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Heterogeneity of Mammalian Plasma Albumin

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포유류 혈장알부민의 이질성

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적 요

소의 혈장으로부터 알부민을 순수 정제하였으며 순도는 면역 화학적 방법을 사용하여 확인하였다. 정제된 알부민에 maleate, iodoacetate, iodoacetamide 및 glutathione의 4가지 thiol 화합물을 각각 반응시켜 그 복합체를 9가지의 상이한 완충용액내에서 초산셀룰로우즈 전기영동한 결과 barbital buffer와 Na-acetate buffer를 제외한 다른 완충용액내에서 albumin-glutathione 복합체는 두 가지의 단백질로 분리되었으며 pH 4.8 citrate buffer 및 pH 4.8 succinate buffer내에서 albumin-iodoacetate 및 albumin-iodoacetamide 복합체는 두 가지의 단백질로 분리되었다.

전기영동상에 나타나는 알부민 분획에는 conformation이 서로 다른 두 가지 이상의 알부민 분자가 존재한다고 사료된다.

INTRODUCTION

The protein albumin in mammals comprises half of the total plasma proteins (Sober, 1974) and has been assigned numerous physiological roles (Peters, 1975). It is synthesized almost exclusively in the liver as a precursor, proalbumin, which has an extra N-terminal hexapeptide (Peters and Reed, 1980).

It has been known for many years that human and bovine plasma albumin preparations are heterogeneous with respect to sulfhydryl content and molecular weight (King, 1961; Janatova *et al.*, 1968; Sollenne *et al.*, 1981). Shrivastava *et al.* (1972) demonstrated the chromatographic and electrophoretic hetrogeneity of human serum albumin by subjecting the protein to ion-exchange chromatograpy on DEAE Sephadex A-50 and to starch gel electrophoresis in pH 8.6 buffer. On the other hand, bovine serum albumin was

suggested by Cann (1966) to interact reversibly with the undissociated buffer component on the basis of the facts that two zones were given after electrophoresis of the protein in phosphate-borate buffer, pH 6.15, on cellulose acetate and that each zone eluted from the two zones again demonstrated two zones under the same conditions. These observations have made the comparative biochemists take one hypothesis on the nature of the plasma albumin whether the protein is heterogeneous *in vivo* and the observed heterogeneity results from later preparative processes, and have made the enzymologists consider the physiological need of the protein in such a large quantity.

Hopkinson (1975), in his electrophoretic experiments with the complexes of isozymes-thiol reagent, cogently suggested that the systematic use of specific thiol reagents and subsequent electrophoresis might offer a more definitive and reliable means of elucidating the structure of a protein.

In the present work with bovine plasma albumin, we have observed the electrophoretic behavior of the albumin-thiol reagent complexes on cellulose acetate and suggested a possibility that at least two proteins with slight conformational differences are contained in the albumin fraction.

MATERIALS AND METHODS

Purification of bovine plasma albumin and preparation of antibody against the purified albumin:

Fresh bovine blood collected immediately after slaughter with Na-EDTA was centrifuged in a PR-2 International Refrigerated Centrifuge at 5,000 g for 30 minutes. The pooled plasma was fractionated using a minor modification of the polyethyleneglycol and ethanol procedure (Jimenez *et al.*, 1974). The bovine plasma treated with polyethyleneglycol (MW=4,000) to the final concentration of 35% (w/v) under continuous stirring at 4°C was centrifuged to discard the pellet. The resulting supernatant was found to contain albumin fraction almost exclusively on the criterion of cellulose acetate electrophoresis. The albumin-polyethyleneglycol mixture was subjected to slow addition of ethanol to a final concentration of 40% at −5°C. The protein precipitate gathered by centrifugation was dissolved in distilled water. Again ethanol was added to reach a final concentration of 40% at −5°C and the pH of the solution was lowered to 5.0. After the centrifugation, the precipitate was pooled and stored at −20°C until further uses.

Protein concentration was measured by the method of Lowry *et al.* (1951). Antisera against the bovine plasma albumin were prepared by injecting 10 mg of the purified albumin with Freund's complete adjuvant (Difco) into each adult rabbits once every week. The other conditions for preparation of antisera were described previously (Park *et al.*, 1976). Using the method of Ouchterlony and Nilsson (1973), the immunodiffusion tests between crude plasma and anti-plasma albumin were carrid out.

Preparation of albumin-thiol reagent complex and cellulose acetate strip electrophoresis:

Four thiol reagents of maleate, iodoacetate, iodoacetamide and glutathione were used in the complex formation and all of them were Sigma products. The albumin-thiol regent complex was made using a slight modification of Hopkinson (1975) method by incubating 1 volume fresh bovine plasma albumin solution (30 mg/ml) with 1 volume 10 mM thiol reagent at pH 6.0 for 1 hr at 37°C, and the complex was then examined by cellulose acetate strip electrophoresis.

The cellulose acetate strip (Millipore ESWPO25FT) was cut in dimension of 2.5×5 cm, prebuffered and placed between two cells bathed in sufficient amount of ice. The buffers used barbital, 0.1 M, pH 8.6; potassium phosphate, 0.1 M, pH 8.0 and pH 6.0; Tris-HCl, 0.1 M, pH 8.0 and pH 7.2; sodium citrate, 0.05 M, pH 6.0 and pH 6.0 and pH 4.8; succinate, 0.2 M, pH 6.0 and pH 4.8; sodium acetate, 0.05 M, pH 5.0; borate, 0.1 M, pH 9.4 and pH 8.6; glycine, 0.1 M, pH 11.4 and pH 9.4; sodium carbonate, 0.1 M, pH 10.2 and pH 9.0. The albumin-thiol reagent complexes were migrated for 50 minutes at 100 volts per 7.5 cm. The proteins were stained with Ponceau S. (Sigma) and allowed to be fixed in diluted acetic acid solution. The other conditions for cellulose acetate strip electrophoresis were described previously (Park and Cho, 1972).

RESULTS

Purity of the bovine plasma albumin:

After each purification step, aliquot of the resulting solution was subjected to cellulose acetate electrophoresis in barbital buffer, pH 8.6, ionic strength 0.075, (Kohn, 1968). The final solution was found to contain only the albumin fraction. In an attempt to get a firmer evidence for the purity, the immunodiffusion tests were performed between the crude bovine plasma and the antibody against the purified plasma albumin. Upon immunodiffusion in agar gel, plasma albumin formed single precipitin line which fused smoothly without spurs (Fig. 1), indicating that the final solution contained only the albumin molecules.

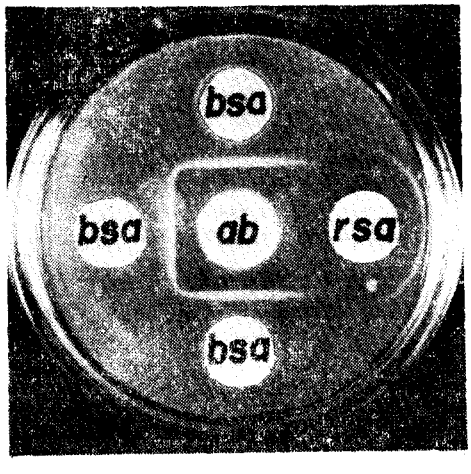

Fig. 1. Immunodiffusion test with antiserum prepared against purified bovine plasma albumin. *ab* means antiserum against the purified bovine plasma albumin; *bsa*, bovine plsama; *rea*, reptilian plasma (*Rhabdophis tigrinus*).

Cellulose acetate electrophoresis of the albumin-thiol reagent complexes in nine different buffers:

Schematized in Fig. 2 are the results of electrophoresis of the albumin-thiol reagent complexes together with the thiol reagent-free albumin in the buffers having maximal buffer capacity at neutral pH. No changes in net charge and in zone number were observed in those complexes of albumin-maleate, albumin-iodoacetate and albumin-iodoacetamide while the albumin-glutathione complex was separated into two proteins with different net charge after electrophoresis in potassium phosphate buffer, pH 8.0 and pH 6.0 and in Tris-HCl buffer, pH 8.0 and pH 7.2. The electrophoresis in barbital buffer, typically used in the cellulose acetate electrophoresis of plasma proteins, showed neither duplicity of albumin-glutathione complex nor separation of the zone of other three albumin-thiol reagent complexes.

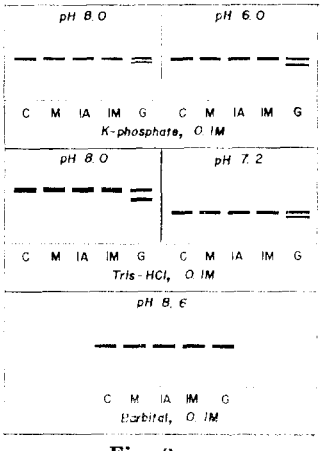

Fig. 2.

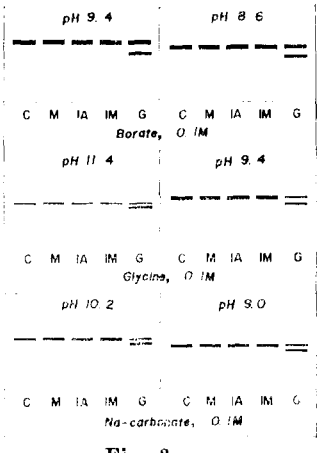

Fig. 3.

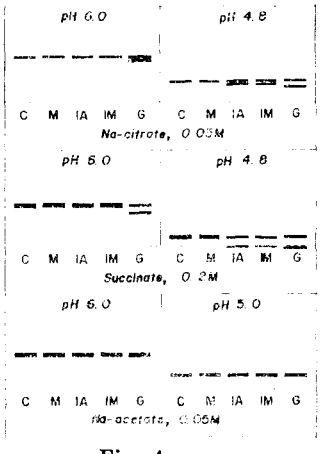

Fig. 4.

Fig. 2. Schematic representations of cellulose acetate electrophoreses of the albumin-thiol reagent complexes in buffers having maximal buffer capacity at neutral pH and in barbital buffer. C means the purified bovine plasma albumin; M, albumin-maleate complex; IA, albumin-iodoacetate complex; IM, albumin-iodoacetamide complex; G, Albumin-glutathione complex. Upper side is the anode.

Fig. 3. Schematic representations of cellulose acetate electrophoreses of the albumin-thiol reagent complexes in alkaline buffers. The complexes were migrated for 20 minutes in glycine buffer, pH 11.4 and in sodium carbonate buffer, pH 10.2 and pH 9.2.

Fig. 4. Schematic representations of cellulose acetate electrophoreses of the albumin-thiol reagent complexes in acidic buffers. No separation of zone was revealed in sodium acetate buffer.

Close inspection of Fig. 3, the results of electrophoresis of those complexes in alkaline buffers, showed that again the separation of albumin-glutathione complex was encountered in borate buffer, pH 9.4 and pH 8.6, glycine buffer, pH 11.4 and pH 9.4 and sodium carbonate buffer, pH 10.2 pH 9.0.

The electrophoresis in acidic buffers, in contrast to those in neutral and alkaline buffers, demonstrated various separation patterns (Fig. 4),

The albumin-glutathione complex was separated into two proteins after the electrophoresis in sodium citrate buffer, pH 6.0 and pH 4.8 and in succinate buffer, pH 6.0 and pH 4.8, whereas only the electrophoresis in sodium citrate buffer, pH 4.8 and in succinate buffer, pH 4.8 were found to separate those complexes of albumin-iodoacetamide into two proteins of which the new one is more positive compared to the native one as in other cases. No separation of the four complexes were revealed in the electrophoresis in sodium acetate buffer, pH 6.0 and pH 5.0.

DISCUSSION

A term microheterogeneity was first used by Foster *et al.* (1965) to describe albumin species which differ with respect to the pH at which they undergo the N-F transition. Removal of tightly bound fatty acids does not eliminate this microheterogeneity (Peters, 1975). McMenamy *et al.* (1971) in their CNBr fragmentation experiments with both fresh and commercial human plasma albumin reported that heterogeneity in disulfide linkages was evident in some preparations. Noel and Hunter (1972) showed the heterogeneity of plasma albumin by fractionation of crystalline bovine albumin on diethylaminoethyl-Sephadex into five fractions.

Based on their experimental results from the electrophoresis of bovine serum albumin, Cann and Goad (1968) formulated a theory of electrophoretic transport for interacting systems of the type

$$P + nHA \rightleftarrows P(HA)n,$$

where Prepresents a protein molecule or other macromolecular ion in solution, and P(HA)n its complex with n moles of a small, uncharged constituent, HA, of the medium, e.g. undissociated buffer acid. It was assumed that the complex migrates in an electric field with an electrophoretic mobility that differs from the mobility of the uncomplexed protein and that equilibrium is established instantaneously. Multiple zones due to protein-buffer interactions have been encountered with carboxylate, glycine, borate, Tris-borate, phosphate-borate and even phosphate buffers; while barbital and Tris buffers appear to be free of these complications (Cann, 1979).

The immunochemical comparison of homologous proteins could provide strong evidences to the similarity of their primary structure (Cocks and Wilson, 1969). Sarich and Wilson (1967), to account for their results of immunological comparisons of primate albumins, proposed the hypothesis that plasma albumin may well serve as an evolutionary clock. This hypothesis has been substantiated by those immunological studies on plasma albumins of iguanid lizards and crocodilians (Gorman *et al.*, 1971) and of some frogs experiencing convergent morphological evolution (Wallace *et al.*, 1971; Maxson and Wilson, 1975). There is, thus, no reason to suspect that the plasma of a typical individual contains albumin species differing in amino acid sequence.

The thiol reagents could alter the net charge of a protein to which it binds. Glutath-

ione, iodoacetate and maleate would make the protein with free sulfhydryl group have more negative charge compared to the native protein (Hopkinson, 1975). Assuming that there are several molecular species differing in amino acid sequence in albumin fraction demonstrated after eletrophoresis and that each of those molecules has different number of free sulfhydryl group, the incubation of albumin with the thiol reagent would result in the separation of electrophoretic zones into up to the number of molecular species. In this experiment, however, only two zones are shown after electrophoresis and the new zone arising after the incubation and subsequent electrophoresis is more positive net charge compared to the original zone, suggesting that there are at least two protcins in the albumin fraction.

It is generally accepted that bovine plasma albumin is a simple protein and a single peptide chain of about 580 residues. Albumin is one of the few extracellular proteins having a free thiol group (Jocelyn, 1972). The reactivity of the sulfhydryl of $CySH_{34}$ is distinctive and deserves consideration in light of its involvement in exchange reactions (Peters, 1975). It might, thus be reasonable to regard the electrophoretic albumin fraction as the molecular entities composed of at least two molecular species with different conformation in which there is intramolecular exchange of disulfide bridges.

SUMMARY

Plasma albumin was purified from the fresh bovine blood using a minor modification of the polyethyleneglycol and ethanol procedure. The resulting protein solution was tested for its purity by both electrophoretic and immunochemical methods and found to contain only the albumin molecules.

Each of the four thiol reagents, maleate, iodoacetate, iodoacetamide and glutathione, was incubated with the purified plasma albumin. The electrophoresis on cellulose acetate of those complexes in various buffers with different component and pH demonstrated that the albumin-glutathione complex was separated into two zones in all buffers used except the barbital and sodium acetate buffers, that the complexes of albumin-iodoacetate and albumin-iodoacetamide also into two zones only in pH 4.8 citrate buffer and in pH 4.8 succinate buffer and that the new zone had more positive net charge compared to the native protein in any case.

These results might suggest a possibility that the electrophoretic albumin fraction is composed of at least two molecular species with different conformation.

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