STRUCTURAL TRANSFORMATIONS IN SERUM ALBUMIN AS DEMONSTRATED BY UREA-PERTURBATION TECHNIQUE*

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A urea-perturbation technique for demonstrating structural transitions in protein is described. Evidence is presented of changes in the structure and stability of BSA originating from exposure to pH extremes, defatting, 'reversible denaturation', reduction and alkylation procedures.

This technique is based on the ability to resolve protein sub-species of identical molecular weight and charge but of differing conformation by gel electrophoresis (Ferris and Katz, 1966; Kitto et al., 1966). The analysis involves the study of the kinetics of the rate and type of sub-species produced by reacting a given protein with a denaturant under defined operational conditions. This analysis as applied to 'modified' protein provides not only a measure of homogeneity but of more consequence reveals possible changes in charge, molar volume (Katz and Ferris, 1966), conformation and stability. The kinetic analysis provides information pertinent to the mechanism(s) of the denaturation process and of the constraints which determine the protein's conformation in solution.

The methodology for urea-perturbation involves pipeting 50 µl protein into 250 µl denaturant so that the final protein concentration was 4 ± 1%. Electro-

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phoretic analyses were performed at intervals ranging from 4 min to 24 hours as a function of reaction rate. Acetate buffer, 0.05 M pH 5.0, was used as solvent unless otherwise stated. The temperature was 24.5 ± 1°. The procedure and electrolyte used for Acrylamide gel electrophoresis have been described previously (Ferris and Katz, 1966). In this study 250 volts was applied reducing the duration of electrophoresis to 3-3.5 hours.

Fatty acid free albumin (FSA) was prepared according to Chen; 0.2 M HCl and NaOH were cooled to 4° prior to pH adjustment (Chen, 1967). The control, FCA, was processed identically but carbon was omitted. Reduced alkylated albumin (RAA) was prepared by reduction with 2-mercaptoethanol in 4 M urea maintained at pH 9.3 with borate buffer and then alkylated with an excess of iodoacetamide (Smithies et al., 1966). This was dialyzed against the buffer used for electrophoresis. The control (RCA) was processed similarly except that treatment with mercaptoethanol and iodoacetamide was omitted. An alkylated mercaptalbumin (MMA) was prepared by reacting bovine mercaptalbumin with iodoacetamide in pH 9.3 borate buffer for 20 min at 24°. Bovine serum albumin (BSA) was purchased from Armour Company; BMA from Mann Research Laboratories, Inc. Urea (Mallinckrodt) was recrystallized prior to use; all chemicals were of the highest purity available.

The kinetics of the perturbation of BSA by urea have been published (Ferris and Katz, 1966). The rate for urea-BSA interaction determined here was lower than that reported initially due to the use of a lower reaction temperature and the specimens used in this study were not subject to freeze-drying. The differences in rate indicate the necessity for maintenance of a standardized procedure for comparative studies. Two major species of BSA(d), S_i and I_j, are demonstrable by gel electrophoresis. The S_i species appears about an hour after reaction with urea at concentrations $\geq$ 5 M with the concentration maximum for this species being at about 7 M urea. The S_i species consists of 6-10 sub-species whose mobilities range from 0-30% of BSA. The mobility of the I_j species is intermediate to S_i and to BSA; this species is

associated with elevated urea concentrations, time and temperatures.

Recently, the preparation of a fatty acid free albumin whose properties were identical to the parent protein by criteria such as O.R.D., analytical ultracentrifugation, binding isotherms, and by several fluorescent parameters was reported (Chen, 1967). This interpretation is seemingly reinforced by the similarity of the gel electrophoretic mobilities found for FSA, FCA, and BSA (see patterns in extreme left, second to the right, and extreme right slots in Fig. 1). The mobility of FSA is about 3% less than BSA as predicted from the charge reduction due to the removal of fatty acids. However, the marked alteration in the urea-perturbation profiles for both FSA and FCA (Figs. 1 and 2) indicates that the treatment has caused structural alterations not

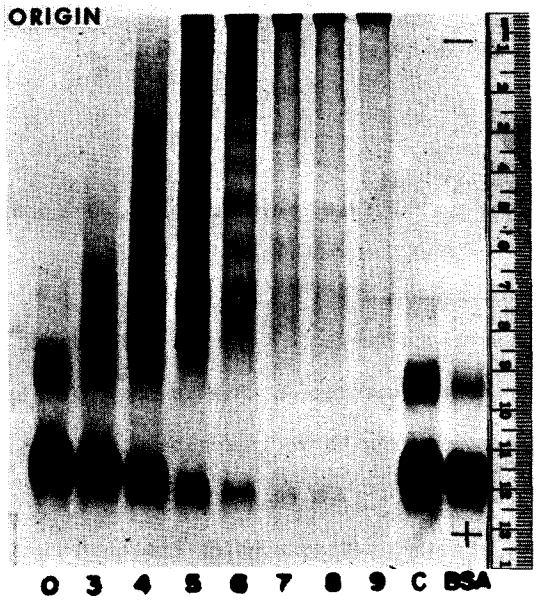

Fig. 1. 24-hour urea-perturbation pattern of 4% fatty acid free BSA in 0.05 M acetate buffer, pH 5.0, exposed to urea solutions for 24 hour at 24.5 ± 0.5° prior to electrophoretic analysis. Urea concentrations are indicated on the figure. The symbol BSA indicates the pattern for 4% BSA in 10% sucrose; C indicates the pattern for the control.

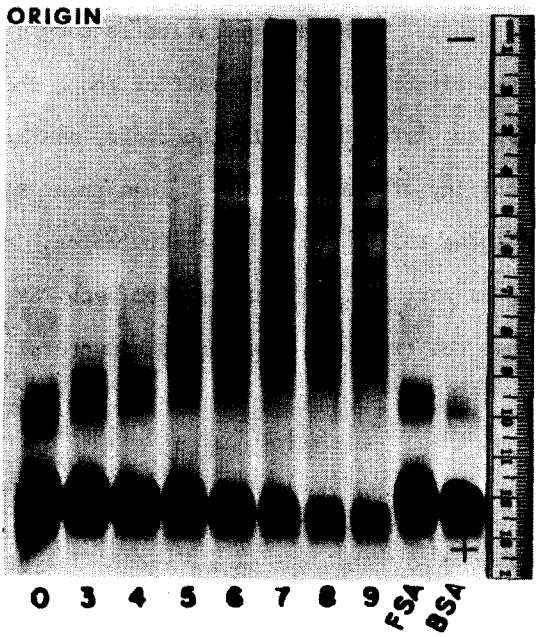

Fig. 2. 24-hour urea-perturbation for control for fatty acid free BSA, FCA. The symbol FSA refers to the fatty acid free BSA. For additional details refer to Fig. 1.

demonstrable by conventional methodology. It is apparent that FSA and FCA are more susceptible to urea denaturation than is BSA. The labile nature of FSA is reflected by its conversion to an aggregate which does not migrate upon exposure to urea concentration $\geq$ 7 M for 24 hr (Fig. 1). In contrast FCA, under similar conditions, produces patterns which simulate that produced by BSA after an exposure to urea for a week. Another departure from the norm is the absence of S_i fractions in these two systems; the slow-moving fractions present are not typical S_i sub-species but are probably polymeric forms of BSA(d).

Reduced-alkylated BSA revealed one fraction with a mobility about 5% greater than BSA and a small amount of material with low mobility (Slot 1

in Fig. 3). The 24-hour urea-perturbation profile for RAA, which was identical to shorter term exposures, indicated no demonstrable action of urea on this derivative. This suggests that RAA, possessing little disulfide or

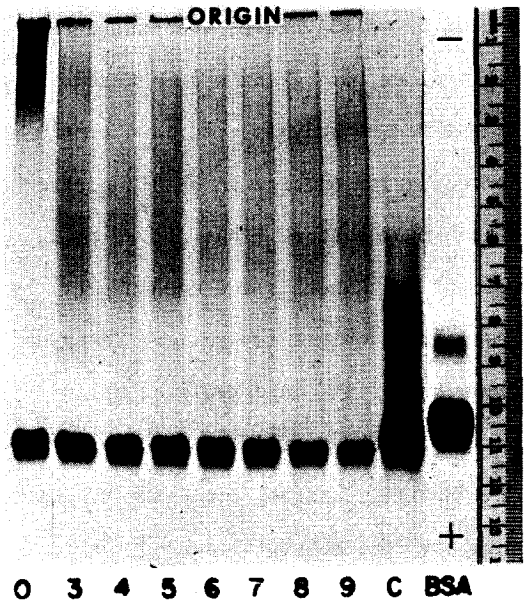

Fig. 3. 24-hour urea-perturbation pattern for reduced alkylated BSA. The control, C, refers to the appropriate control for this system.

helical content (McArdle, 1966) and existing as a random coil, cannot be further altered by urea. Parenthetically, the enhanced mobility of RAA relative to BSA supports the hypothesis advanced previously that the migration rate of the random-coil form of BSA in gel is comparable to the native or globular form (Katz and Ferris, 1966). The control consisted of two major and three minor fractions (Slot 9 in Fig. 3). Apparently, the reduction-alkylation procedure results in the conversion of these five entities to one species. the 1 hour urea-

perturbation profile for RCA showed no change from the starting material; however, the 24-hour urea-perturbation profile resembles that produced by BSA except that no S_i fractions were demonstrable.

Perturbation studies were also performed with mercaptalbumin and alkylated mercaptalbumin. The BMA as received consisted of 5 fractions with most of the protein being distributed equally in the 2 leading fractions whose mobilities were identical to their counterparts in BSA. The urea-perturbation profile for BMA was roughly similar to BSA except that the decay rate for the second component, probably the dimer, was greater than that of the monomer, the leading fraction. While BSA was relatively resistant to 4 M urea at pH 5, it was denatured by this concentration of urea in pH 9.3 borate buffer; in contrast BMA was susceptible to 4 M urea at pH 5 but not at pH 9.

A detailed report of the kinetics and mechanism(s) of these reactions is in preparation; consequently, only a few salient features will be mentioned. Since the molecular weights of the intermediates are unchanged and the effect of the medium on the pK's of the protein's prototropic groups is relatively small, the substantial mobility changes found for BSA(d) must be indicative of conformational changes. The multiple forms of BSA(d) can be attributed to the existence of proteins with differing secondary and tertiary content or, alternatively, that the sub-species of a given protein have essentially the same content of organized structure but the distribution per molecule is different. Either of these states can arise from the disruption of hydrophobic and hydrogen bonds and possible intra-bond exchange. Studies are being conducted to determine the possible contribution of the disruption of intra-disulfide bonds and weak electrostatic forces of the type proposed by Vijai and Foster (1967) with respect to the formation of multiple species of denatured albumins.

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