The Microheterogeneity of Plasma Albumin. VII. An Investigation by the Equilibrium Salting-Out Method of the Origins of Microheterogeneity

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ABSTRACT: The solubility–Σ method, described in the preceding paper, has been utilized in attempts to determine the structural basis of the microheterogeneity of bovine plasma albumin. Monomeric charcoal-defatted crystallized bovine plasma albumin, with the sulfhydryl groups blocked to prevent disulfide interchange, was denatured in 6 M guanidine hydrochloride and renatured by exhaustive dialysis against progressively decreasing concentrations of the denaturant. The solubility–Σ profile was not affected by this treatment. Two subfractions of the same protein, prepared by fractional precipitation in $(NH_4)_2SO_4$, showed distinctly different solubility–Σ profiles and retained their identity through this denaturation–renaturation treatment. These results support the earlier conclusion, based on the solubility–pH method, that microheterogeneity is not due to three-dimensional folding *per se*, but must have a covalent origin. Bovine plasma albumin undergoes a broadening of the solubility–Σ profile under conditions conducive to disulfide interchange. Subfractions of bovine plasma albumin each undergo such a broadening of their profile but they fully retain their identity. There appears to be no tendency for the profiles to merge during disulfide randomization. It is tentatively concluded from this result that the differences between subfractions cannot be ascribed to variations in disulfide pairing alone and other differences in covalent structure must exist in the microheterogeneous population. A sample of bovine plasma albumin defatted by heptane–acetic acid extraction, reported by McMenamy and Lee to be free of microheterogeneity, was subfractionated into two fractions having drastically different solubility in ammonium sulfate.

In the preceding paper of this series (Wong and Foster, 1969) we have described a membrane equilibrium salting-out method for the demonstration of microheterogeneity in proteins. Data were plotted as f_s *vs.* Σ, where f_s is the fraction of the protein remaining in solution and Σ the concentration of $(NH_4)_2SO_4$ in dialytic equilibrium with the partially precipitated protein solution. These plots, termed solubility–Σ profiles, appear to measure intrinsic microheterogeneity of BPA; that is, they are insensitive to bound impurities such as free fatty acids.

The basic structural cause of microheterogeneity in BPA remains uncertain. Moore and Foster (1968) showed that the distribution of species, as measured by the earlier solubility–pH profile method, is not altered by reversible denaturation in 6 M guanidine hydrochloride and concluded that microheterogeneity is not due to differences in three-dimensional folding *per se*. Sogami *et al.* (1969) showed that the distribution of species is broadened under conditions favoring sulfhydryl-catalyzed disulfide interchange and suggested that randomization of disulfide pairing might be at least a partial cause of microheterogeneity. The present study takes advantage of the solubility–Σ method to explore these questions more carefully. The results support the conclusions of Moore and Foster (1968) and of Sogami *et al.* (1969) but suggest further that while disulfide pairing may contribute it cannot be the sole cause of microheterogeneity. By implication there must exist covalent differences between microheterogeneous species other than variations in disulfide pairing.

Experimental Section

Materials. Mann's Ultra-Pure grade guanidine hydrochloride was used for all denaturation experiments. A 6.0 M solution had an absorbance of less than 0.2 down to 225 mμ. Crystallized BPA (Armour Lot B70411) was charcoal defatted and freed of dimer and higher polymers by Sephadex gel chromatography as described in the previous paper (Wong and Foster, 1969). All other chemicals were the same as described in that paper.

Denaturation and Renaturation. BPA was denatured with 6 M guanidine hydrochloride. Most experiments were conducted with 0.1–0.5% BPA solutions at room temperature. Relatively concentrated solutions of the denaturant were prepared (approximately 8 M), the solvent being water or 0.1 M $(NH_4)_2SO_4$, and the pH was adjusted to 5.3–5.4. The calculated amount of concentrated protein solution was then added to give a BPA solution which was 6 M guanidine hydrochloride, 0.1 M $(NH_4)_2SO_4$, and of pH 5.3–5.4. Air was excluded by flushing the reaction vessel with nitrogen gas during the denaturation period, which was usually 3 hr.

Renaturation of the protein was achieved by dialysis against fivefold volumes of, successively, 5, 4, 3, and 2 M guanidine

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[1] Abbreviations used that are not listed in *Biochemistry 5*, 1445 (1966), are: BPA, bovine plasma albumin; IA, iodoacetamide; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid).

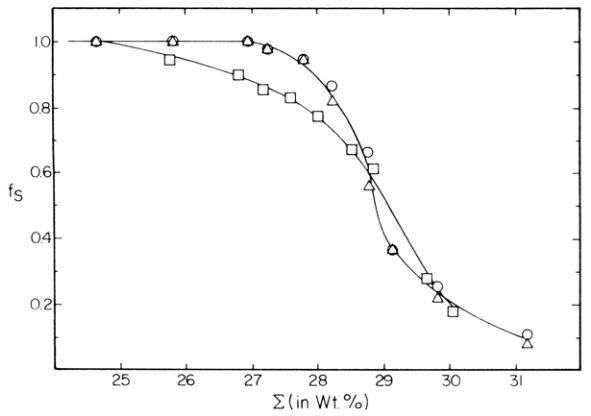

FIGURE 1: Comparative solubility–Σ profiles of BPA showing effect of sulfhydryl blocking. Circles, control nonblocked BPA; triangles, BPA with sulfhydryl blocked by iodoacetamide; rectangles, BPA with sulfhydryl blocked by exchange with cystine.

hydrochloride solution, each dialysis being for 4 hr at room temperature. The protein was then dialyzed exhaustively against a 40-fold volume of 0.1 M $(NH_4)_2SO_4$ solution in the cold room with four changes of 0.1 M $(NH_4)_2SO_4$. Mechanical agitation to facilitate the dialysis process was achieved by the use of magnetic stirrers. Concentration of guanidine hydrochloride was determined by refractive index, employing an Abbé-type precision sugar refractometer manufactured by Bausch & Lomb Optical Co. A calibration curve was established using data reported earlier (Kielly and Harrington, 1960).

Alkaline Aging of BPA. Aging experiments were conducted on 0.5% isoionic protein solutions by adjusting the pH to approximately 7.5 with dropwise addition of 0.1 M KOH. The solution was then kept in a cold room for 3 days.

Sulfhydryl-Blocked BPA. Two methods were used to prepare sulfhydryl-blocked BPA. The blocking of the reactive sulfhydryl group by cystine was based on the study of Andersson (1966). A threefold molar excess of cystine was dissolved in a 3–4% isoionic solution of BPA. Enough Tris was added to make the solution 0.2 M. Dilute HCl was added simultaneously to adjust the solution to pH 8.0. The solution was stirred slowly for 4 hr at room temperature and then kept in a cold room for another 7 or 8 days. Excess cystine was removed by dialysis against a 30-fold volume excess of 0.1 M $(NH_4)_2SO_4$. Blocking with IA was done in the following manner: To a 1% BPA solution in 0.1 M $(NH_4)_2SO_4$ and at pH 7.2 was added the desired amount of IA (usually 1.4 moles/mole of protein). After standing for 20–24 hr in the cold room, this solution was exhaustively dialyzed against 0.1 M $(NH_4)_2SO_4$. The final pH was adjusted to 5.3 by addition of 0.1 M H_2SO_4. The sulfhydryl content after blocking was assayed by the DTNB method as described earlier (Wong and Foster, 1969). All preparations showed virtually no free sulfhydryl to be present.

Other Techniques. The details of the procedure for determination of the solubility–Σ profiles as well as other techniques employed have been described in the preceding paper (Wong and Foster, 1969). The profiles are presented as f_s, the fractional amount of the protein remaining in solution, as a function of Σ, the weight concentration of $(NH_4)_2SO_4$ in an aqueous solution in dialytic equilibrium with the protein solution.

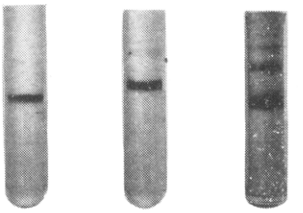

FIGURE 2: Disc gel electrophoresis patterns of BPA and sulfhydryl-blocked BPA following the denaturation–renaturation treatment described in the text. (A) IA-blocked BPA; (B) BPA blocked by exchange with cystine; (C) unblocked BPA. All gels contained approximately 40 μg of protein. Migration was from the top (anode) toward the bottom (cathode) for approximately 50 min at 4–5 mA/tube.

Results

Effect of Blocking of the Sulfhydryl Group on the Solubility–Σ Profile. Before subjecting BPA to denaturation in concentrated guanidine hydrochloride it is essential that the sulfhydryl group be blocked to prevent drastic broadening of the microheterogeneity due to sulfhydryl-catalyzed disulfide interchange. Moore and Foster (1968) employed blocking with IA for this purpose, and found that such blocking did not alter the solubility–pH profile of the protein. Since the "natural" blocking reagent present in the bulk of the nonmercaptalbumin is cysteine, it might seem preferable to employ disulfide interchange between the free sulfhydryl groups and cystine (Andersson, 1966) as a means of blocking these groups. Figure 1 shows solubility–Σ profiles for BPA blocked in both of these ways in comparison with the profile for nonblocked BPA. Blocking with IA has no discernible effect on the solubility–Σ profile, as was anticipated from the work of Moore and Foster (1968). Surprisingly, cystine blocking results in a drastic broadening of the profile, implying a substantial increase in the microheterogeneity of the protein. No attempt was made to elucidate the cause of this effect; however, it may be conjectured that it results from intramolecular disulfide interchange catalyzed by the cysteine which is a product of the primary exchange reaction. Alternatively, it is possible that other unknown reactions are taking place with other reactive groups in the protein. It is of interest that no detectable change in the disc gel electrophoresis pattern of the protein accompanied this alteration. BPA blocked by either method yielded a single band on disc electrophoresis. Disc gel patterns obtained on these derivatives following denaturation–renaturation as described in the Experimental Section are shown in Figure 2.

Reversible Denaturation in 6 M Gu·HCl. Figure 3 shows solubility–Σ profiles for BPA and for two subfractions obtained therefrom, all samples being in the IA-blocked form. In all cases results for undenatured controls are shown together with results on the corresponding samples which had been subjected to reversible denaturation and renaturation as described in the Experimental Section. It is worth noting that in all cases disc gel electrophoresis showed a single sharp band, both before and after denaturation. By contrast, denaturation and renaturation of an unblocked sample led to a multiplicity of bands. Disc gel patterns obtained following denaturation–renaturation are shown in Figure 2. Thus, in accord with the findings of Moore and Foster (1968), IA blocking is suffi-

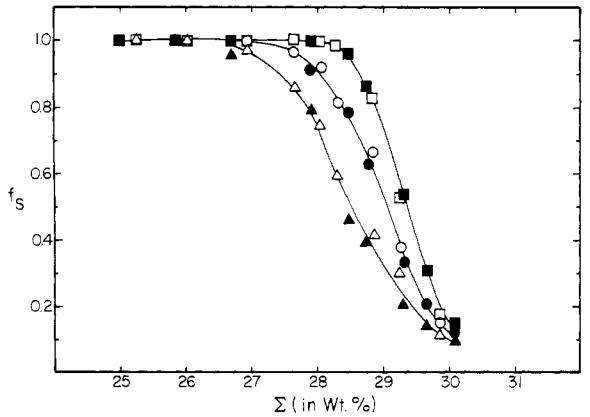

FIGURE 3: Comparative solubility–Σ profiles of IA-blocked BPA before and after reversible denaturation in 6 M guanidine hydrochloride. Open circles, control sample; filled circles, regenerated control BPA; open rectangles, subfraction III; filled rectangles, regenerated subfraction III; open triangles, subfraction I; filled triangles, regenerated subfraction I.

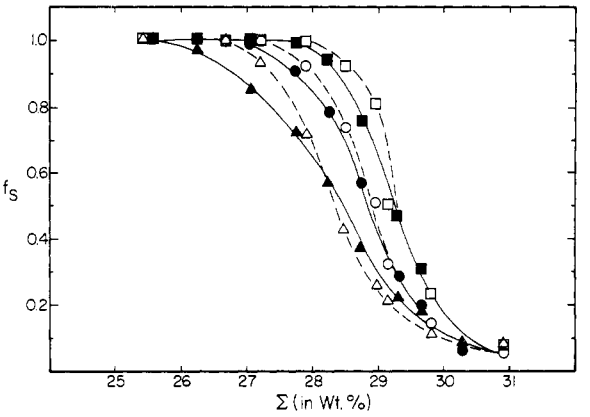

FIGURE 5: Comparative solubility–Σ profiles of nonblocked BPA and subfractions showing effect of aging. Open circles, control sample; filled circles, aged control sample; open rectangles, subfraction III; filled rectangles, aged subfraction III; open triangles, subfraction I; filled triangles, aged subfraction I.

cient to prevent aggregation during the denaturation–renaturation procedure. Figure 3 demonstrates clearly that in each case denaturation is truly reversible as judged by the solubility–Σ profile method. Similar results obtained with BPA blocked by exchange with cystine and two subfractions obtained from this sample are shown in Figure 4. The cystine-blocked protein is extremely microheterogeneous, as mentioned above, but again the denaturation process appears to be fully reversible.

Effect of Aging on the Solubility–Σ Profile. Sogami *et al.* (1969) have called attention to the fact that a substantial broadening of the solubility–pH profile of BPA may occur on aging the protein in solution, especially at slightly alkaline pH and at low ionic strength. Using the solubility–Σ criterion we have found that unblocked protein experienced a substantial increase in microheterogeneity on aging at pH 7.5 and IA blocking successfully prevented the alteration. The results of similar aging experiments conducted on two extreme subfractions of BPA are shown in Figure 5. It was anticipated that if variations in disulfide pairing were the main or sole cause of microheterogeneity, solubility profiles of the subfractions might tend to merge on aging. This is clearly not so. To a first approximation, aging appears to result in a broadening of each subpopulation about its midpoint. The two subfractions and the control all preserve their identity on subsequent aging.

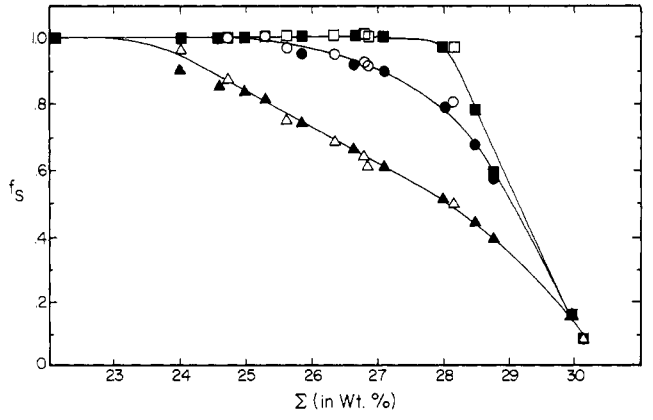

FIGURE 4: Comparative solubility–Σ profiles of Cys-blocked BPA before and after denaturation in 6 M guanidine hydrochloride. Open circles, control sample; filled circles, regenerated control BPA; open rectangles, subfraction III; filled rectangles, regenerated subfraction III; open triangles, subfraction I; filled triangles, regenerated subfraction I.

Microheterogeneity of Heptane–Acetic Acid Extracted BPA. McMenamy and Lee (1967) have contended that the microheterogeneity of BPA is due to bound contaminants and that protein which they defatted by means of heptane–acetic acid extraction is not microheterogeneous. We have examined a sample of protein so purified, kindly supplied by Dr. McMenamy, by means of the solubility–Σ method. Unfortunately, the amount of protein available did not permit as complete a study as would have been desirable. However, this protein was separated into two subfractions by equilibration against an ammonium sulfate solution of Σ = 28.2. A portion of the unfractionated protein and each of the subfractions were then dialyzed against a common solution of ammonium sulfate (Σ = 27.66) and the fraction of protein remaining in solution was determined. The results, shown in Table I, clearly show that the subfractions differ in their solubility, one being considerably more soluble and the other less soluble than the

TABLE I: Demonstration of Microheterogeneity in Hexane–Acetic Acid Extracted BPA Sample of McMenamy.[a]

Sample	f_s (at Σ = 27.66)
Control (unfractionated)	0.85
Fraction I	0.64
Fraction II	0.99

[a] Two fractions were prepared by equilibration of the sample against $(NH_4)_2SO_4$ solution of Σ = 28.2. The two subfractions and the unfractionated control were then equilibrated against a common solution of Σ = 27.66.

control (unfractionated) sample. Clearly the solubility–Σ method is capable of demonstrating substantial microheterogeneity in this preparation.

Discussion

The concept that the primary structure of a protein completely dictates a unique three-dimensional conformation has received widespread acceptance among protein chemists. However, it is not inconceivable that some proteins might possess a multiplicity of pseudostable structures and that the distribution among these would be kinetically controlled. Indeed, we have suggested this as one of the possible causes of the microheterogeneity of plasma albumins (Foster *et al.*, 1965). This possibility was investigated by Moore and Foster (1968) by subjecting BPA and subfractions of BPA to exhaustive denaturing conditions and renaturing. These results indicated that so long as disulfide interchange is prevented by blocking the sulfhydryl group of the protein, denaturation is fully reversible as judged by solubility–pH profiles. In particular, various subfractions were found to retain their identity even on denaturation in 6 M guanidine hydrochloride. Tanford and coworkers (Tanford *et al.*, 1967a,b; Nozaki and Tanford, 1967; Lapanje and Tanford, 1967) have presented a variety of experimental data to support their contention that 6 M guanidine hydrochloride completely destroys the three-dimensional structure of most proteins, including BPA. Moore and Foster were thus led to the conclusion that microheterogeneity has its origin in covalent structural features which are not affected by the denaturation media.

The present results confirm and strengthen this conclusion. The differences in solubility–Σ profile between the subfractions both before and after denaturation, as shown in Figure 3, are unmistakable. This is particularly true since these profiles were obtained by the comparative solubility method; that is, the various preparations were dialyzed simultaneously against the same Σ solutions to provide better control of experimental conditions (Wong and Foster, 1969). In addition, the renaturation conditions employed in the present study were designed to provide a more adequate opportunity for release and removal of any noncovalently bound contaminants. This was done by dialyzing progressively against decreasing concentrations of the denaturant. To the extent to which 6 M guanidine hydrochloride does indeed erase all memory of the native folded structure the conclusion appears to be inescapable that microheterogeneity resides in differences in covalent structure.

One covalent structural feature which has been suspected of contributing to the microheterogeneity of BPA is the pairing of half-cystine residues through disulfide linkage. Since this protein contains a large number (17–18) of disulfide bonds and a single unpaired cysteine residue the number of conceivable pairings is very large indeed. We have presented evidence (Sogami *et al.*, 1969) that the microheterogeneity of BPA can be increased dramatically under conditions which are conducive to sulfhydryl-catalyzed disulfide interchange, namely, aging in solution at pH somewhat above neutrality. Indeed, some broadening of the solubility–pH profiles was demonstrated at the isoionic pH (near 5.0) in salt-free solutions of carefully defatted protein. The effect was shown to be largely eliminated by blocking the free sulfhydryl group of the protein.

The present results reinforce these earlier conclusions. Even more importantly, however, they provide suggestive evidence that disulfide pairing may not be the only feature of covalent structure which is responsible for microheterogeneity. It seems clear that if variations in disulfide pairing constituted the sole difference between various species of the microheterogeneous population, different subfractions should tend to lose their identity upon undergoing disulfide interchange. It would be expected that different subfractions ultimately would tend to approach the same most probable distribution of disulfide pairings. The results presented in Figure 5 suggest that this does not happen. Each fraction undergoes broadening, but there is no tendency for the curves to merge.

We conclude that the microheterogeneity of BPA can potentially arise at three different levels. Unquestionably, there are extrinsic contributions from bound fatty acids or other contaminants (McMenamy and Lee, 1967; Sogami and Foster, 1968). The present studies strongly suggest, however, that there are other "intrinsic" variations which resist exhaustive denaturation by guanidine hydrochloride and which must be covalent in origin. In part these may be variations in disulfide pairing, at least in protein samples which have had the opportunity to undergo disulfide interchange. The present results suggest, moreover, that there must exist additional variations in covalent structure. Possibly these variations result from covalent modifications of functional groups. A circulating protein such as plasma albumin might be a prime candidate for such "molecular aging." One interesting example of such a modification, namely, acetylation of human plasma albumin when exposed to acetylsalicylic acid under physiological conditions, has recently come to light (Hawkins *et al.*, 1968).

Finally, however, the possibility of variations in the primary amino acid sequence *per se* should not be ignored. This could arise through the mechanism proposed by Von Ehrenstein (1966) who finds variations in the sequence of the α chain of rabbit hemoglobin. Alternatively, however, the possibility must be considered that primary sequence variations result from individual or genetic variations since our experiments were performed with a protein sample obtained from pooled blood representing many individual animals. We have concluded previously (Foster *et al.*, 1965; Foster, 1968) that albumin preparations from individuals are at least as microheterogeneous as those from pooled blood samples. However, it is clear now that this might have resulted from extrinsic contributions (such as fatty acid contaminants) or from variations in disulfide pairing which could have masked contributions of the primary structure *per se*. Clearly, it is now desirable to extend studies, by the highly sensitive technique used here, to properly purified albumin samples obtained from single individuals.

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Computed Circular Dichroism Spectra for the Evaluation of Protein Conformation*

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ABSTRACT: Circular dichroism curves of poly-L-lysine containing varying amounts of α helix, β-pleated sheet, and random coil segments have been computed in the 190–250-mμ region. The application of these curves for determining protein conformation is discussed. The circular dichroism curves of several proteins, whose three-dimensional structures are known from X-ray diffraction studies, have been fitted by a linear combination of the three reference structures in the 208–240-mμ region. The results show that these computed curves are very useful in predicting protein structure, and if the protein possesses a high degree of secondary structure, the agreement between the calculated and the X-ray diffraction determined structure is extremely good. If the protein is largely nonregular, the results are less satisfactory but are still informative. The results show that the use of circular dichroism is a decided improvement over the use of optical rotatory dispersion for the evaluation of protein conformation.

In an attempt to evaluate the conformation of proteins we have previously reported computed optical rotatory dispersion curves for theoretical combinations of experimentally obtained curves for the α helix, the random coil, and the antiparallel pleated-sheet (β structure) conformations of poly-L-lysine (Greenfield *et al.*, 1967). These generated curves were compared with experimental spectra of lysozyme and myoglobin and it was found that curves which gave the best fit overestimated the β content and underestimated the α-helix composition, known from X-ray diffraction studies. The differences observed were attributed to aromatic side-chain chromophores, disulfide-bridge contributions, prosthetic group contributions, and possibly contributions from conformations of the amide bonds other than those in the three reference conformations. More recently Magar (1968) has used a more precise method of minimizing the variance between theoretical and experimental optical rotatory dispersion curves, but he essentially reached the same conclusions as Greenfield *et al.* (1967).

In this paper the computed spectra for conformational variation are reported for the circular dichroism spectra of poly-L-lysine based on the experimentally obtained curves for the three standard conformations: α, β, and random coil. These computed spectra are compared with the experimentally obtained spectra of several proteins whose conformations in the crystal state are known from X-ray diffraction studies. It was hoped that by using circular dichroism, which has less overlap of transitions than does optical rotatory dispersion, it would be possible to obtain closer fits of the spectra to those calculated from the crystal state conformations. The use of circular dichroism in the study of proteins has been recently reviewed by Beychok (1968).

Since our earlier optical rotatory dispersion study there have been several theoretical papers on the optical rotatory dispersion properties of polypeptides and simpler amides and there has been considerable doubt expressed that proteins can be analyzed adequately by use of only three parameters. Woody and Tinoco (1967) have calculated the theoretical optical activity of the α helix and observed that the rotatory strength of the π–π^* transition should be greatly dependent upon chain length. Scheraga's group (Vournakis *et al.*, 1968) and Tinoco *et al.* (1963) have calculated that the rotational strength of the n–π^* transition at 222 mμ of the α helix should also be chain length dependent. Urry (1968a) did not

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