Biochem. J. (1986) 236, 307–310 (Printed in Great Britain)

Direct evidence for the involvement of domain III in the N–F transition of bovine serum albumin

M. Yahiya KHAN
Department of Biochemistry, School of Life Sciences, North-Eastern Hill University, Shillong-793014, India

The domain III of bovine serum albumin containing residues 377–582 of the protein sequence was isolated and its behaviour in acid solution was studied. The fragment was found to undergo structural transformations over the pH range 3.5–4.5 known to cause N–F transition in serum albumin. On the other hand, an albumin fragment that was devoid of domain III was unable to exhibit such a transition. These results were consistent with a mechanism where N–F transition involves the separation of domain III from the rest of the albumin molecule as well as the separation of the subdomains of domain III from each other. The structural transition starts at about pH 4.3 and is completed at pH 3.5.

INTRODUCTION

It is well established that a decrease in pH of bovine serum albumin (BSA) solution from about 4.5 to 3.5 causes a transition from a native or 'N' form to a faster-migrating or 'F' form of the protein, a phenomenon known as the 'N–F transition' (Foster, 1977; Peters, 1975, 1980). In spite of the extensive studies made in this area, the exact mechanism of N–F transition remains an enigma. Since the primary structure of BSA comprises three structural domains (Brown, 1976), the occurrence of the N–F transition may be correlated with the separation of domains (Reed *et al.*, 1975). Fairly recently we showed that an albumin fragment that lacked domain III of its parent molecule was unable to exhibit N–F transition (Khan & Salahuddin, 1984). This, along with other observations (Hilak *et al.*, 1974; Peters, 1980), made us postulate that the N–F transition involved the unfolding of domain III and/or its separation from the rest of the albumin molecule (Khan & Salahuddin, 1984). In an attempt to substantiate this hypothesis further, I have now isolated a tryptic fragment of BSA that represents domain III of the protein. The behaviour of the tryptic fragment in acid solution was studied, and it is shown that domain III is responsible for the N–F transition in the intact BSA molecule. A mechanism for the transition is proposed that explains all the observations made on the transition up to the present time.

Some of the work described here was presented at the 13th International Congress of Biochemistry held in Amsterdam 25–30 August 1985.

MATERIALS AND METHODS

BSA (lot no. 10C-8080), tosylphenylalanylchloromethane ('TPCK')-treated trypsin and all other proteins were purchased from Sigma Chemical Co. The commercial BSA was routinely passed through a Sephadex G-100 column to remove dimer and other aggregated forms of the protein. The monomeric fraction (M_r 68000) of albumin thus obtained was used for all subsequent studies. Protein concentrations were determined from the absorbance of their solutions at 278 nm. Specific absorption coefficients ($A^{1\%}_{1cm}$) of 6.66 and 3.2 (Khan, 1982) were used for the albumin and its fragment respectively.

The tryptic fragment comprising domain III of albumin was isolated from tosylphenylalanylchloromethane-treated-tryptic digests obtained by hydrolysing the protein for 1 h in 0.01 M-sodium phosphate buffer, pH 8.2, at an albumin/enzyme ratio of 100:1. The incubation mixture was stirred gently at 40 °C, the pH being kept constant at 8.2 by adding 1 M-NaOH. The digestion mixture thus obtained was subjected to Sephadex gel and DEAE-cellulose chromatography to give the desired fragment (Habeeb & Atassi, 1976).

The amino acid composition of the proteins was determined on an LKB Biochrom automatic amino acid analyser (model 4101) by using the procedure recommended by the manufacturer.

Identification of the *N*-terminal amino acid residue was done by the dansylation method described by Gray (1967). The analysis of the *C*-terminal amino acid residue was done by using the enzymic method described by Narita (1970).

The frictional ratio and Stokes radius of the fragment were calculated from the data obtained by gel filtration on a Sephadex G-200 column (80 cm × 2.74 cm) equilibrated with 0.06 M-sodium phosphate buffer, pH 7.0, at 25 °C by the method of Baig & Salahuddin (1978). The intrinsic viscosity, $[\eta]$, was calculated with the help of following equation (Tanford, 1955):

$$[\eta] = \lim_{c \to 0} (\eta - \eta_0)/\eta_0 \cdot c \quad (1)$$

$$[\eta] = \lim_{c \to 0} [(t - t_0)/t_0 \cdot c] + [(1 - \bar{v} \cdot \rho_0)/\rho_0] \quad (2)$$

where η_0 and η are the viscosities in poise [1P = 10^{-1} Pa·s (the SI unit)] of the solvent and protein solution respectively, c the concentration of the protein solution (g/ml) and ρ_0 the density of the solvent (0.06 M-sodium phosphate buffer, pH 7.0) in g/cm^3. The partial specific volume, $\bar{v}$, of the protein (in ml/g) was calculated by the method of Haschemeyer & Haschemeyer (1973). The time of fall of the solvent, t_0, and that of the protein solution,

Abbreviations used: BSA, bovine serum albumin; dansyl, 5-dimethylaminonaphthalene-1-sulphonyl.

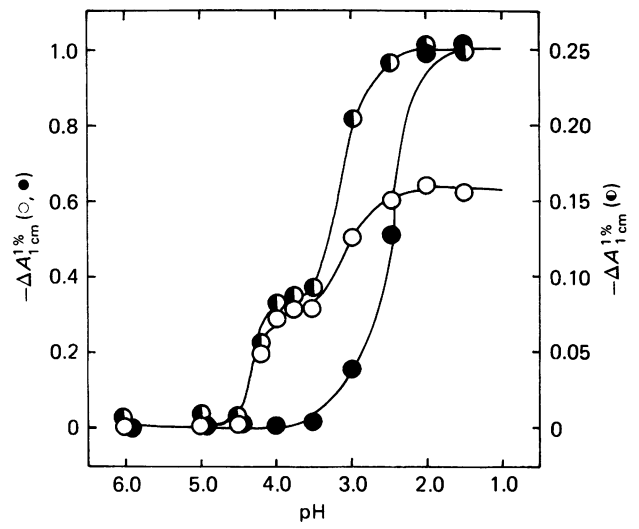

Fig. 1. Effect of pH on the difference absorption ($-\Delta A^{1\%}_{1cm}$) of bovine serum albumin (○), and its fragments BSA-P_{1-385} (●) and BSA-$T_{377-582}$ (◐)

Difference in the absorption at 287 nm was measured at 25 °C by taking two well-matched silica cells of 1 cm path length. The pH of the sample, in 0.2 M-KCl, was adjusted to the desired value by adding constant-boiling HCl. The protein concentration was in the range 2–3 mg/ml.

t (in s), were recorded at 25 °C in a Kimax G-46 (size 25) viscometer as described previously (Ahmad & Salahuddin, 1974).

The difference spectra of the acid-induced transition of the proteins were recorded in 0.2 M-KCl on a ratio recording spectrophotometer by a method described previously (Khan & Salahuddin, 1984). Since the change in absorbance was maximum at 287 nm, the latter was used to monitor the acid-induced transition.

RESULTS AND DISCUSSION

The tryptic fragment isolated in the present study was homogeneous with respect to charge as well as size. *N*- and *C*-Terminal amino acid residues of the fragment were identified to be histidine and alanine respectively. These observations, taken together with the M_r and amino acid composition (Khan, 1982), led me to name the fragment 'BSA-$T_{377-582}$' (where T indicates the mode of cleavage, i.e. trypsin, and the subscript 377–582 indicates the position of the fragment in the primary structure of BSA). This is at variance with the results of Habeeb & Atassi (1976), who isolated the fragment 'Peptide 377–571' under similar conditions. The reason for this difference is not clear at present. However, as pointed out above, the M_r, amino acid composition and end-group analysis clearly established that the tryptic fragment isolated by

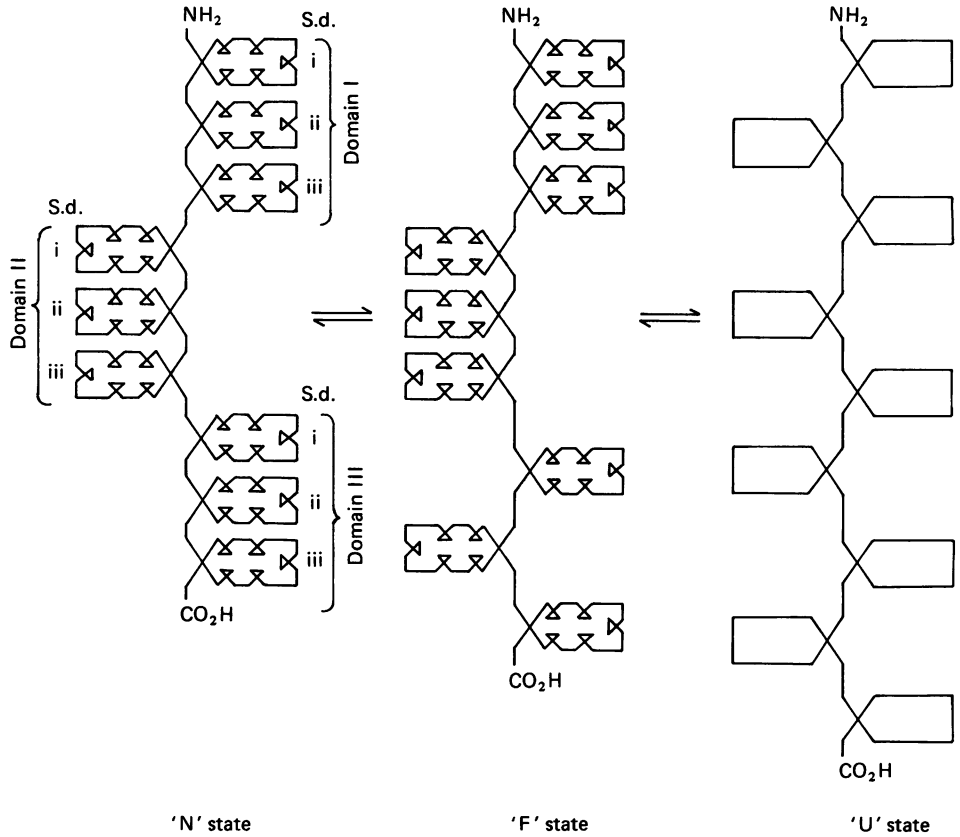

Fig. 2. Schematic representation of N–F transition in bovine serum albumin

Each of the three domains (I, II and III) is made up of three subdomains marked S.d. i, ii and iii. For the definition of 'N', 'F' and 'U' states see the text. The depiction of the acid-unfolded state is arbitrary, because its formation does not involve the total loss of the helical structure (as has been shown in the Figure). The residual structure retained by the acid-denatured state, 'U', of the albumin may, however, be removed by stronger denaturants like guanidinium chloride to give cross-linked random coil (Tanford, 1968).

me consists of residues 377–582 of the albumin molecule. This fragment is similar to the fragment isolated by Peters & Feldhoff (1975) (named 'T-A' by them) by using immobilized albumin.

The values for Stokes radius and frictional ratio were determined to be 2.39 nm and 1.05 respectively. The intrinsic viscosity of the fragment was found to be 3.13 cm^3/g, which was well within the range (3.0–4.0 cm^3/g) expected for native proteins having a compact and globular conformation (Tanford, 1968). The Stokes radius of the tryptic fragment was also calculated from its measured intrinsic viscosity (Tanford, 1961). The value (2.22 nm) thus obtained was in excellent agreement with 2.39 nm determined by gel filtration. All these hydrodynamic parameters of the fragment therefore indicated that it had retained its native globular conformation. This contention is supported by the observations made by Reed *et al.* (1975) on a similar fragment, for which they have shown that the secondary structure of the native albumin is retained in the fragment.

The results on the difference-spectral measurement of the tryptic fragment are shown in Fig. 1. For comparison, data are also presented for intact albumin and one of its peptic fragments, BSA-P_{1-385} (Khan & Salahuddin, 1984). It is seen that there is a significant decrease in the absorbance of the tryptic fragment BSA-$T_{377-582}$ over the pH range 4.5–3.5 that causes N–F transition in intact serum albumin. Further decrease in pH (below 3.5), as with intact BSA, caused an acid-induced unfolding of the fragment. On the other hand, the peptic fragment, BSA-P_{1-385}, was unable to show any sign of alteration in its structure over the pH range 3.5–4.5. These results clearly indicate that N–F translation is confined to the *C*-terminal one-third of the molecule, i.e. domain III of the albumin molecule. On the basis of these observations and the fact that domain III is more susceptible to peptic digestion (King, 1973; Hilak *et al.*, 1974; Feldhoff & Peters, 1975), a mechanism for N–F transition in serum albumin is proposed (see Fig. 2).

N–F transition in albumin is the first step in acid-induced expansion of the protein. The onset of N–F transition below pH 4.5 involves the separation of domain III from the rest of the albumin molecule as well as the separation of the three subdomains of domain III from each other. The transition is complete at about pH 3.5. Further decrease in pH below 3.5 causes acid-induced unfolding of albumin, during which the 'F' form is transformed into the 'U' or acid-unfolded form of the protein. The overall sequence of events will therefore become N→F→U.

The connecting peptide link between domains II and III (Brown, 1977; Reed *et al.*, 1980) of the albumin molecule is particularly susceptible to peptic attack at acid pH (Peters, 1975). Further, Luzzati *et al.* (1961) have shown through low-angle X-ray scattering that, at pH 3.6, about one-third of the albumin molecule unwinds to take up a loose structure around the remaining two-thirds, the latter fraction still retaining its compact globular structure (for a review see Franglen, 1974). In one of the most significant reports on N–F transition in human serum albumin, Geisow & Beaven (1977) concluded that the transition probably occurs as a result of the unfolding or separation of the *C*-terminal third of the protein from the remainder of the molecule. Taken together, these results suggest structural transformations within domain III and its separation from the rest of the albumin molecule during N–F transition, a contention that is the backbone of the mechanism proposed in Fig. 2.

About 60% of the total amino acids present at the interface of domains II and III, and in the connecting segments of the three subdomains (i.e. i and ii, and ii and iii) of domain III, are hydrophobic (alanine, phenylalanine, valine, leucine, isoleucine, tyrosine, proline) in nature (Brown, 1976). These amino acids are exposed to the outer environment during the N–F transition and thus account for the substantial decrease in the aqueous solubility of the 'F' form of the protein (Foster, 1960; Peters, 1975). Similarly, the exposure of many scissible peptide bonds that are present in the connecting segments of the subdomains of the domain III explains the enhanced susceptibility of domain III for peptic cleavage at pH 3.5 (King, 1973; Hilak *et al.*, 1974; Feldhoff & Peters, 1975). Likewise, the observation that the peptic fragment which is devoid of domain III does not undergo N–F transition (Geisow & Beaven, 1977; Khan & Salahuddin, 1984) is also explained by the above mechanism (see Fig. 2). The real test of the proposed mechanism will, however, be possible only after the three-dimensional structure of serum albumin is worked out.

Financial help from University Grants Commission, New Delhi, and the facilities extended by Aligarh Muslim University (during my stay there as a Research Fellow) and North-Eastern Hill University are gratefully acknowledged.

REFERENCES

Ahmad, F. & Salahuddin, A. (1974) Biochemistry **13**, 245–249
Baig, M. A. & Salahuddin, A. (1978) Biochem. J. **171**, 89–97
Brown, J. R. (1976) Fed. Proc. Fed. Am. Soc. Exp. Biol. **35**, 2141–2144
Brown, J. R. (1977) Proc. FEBS Meet. 11th 1–10
Feldhoff, R. C. & Peters, T., Jr. (1975) Biochemistry **14**, 4508–4515
Foster, J. F. (1960) in The Plasma Proteins (Putnam, F. W., ed.), vol. 1, pp. 179–239, Academic Press, London and New York
Foster, J. F. (1977) in Albumin Structure, Function and Uses (Rosenoer, V. M., Gratz, M. & Rothschild, M. A., eds.), pp. 53–84, Pergamon Press, New York
Franglen, G. (1974) in Structure and Function of Plasma Proteins (Allison, A. C., ed.), vol. 1, pp. 265–281, Plenum Press, London
Geisow, M. J. & Beaven, G. H. (1977) Biochem. J. **163**, 477–484
Gray, W. R. (1967) Methods Enzymol. **11**, 139–151
Habeeb, A. F. S. A. & Atassi, M. Z. (1976) J. Biol. Chem. **251**, 4616–4621
Haschemeyer, R. H. & Haschemeyer, A. E. V. (1973) Proteins: A Guide to Study by Physical and Chemical Methods, pp. 160–164, John Wiley and Sons, New York
Hilak, M. C., Harmsen, B. J. M., Bram, W. G. M., Joordens, J. J. M. & Van Os, G. A. J. (1974) Int. J. Pept. Protein Res. **6**, 95–101
Khan, M. Y. (1982) Ph.D. Thesis, Aligarh Muslim University
Khan, M. Y. & Salahuddinin, A. (1984) Eur. J. Biochem. **141**, 473–475
King, T. P. (1973) Arch. Biochem. Biophys. **156**, 509–520
Luzzati, V., Witz, J. & Nicholaieff, A. (1961) J. Mol. Biol. **3**, 379

Narita, K. (1970) in Protein Sequence Determination (Needleman, S. B., ed.), pp. 25–90, Chapman and Hall, London
Peters, T., Jr., (1975) in The Plasma Proteins (Putnam, F. W., ed.), vol. 2, pp. 133–181, Academic Press, London and New York
Peters, T., Jr. (1980) Albumin: An Overview and Bibliography, pp. 1–34, Miles Laboratories, Elkhart
Peters, T., Jr. & Feldhoff, R. C. (1975) Biochemistry **14**, 3384–3390
Reed, R. G., Feldhoff, R. C., Clute, O. L. & Peters, T., Jr. (1975) Biochemistry **14**, 4578–4583
Reed, R. G., Putnam, F. W. & Peters, T., Jr. (1980) Biochem. J. **191**, 867–868
Tanford, C. (1955) J. Phys. Chem. **59**, 798–799
Tanford, C. (1961) Physical Chemistry of Macromolecules, John Wiley and Sons, New York
Tanford, C. (1968) Adv. Protein Chem. **23**, 121–202

Received 30 October 1985/4 March 1986; accepted 12 March 1986