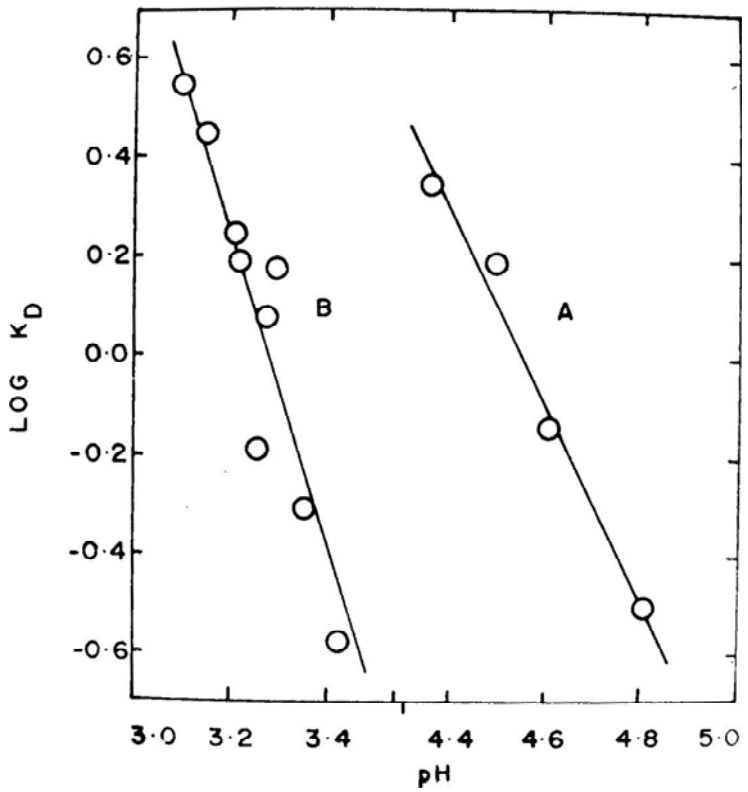

Figure 6. dependence of the equilibrium constant, K_D, for the acid induced unfolding of the fragment BSA-$T_{377–382}$ as followed by difference spectral measurements. (**A**), pH dependence of K_D for the N_F-X_F transition and (**B**), pH dependence of K_D for the X_F-D_F transition,

transitions were reversible. The two cyanogen bromide fragments underwent a single step transition involving only two major states (native and acid denatured) whereas the tryptic fragment, representing domain III of BSA, showed two step transition involving atleast one stable intermediate state, X_F. As in intact albumin, the unfolding of the fragments BSA-$CNBr_{1–183}$ and BSA-$CNBr_{184–582}$ followed by viscometry and spectroscopy, did not appear to be completely concomitant processes. However, the transitions of the fragment BSA-$CNBr_{1–183}$ measured by the two techniques are comparable with each other and they are closer to the second transition of intact albumin (see table 3). Likewise, the acid induced transitions (measured by both the techniques) of the fragment BSA-$CNBr_{184–582}$ and the second $X_F \rightleftharpoons D_F$) transition of the fragment BSA–$T_{377–582}$ are closer to the second transition of intact albumin. Furthermore, the first transition ($N \rightleftharpoons X_F$) of BSA-$T_{377–582}$ although starts at slightly higher pH, is closer to $N \rightleftharpoons X'$ transition of BSA. It is therefore, possible that the second acid induced transition in BSA might represent the gross unfolding of the protein molecule during which a part of its structure is lost. On the other hand, the first transition (presumably the $N \rightleftharpoons F$ transition) of BSA is likely to be caused due to the partial unfolding of its domain III (represented by fragment BSA–$T_{377–382}$) and/or the separation of the sub-

domains of domain III from each other as well as from the rest of the albumin molecule. The second transition of BSA–$T_{377–582}$ *($X_F \rightleftharpoons D_F$)* represents its gross unfolding which is observed in the form of second transition of BSA which also represent the unfolding of domain I and II of the intact albumin. The separation of the sub-domains of domain III (Brown, 1976) from each other (which in our opinion is the cause of $N \rightleftharpoons F$ transition in BSA and the first transition in BSA-$T_{377–582}$) , may be facilitated to some extent when it (domain III) is isolated from the rest of the albumin molecule. This explains the early start of the first transition of BSA–$T_{377–582}$ as compared to the first transition of BSA. It is interesting to recall here that a fragment representing domain I + II (BSA–$P_{1–385}$) of BSA was unable to undergo $N \rightleftharpoons F$ transition and exhibited a single step acid induced transition comparable to the second transition of intact albumin (Khan and Salhuddin, 1984). Furthermore, the reduced solubility of BSA (Khan *et al.,* 1985) and its increased susceptibility towards peptic digestion (Hilak *et al.,* 1974) at about pH 3·6 (known to cause $N \rightleftharpoons F$ transition) without a major structural change, can be explained in terms of the exposure of hydrophobic amino acid residues present at the interface of domains II and III and in the connecting segments of the sub-domains of domain III. Incidently, such residues constitute about 60% of the total amino acids present in these regions of the albumin molecule (Brown, 1976). The reason why BSA-$CNBr_{184–582,}$ which also includes domain III as its constituent, shows a single-step transition is not explainable at this stage. However, one possibility could be that the structural alterations associated with the first transition of domain III had already occurred in fragment BSA-$CNBr_{184–582}$ (domain II + III) during course of its isolation making the latter unable to exhibit a two step-transition. Indeed, our results on hydrodynamic behaviour of the 3 fragments, as described earlier, have shown that fragment BSA-$CNBr_{184–582}$ is asymmetric in nature.

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