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Different Types of Microheterogeneity of Human Thyroxine-Binding Prealbumin†

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Received June 17, 1986; Revised Manuscript Received December 9, 1986

ABSTRACT: Human thyroxine-binding prealbumin or transthyretin has been isolated by three different methods. In all cases, the isolated material is microheterogeneous in an isoelectric focusing system, revealing a pattern of at least 10 bands. These subforms represent tetramers. Dissociation of the protein in serum samples and dissociation in the presence of urea or sodium dodecyl sulfate (SDS) reveal eight other forms, differing in isoelectric point and concluded to be monomers. Two different sets of dissociated forms are identified, one from urea treatment and the other from SDS treatment. The latter set is apparently also present in serum. Interpretations are complicated by multiplicities of all forms, atypical electrophoretic migrations, nonidentical effects of urea and SDS, and the absence of dimer under most conditions. However, reassociations of monomers and formation of interspecies hybrid molecules identify dimers and clarify interpretations of the dissociated sets. In at least five of the forms likely to represent monomers, the difference is dependent on the nature of the SH group at Cys-10, which can be oxidized, can be reduced, or can be in a mixed disulfide, probably with glutathione and its degradation products. Amino acid sequence analysis reveals an additional N-terminal heterogeneity (with start at positions 1, 2, and 3) of the three most abundant monomers, but this does not explain the observed differences among monomeric forms. It is concluded that prealbumin exhibits different types of microheterogeneity, the major pattern of which is explained by the status of the subunit SH group and the ability of prealbumin to dissociate into monomers. A relation appears to exist between SH group status on the one hand, and retinol-binding protein interaction, the ability to dissociate into monomers, and possibly also binding of thyroxine on the other. These interactions suggest complex functional relationships for prealbumin in serum.

Three different binding proteins for the thyroid hormone thyroxine occur in blood, thyroxine-binding globulin, thyroxine-binding prealbumin, and albumin. Of these three proteins, thyroxine-binding globulin has long been considered to be the most important thyroxine carrier (Woeber & Ingbar, 1968; Gordon & Coutsoftides, 1969; Pages et al., 1973; Wosilait, 1977; Cheng et al., 1977; Andrea et al., 1980; Yamamoto et al., 1980). However, it is now known that, under physiological conditions, prealbumin and thyroxine-binding globulin are of almost equal importance for thyroxine transport; 40–45% of the total thyroxine is bound to prealbumin, 45–50% to thyroxine-binding globulin, and 5–10% to albumin (Pettersson & Carlström, 1979). This suggests a higher affinity of prealbumin for thyroxine than has been anticipated from in vitro studies. The discrepancy calls for further studies of prealbumin and its interactions.

The prealbumin tetramer consists of four identical subunits (Blake et al., 1978). The amino acid sequence of the 127-residue subunit is known (Kanda et al., 1974). Two genetic variants involving amino acid substitutions (Val-30 → Met, Gly-49 → Thr) have been reported in patients suffering from familial amyloidotic polyneuropathy (Dwulet & Benson, 1983; Pras et al., 1983). An electrophoretic microheterogeneity of human prealbumin has also been reported (Altland et al., 1981; Felding & Fex, 1984). X-ray chrystallographic studies show that thyroxine is bound in a channel passing through the tetramer (Blake et al., 1978) and suggest the presence of two identical binding sites per tetramer, one at each end of the channel. However, thyroxine is known to bind to the prealbumin tetramer in a molar ratio of 1:1, suggesting a negative cooperativity (of unknown nature) between the two sites (Ferguson et al., 1975). In this respect, the complete identity of the subunits could be questioned. This is further substan-

† This work was supported by the Swedish Medical Research Council (Projects 03X-6231 and 03X-3532) and the Swedish Cancer Society (Project 1806).

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0006-2960/87/0426-4572$01.50/0

tiated by the different types of microheterogeneity of prealbumin now reported.

The aim of this work has been to investigate whether the human prealbumin behaves differently in the serum environment than in the purified form, with special regard to quaternary structure, thyroxine binding, thiol reactivity, and retinol-binding protein interaction. The microheterogeneity has been studied under nondenaturing conditions or in the presence of denaturing agents (urea or SDS[1]) and has been related to the behavior of the protein in the presence of serum. Finally, the observed heterogeneities have been found to be related to the status of the SH group of Cys-10 of the prealbumin subunit. The studies have required combinations of different electrophoretic techniques, such as isoelectric focusing, crossed immunoelectrophoresis, and gradient polyacrylamide gel electrophoresis in two-dimensional runs involving nondenaturing and denaturing (urea or SDS) conditions, or the presence of serum.

Materials and Methods

Protein and Pretreatments

Isolation of Human Thyroxine-Binding Prealbumin. Prealbumin was purified from pooled human serum by three methods.

Method 1 essentially followed the method introduced by De Nayer et al. (1966) utilizing phenol precipitation as the first step. The optimal concentration of phenol was 4.6% w/v, and the addition of phenol to the serum mixed with salt (200 g of NaCl/L) was made dropwise under vigorous stirring at 0 °C. All further steps were made at 4 °C. Anion-exchange chromatography on DEAE-Sepharose (Pharmacia Fine Chemicals) was performed in 0.05 M Tris-HCl, pH 7.2, with a gradient of NaCl from 0.05 to 0.32 M. The final step was preparative polyacrylamide gel electrophoresis in short columns with a discontinuous buffer system (concentrating gel, 3% polyacrylamide, 0.0575 M Tris–H_3PO_4, pH 7.2; resolving gel, 12.5% polyacrylamide, 0.375 M Tris-HCl, pH 8.9; cathode buffer, 0.05 M Tris–glycine, pH 8.9; anode buffer, 0.1 M Tris-HCl, pH 8.1; eluting buffer, 0.2 M Tris-HCl, pH 8.1).

Method 2 was based on direct affinity chromatography on human retinol-binding protein attached to Sepharose 4B (Pharmacia Fine Chemicals). The retinol-binding protein was isolated by affinity chromatography on Sepharose–prealbumin (Fex & Hansson, 1979) of urine collected from patients with renal failure; this binding protein was saturated with retinol before coupling. The column was equilibrated with 0.04 M Tris-HCl, pH 7.4, 0.5 M NaCl, and 2.0 mM ethylenediaminetetraacetic acid (EDTA). Serum was directly applied to the column which was then washed with the equilibration buffer until the absorbance at 280 nm was constant. Prealbumin bound was eluted with distilled water and was further purified by preparative polyacrylamide gel electrophoresis as above.

Method 3 utilized direct agarose electrophoresis in 0.075 M barbital buffer, pH 8.6 (Johansson, 1972). The material in the position corresponding to prealbumin was cut out, and the protein was eluted or directly submitted to isoelectric focusing.

Isolation of Homogeneous Prealbumin Tetramers. Prealbumin, purified according to method 1 was submitted to isoelectric focusing, pH 6.5–4.0, in the presence of 7.0 M urea, producing monomeric prealbumin forms (cf. below). The fractions at isoelectric points 5.7 (M1; cf. nomenclature below) 5.3 (M2), and 5.45 (M3) were cut out and separately eluted with 0.04 M Tris-HCl, pH 7.4, 0.5 M NaCl, and 2.0 mM EDTA. Urea and ampholytes were removed by affinity chromatography on retinol-binding protein–Sepharose, producing reassembled prealbumin tetramers homogeneous in M1, M2, or M3, respectively.

Carboxymethylation. Carboxymethylation of Cys-10 before and after reduction with dithiothreitol (10-fold molar excess over total SH, 4 h, 25 °C, under N_2) was carried out in 0.1 M Tris-HCl, pH 8.15, and 2 mM EDTA, by incubation for 3 h at 37 °C with iodoacetate (40–120-fold molar excess over total SH). Reagents were removed by exclusion chromatography on Sephadex G-25 (fine) in water. ^{14}C-Labeled iodoacetic acid was from New England Nuclear.

Analytical Methods

Isoelectric Focusing. This was performed in 1% Iso-Gel agarose (Marine Colloids), agarose Z (LKB), or a mixture of both (in a ratio of 1:1, for gels containing urea). Ampholytes were from Pharmacia Fine Chemicals (Pharmalyte series with pH intervals 6.5–4.0 or 5.4–4.5). The electrode solutions were 0.1 M β-alanine for the cathode and 0.1 M glutamic acid and 0.5 M phosphoric acid for the anode. Focusing was performed under nondenaturing or denaturing (7.0 M urea) conditions, and the gradient was always prefocused (2 h) before application of samples. The urea for the focusing gel was of analytical grade (Merck) and that for incubation of samples was of ultrapure quality (BDH, Aristar). The focusing gel was fixed for 3 h in sulfosalicylic acid (5%) and trichloroacetic acid (10%) and stained with 0.4% (w/v) Coomassie Brilliant Blue R (Sigma) in 35% ethanol and 20% acetic acid. For a second dimension of isoelectric focusing, crossed immunoelectrophoresis, or gradient polyacrylamide gel electrophoresis, unfixed strips were cut out and transferred to the second gel.

Crossed Immunoelectrophoresis. Serum submitted to isoelectric focusing was analyzed for prealbumin by crossed immunoelectrophoresis against specific anti-prealbumin antiserum (Dakopatt immunoglobulin) in 0.8% agarose gels (Sea Kem Me agarose; Marine Colloids) with 0.075 M barbital buffer, pH 8.6, containing calcium lactate and azide (Ganrot, 1972). The agarose type used for crossed immunoelectrophoresis differs in electroendosmosis from that used in isoelectric focusing (above). The different types are essential for optimal performance of the two methods. For the crossed immunoelectrophoresis, the protein from the isoelectric focusing strip was first transferred by a run for 20 min into an antibody-free section of the immunoelectrophoresis gel. Subsequently, the isoelectric strip was replaced by Sea Kem Me agarose before continuation of the immunoelectrophoresis.

Immunofixation. Agarose (0.8% Sea Kem Me agarose in 0.075 M barbital buffer, pH 8.6), containing antiserum, 0.2 mL/cm^2, was cast (1 mm thick) and placed on top of the isoelectric focusing gel (also in the case of the urea-containing gel). Diffusion was allowed for 24 h in a moist chamber at room temperature. The focusing gel was then washed several times in 0.9% NaCl before staining.

Gradient Polyacrylamide Gel Electrophoresis. Gradient (10–15%) polyacrylamide gel electrophoresis was carried out with a discontinuous buffer system as described (Laemmli, 1970), under both nondenaturing and denaturing conditions (with SDS or urea).

Two-dimensional electrophoresis (isoelectric focusing–gradient polyacrylamide gel electrophoresis) was performed by

[1] Abbreviations: DEAE, diethylaminoethyl; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); EDTA, ethylenediaminetetraacetic acid; Gdn-HCl, guanidine hydrochloride; GSH, reduced glutathione; GSSH, oxidized glutathione; SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)-aminomethane.

Table I: Isoelectric Points and Designations of Prealbumin Forms in Purified Preparations and in Serum under Denaturing and Nondenaturing Conditions (Dominating Patterns in Italics)

isoelectric points	serum and purified PA, denaturing conditions[a]	serum PA, nondenaturing conditions[b]	purified PA, nondenaturing conditions[c]
5.7	*M1*		
5.45	M3		
5.4		*M1a*	M1a
5.3	*M2*		
5.25		*M1b*, M1c	M1c
5.2		M2a	
5.15		M2b	M2b
5.1	M4	M2c	
4.95–4.6		tetramers	*tetramers*

[a] Pattern in Figure 2. [b] Pattern in Figure 1B, bottom, and Figure 7. [c] Pattern in Figure 1A.

placing agarose strips from the focusing gel onto the spacer gel of the gradient polyacrylamide gel.

Chromatography on Sephacryl S-200. Exclusion chromatography on Sephacryl S-200 (Pharmacia Fine Chemicals) under denaturing (3.0 M urea) and nondenaturing conditions was carried out at pH 7.4 and 6.5 (0.05 M phosphate, 0.15 M NaCl) and pH 5.5 and 4.8 (0.05 M acetate, 0.15 M NaCl). Molecular size markers were albumin and retinol-binding protein.

Hybridization. Purified human and porcine prealbumins were incubated at equal concentrations in 0.1 M Tris-HCl buffer, pH 8.0, in the absence or presence of 1 M urea for 6, 12, 24, or 48 h. The hybrids were resolved and separated from the incubation mixture by agarose electrophoresis at pH 8.6.

Amino Acid Sequence Analysis. Degradations were performed in a Beckman 890 D liquid-phase sequencer in the presence of glycine-precycled polybrene and utilizing reverse-phase high-performance liquid chromatography for identification of phenylthiohydantoin derivatives.

Nomenclature

Purified prealbumin refers to the protein isolated from serum by one of the three methods described above.

Serum prealbumin refers to the protein as it appears when serum is directly submitted to nondenaturing isoelectric focusing.

Native prealbumin tetramers are of heterogeneous composition in terms of the different monomeric forms. *Homogeneous prealbumin tetramers* are obtained after reassembling identical monomeric forms isolated under denaturing conditions. Denaturing and nondenaturing conditions refer to the presence or absence of urea if nothing else is stated.

The observed forms of prealbumin are summarized in Table I, together with the isoelectric points. Tetrameric forms occur within the pH range 4.95–4.60. Other forms are designated M1, M2, M3, and M4 (monomers, cf. below) in the urea denaturing system and M1a, M1b, M1c, M2a, M2b, and M2c (also concluded to represent dissociated forms, cf. below) in the nondenaturing system, respectively. The latter forms are named because of relationships with M1 and M2, respectively.

RESULTS

Multiple Forms of Prealbumin

Charge Differences under Nondenaturing Conditions. Purified prealbumin is microheterogeneous, with at least 10 fractions in the pH range 4.95–4.60 upon isoelectric focusing (Figure 1A). Expansion of the pH gradient reveals each of the 10 subfractions to be further heterogeneous; up to 32 bands

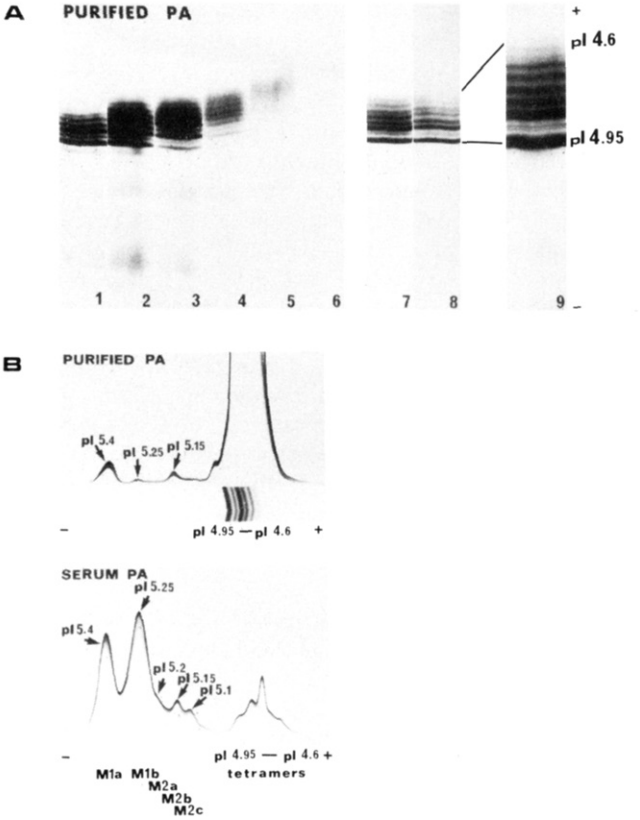

FIGURE 1: Microheterogeneity patterns of prealbumin (PA) under nondenaturing conditions. (A) Isoelectric focusing of different prealbumin preparations in the pH gradient 6.5–4.0 (lanes 1–8) or expanded to pH 5.4–4.5 (lane 9). Lanes 1–6 represent six consecutive fractions of prealbumin eluted from the DEAE chromatography column utilized in isolation method 1. Lanes 7 and 9 represent prealbumin isolated according to method 2 (affinity chromatography), and lane 8 represents prealbumin isolated according to method 3 (agarose electrophoresis). (B) Crossed immunoelectrophoresis against specific antiserum toward human serum prealbumin after initial isoelectric focusing (horizontal) of purified prealbumin (upper part) in the pH gradient 6.5–4.0. Below, the heterogeneity pattern of serum directly submitted to isoelectric focusing.

can be separated (Figure 1A). The method of isolation of prealbumin does not change the major features of the microheterogeneity pattern. However, there is a quantitative difference (Figure 1A). Prealbumin isolated by method 2 (involving affinity chromatography) or method 3 (agarose gel electrophoresis) contains more of the less acidic fractions than prealbumin isolated according to method 1 (the traditional procedure including three steps of purification). Repeated isoelectric focusing under nondenaturing conditions shows the heterogeneity to be genuine and not explained by differential complexing of ampholytes. All 10 subfractions react with the anti-prealbumin antiserum and are concluded to represent tetramers as shown below. In addition, crossed immunoelectrophoresis reveals minor fractions with isoelectric points at higher pH (Figure 1B) corresponding to the forms M1a, M1c, and M2b (below).

Undiluted serum examined by nondenaturing isolectric focusing and crossed immunoelectrophoresis is also heterogeneous but shows very little prealbumin in the pH range 4.95–4.60. Instead, most of the prealbumin appears at five positions representing forms M1a, M1b, M2a, M2b, and M2c as shown in Figure 1B.

Charge Differences under Denaturing Conditions. Isoelectric focusing of purified prealbumin in 7.0 M urea shows another heterogeneity pattern: four dominating fractions representing forms M1, M2, M3, and M4 (in order of relative

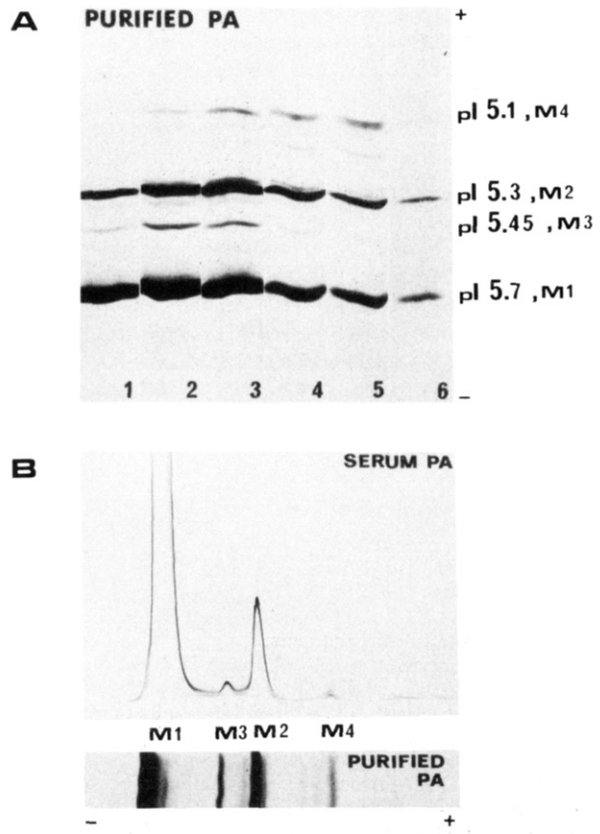

FIGURE 2: Microheterogeneity patterns of prealbumin (PA) in 7 M urea. (A) Purified prealbumin (the same six fractions from DEAE chromatography as those in Figure 1A) submitted to isoelectric focusing. (B) Serum sample analyzed by direct isoelectric focusing followed by crossed immunoelectrophoresis against specific antiserum toward human prealbumin (vertical) compared with the pattern of purified prealbumin (horizontal). The identity between the patterns of purified prealbumin and serum prealbumin is shown.

abundance) are visible (Figure 2A). Repeated isolectric focusing in 7.0 M urea shows that each fraction is stable.

Isoelectric focusing of serum under denaturing conditions and detection of prealbumin by crossed immunoelectrophoresis show the same forms in serum as in purified prealbumin (Figure 2B).

Dissociation

Purified Prealbumin. The relationship between the native forms and the denatured forms of purified prealbumin was studied by two-dimensional isoelectric focusing without urea in the first dimension and with urea in the second dimension. The native fractions in the pH range 4.95–4.60 (Figure 1) split into the two main subfractions of the denatured pattern M1 and M2 (Figure 2) as shown in Figure 3A. Densitometric evaluation shows that the three dominating subfractions of the native pattern (indicated by arrows in Figure 3A) are composed of M1 and M2 in the quantitative relations of 100% M1 (left arrow), 75% M1 and 25% M2 (middle arrow), and 50% M1 and 50% M2 (right arrow). This pattern is consistent with dissociation of tetramers into monomers upon the urea treatment.

Serum Prealbumin. The same type of two-dimensional, isoelectric focusing of serum prealbumin shows that the second step of denaturing isoelectric focusing demonstrates a conversion of M1a and M1b essentially to M1. Quantification of conversions in the second step by crossed immunoelectrophoresis also reveals minor conversions of both M1a and M1b to M2 (not visible in the two slices shown in Figure 3B, but visible in the adjacent sections).

Fractions M2a, M2b, and M2c all convert to M2 in denaturing media (Figure 3B). None of the serum forms observed under nondenaturing conditions split upon denaturation into M1 and M2 in specific ratios (Figure 3B), as in the case of purified prealbumin in Figure 3A, which would be expected if they represented heterodimers or heterotetramers. This indicates that the serum forms M1a, M1b and M2a–M2c could be homotetramers, homodimers, or monomers, but in all cases of a different type than the tetramers obtained from purified prealbumin or the monomers M1 and M2 obtained under urea denaturing conditions, respectively. Furthermore, nondenaturing isoelectric focusing of purified prealbumin that was preheated (10 min, 95 °C) in SDS with or without reduction shows that, at a critical SDS concentration (10 mM), a conversion of the prealbumin tetramers into forms corresponding to the serum prealbumin forms M1a, M1b and M2a–M2c takes place as shown in Figure 4. This shows that the M1a, M1b, M2a–M2c patterns are obtained not only from serum prealbumin under nondenaturing conditions but also from purified prealbumin upon SDS denaturation. Since in the latter case, parent tetrameric forms of purified prealbumin contain heterotetramers (Figure 3A), the M1a, M1b, M2a–M2c forms, if still tetrameric (or dimeric), cannot be homotetramers (homodimers) as suggested from one of the alternative conclusions above (Figure 3B). However, the other alternative concluded from the results in Figure 3B, that the forms M1a, M1b and M2a–M2c are monomers, is compatible also with the results of Figure 4. Consequently, the combined results of Figures 3 and 4 suggest that the serum prealbumin forms M1a, M1b and M2a–M2c are monomers. Obviously, denaturation in urea (Figure 2) and SDS (Figure 4) gives different types of monomers, the latter also present in serum (Figure 1) and of a type that can convert to the former (Figure 3B).

Conclusions. Three different patterns of prealbumin heterogeneity are observed: one encompasses the forms in the pH range 4.95–4.60 (seen under nondenaturing conditions of purified prealbumin and since long concluded to represent tetrameric forms). The second pattern encompasses the forms M1, M2, M3, and M4 (seen upon isoelectric focusing under urea denaturing conditions of both serum and purified prealbumin). They are clearly derived from dissociation into monomers. The third pattern encompasses the serum forms M1a, M1b, M2a, M2b, and M2c (also seen under nondenaturing conditions). Expansion of the gradient reveals a further monomeric form, M1c (cf. below, Figure 7). These forms were initially concluded to represent another set of tetramers, dimers, or monomers but could be shown by heating in the presence of SDS (a treatment known to dissociate the prealbumin tetramer into monomers; cf. below, Figure 5A) to be likely to represent monomers of another type. Consequently, a novel set of forms has been detected and found probably to constitute a set of monomers that are present also in serum. The forms M3 and M4, still different from M1 and M2, appear mainly to be derived from the tetramers represented by the nondenatured pattern of serum prealbumin (Figure 3B), indicating that these two monomeric forms under native conditions participate in more stable tetramers. In summary, at least eight forms (M1a–M1c, M2a–M2c, M3, and M4) likely to represent different native monomers have been identified and the relationships to traditional tetramers and the urea-dissociated monomers have been traced.

Dissociation and Reassociation of Tetramers

Two-Dimensional Isoelectric Focusing. Analysis of serum by this method under nondenaturing conditions, followed by

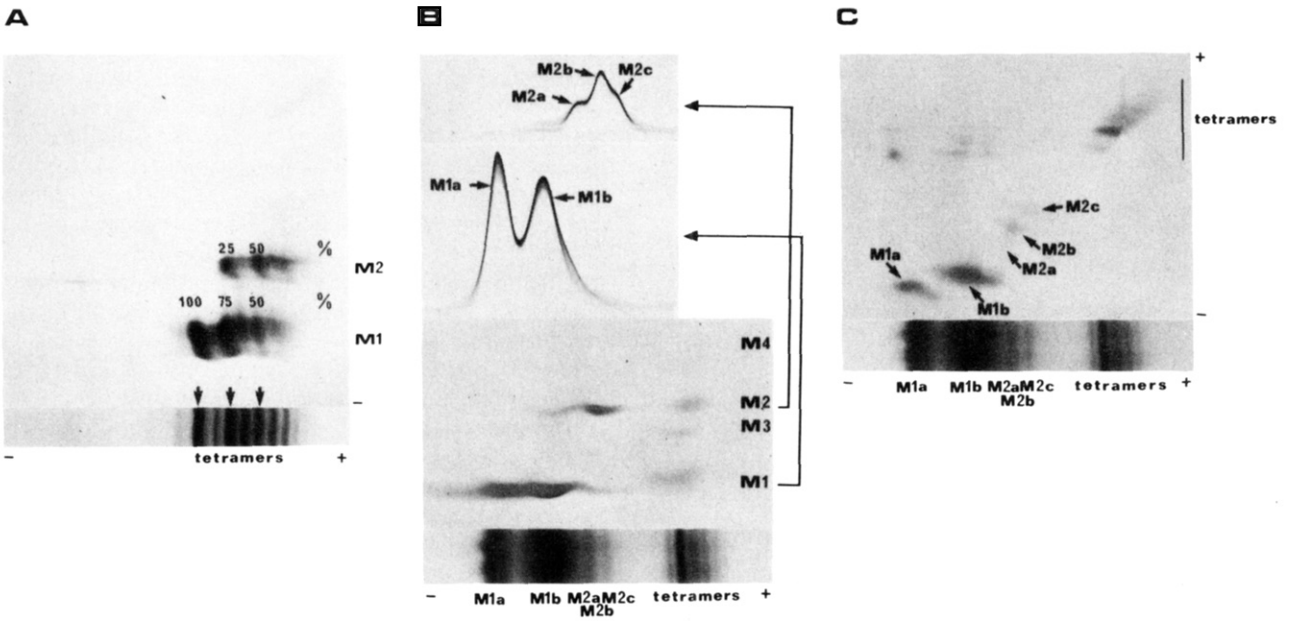

FIGURE 3: Relationship between microheterogeneity patterns of prealbumin under nondenaturing and denaturing conditions examined by two-dimensional isoelectric focusing (first dimension, horizontal, without urea; second dimension, vertical, with urea in A and B and without urea in C). (A) Purified prealbumin analyzed under nondenaturing conditions in the expanded pH gradient 5.4–4.5 and under denaturing conditions in the pH gradient 6.5–4.0. The dominating native subfractions (arrows) are composed of M1 and M2 in quantitative relations of 100% M1 (left arrow), 75% M1 and 25% M2 (middle arrow), and equal amounts of M1 and M2 (right arrow). This is consistent with a complete dissociation of original tetramers into monomers (M1 and M2). (B) Serum submitted to isoelectric focusing under nondenaturing conditions in the pH gradient 6.5–4.0, followed by denaturing conditions in the same gradient; (detection of prealbumin in both dimensions was made qualitatively by immunofixation). For quantification in the second dimension, the gel was sliced and each slice was submitted to a crossed immunoelectrophoresis; the results with the two slices corresponding to M1 and M2, indicated by arrows, are shown (top). M1 corresponds to M1a and M1b; M2 corresponds to M2a, M2b, and M2c, while M3 and most of M4 are found to be constituents in the tetramers of serum prealbumin. (C) Two-dimensional nondenaturing isoelectric focusing of serum. The experiment shows a reassociation of the forms M1a and M1b into tetramers in the second dimension (detection by immunofixation).

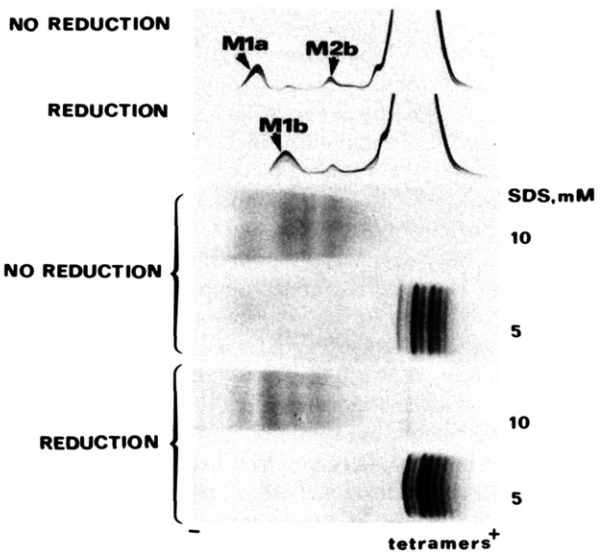

FIGURE 4: Conversion of purified prealbumin into serum prealbumin forms (M1a, M1b and M2a–M2c) by heating in the presence of SDS (sodium dodecyl sulfate). Purified prealbumin (20 μM) in 0.1 M Tris-HCl, pH 8.0, was heated for 10 min at 95 °C in SDS (5 or 10 mM as indicated), with or without reduction, and submitted to nondenaturing isoelectric focusing (lower two sections). The SDS-dissociated forms are compared with the patterns of untreated purified prealbumin (upper two sections, corresponding to the patterns in the upper part of Figure 1B).

detection of prealbumin by immunofixation, shows the forms M1a and M1b to give rise to some tetramers in the second dimension (Figure 3C). This can be concluded from the fact that the reformed fractions are on line with the original tetramers. This indicates an equilibrium between M1a, M1b, concluded to be monomers, and the tetramers (Figure 3C). The reformed tetramers are not homogeneous, however, because of some spontaneous conversions of M1b to M1a and both M1a and M1b to M2 (cf. below).

SDS/Gradient Polyacrylamide Gel Electrophoresis. The effects of SDS, heating, reduction, and alkylation on purified prealbumin show the SH groups of prealbumin not to be associated in interchain disulfide bridges. Dissociation into monomers can be achieved by heating in the presence of SDS, without reducing agent (Figure 5A, left).

Samples of purified prealbumin were also preincubated at different SDS concentrations (0.05–55.4 mM) without heating and submitted to seven different polyacrylamide gradient gels containing varying SDS concentrations from 0.0 to 7.0 mM (the gel containing 3.5 mM corresponding to 0.1% (w/v) SDS is shown in Figure 5A, right). When the SDS concentration was increased during preincubation, a dissociation of the prealbumin tetramer into subunits, dimers, or monomers, was anticipated. The experiment shows that the prealbumin of the SDS-preincubated samples moves in the same position as tetrameric prealbumin not exposed to SDS, more or less corresponding to that expected for the dimer as judged by the migrations of standard proteins (Figure 5). At higher SDS concentrations, monomers appear, but dimers are not resolved (for the dimer position, see Figure 5B, right). Almost no material is found at the position expected for the tetramer (Figure 5A, right), but the sensitive trans-blot procedure reveals faint bands in this region (not shown). These latter forms cannot be seen when the prealbumin is saturated with thyroxine. The nature of these forms has not been separately investigated, but their position (cf. mobility of monomers and dimers in Figure 5B, left) and their behavior with thyroxine (see below) may indicate that they represent monomers or dimers.

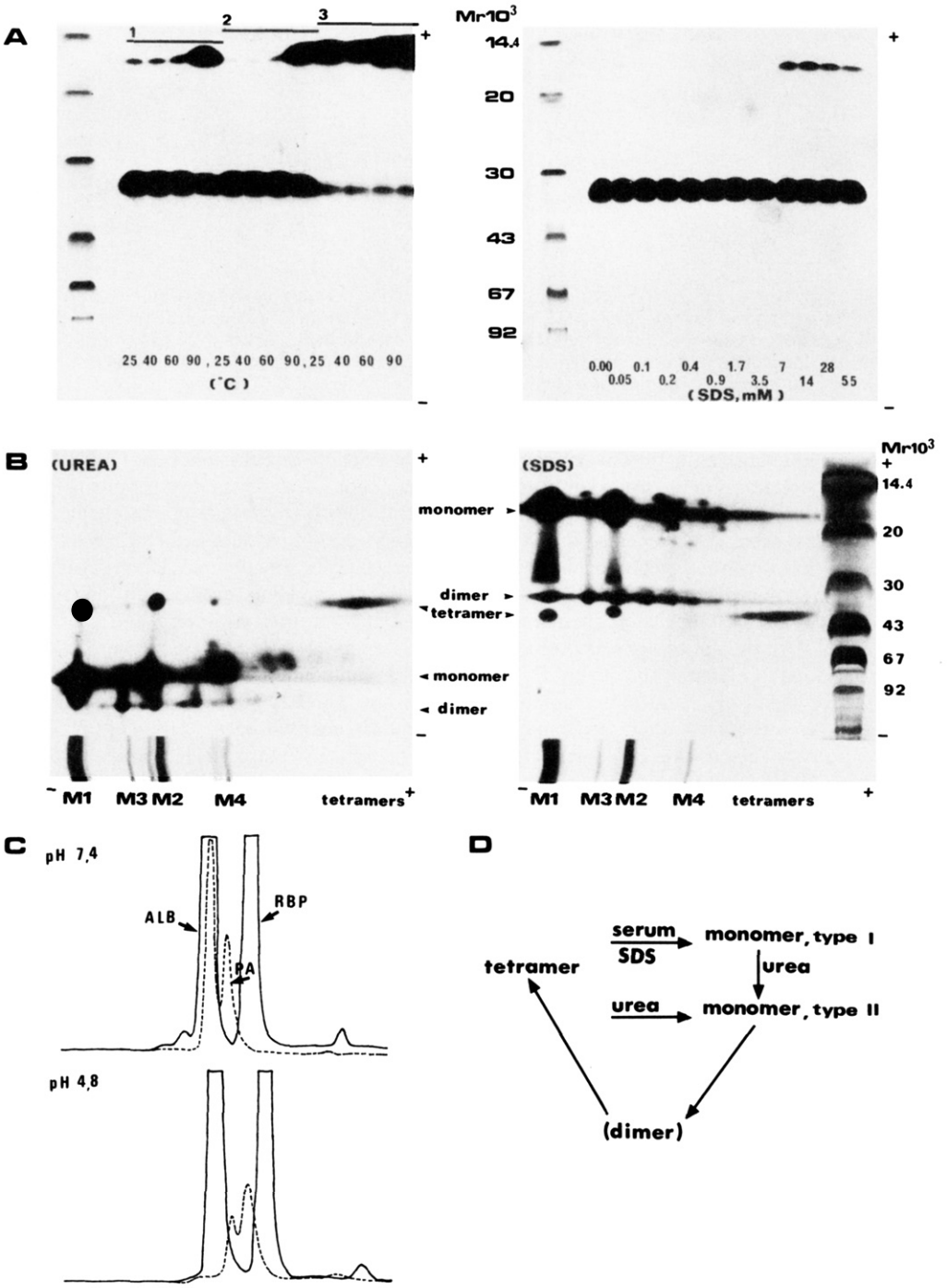

FIGURE 5: Dissociation and reassociation of purified prealbumin tetramers. (A) 0.1% SDS/gradient polyacrylamide gel electrophoresis of purified prealbumin (200 μM) after different pretreatments. Left: incubation (10 min) in 0.1 M Tris-HCl, pH 8.0, and 0.6% SDS at different temperatures (degrees Celsius) as shown, without (1) and with (2) reduction (dithiothreitol, 30 mM) and with (3) alkylation (iodoacetamide) before electrophoresis. Right: incubation for 12 h in 0.1 M Tris-HCl, pH 8.0, at room temperature in different SDS concentrations (millimolar) as shown, without reduction and alkylation before electrophoresis (molecular weights of marker proteins are indicated). (B) Two-dimensional electrophoresis of purified prealbumin. The first dimension (horizontal, Coomassie Brilliant Blue stained) is isoelectric focusing under denaturing conditions in 7 M urea, and the second dimension (vertical) is gradient polyacrylamide gel electrophoresis in the presence of 6.0 M urea [left, silver stained according to Merril et al. (1982)] and 0.05% SDS (right, silver stained). M1, M2, and M4 appear mainly as monomers and tetramers, while M3 appears predominantly as a dimer upon reassociation (molecular weights of marker proteins are indicated as in A). (C) Chromatography on Sephacryl S-200 of purified prealbumin (PA) in two different systems: 0.05 M phosphate buffer, pH 7.4, and 0.15 M NaCl; and 0.05 M acetate buffer, pH 4.8, and 0.15 M NaCl; both in 3.0 M urea. The prealbumin appears in two peaks (dashed) with apparent molecular sizes between 66 000 (albumin, ALB) and 21 000 (retinol-binding protein, RBP). Vertical scale unmarked; absorbancy at 280 nm. (D) Schematic representation of the dissociation (SDS, urea, or serum mediated) and the reassociation (as shown by the experiments in B) of the prealbumin tetramer.

Two-Dimensional Electrophoresis Combining Denaturing Isoelectric Focusing and Urea- or SDS-Containing Polyacrylamide Gel Electrophoresis. In the first dimension purified prealbumin is either completely (in 7.0 M urea) or partially (in 4.0 M urea) dissociated into monomers (the former case shown in Figure 5B). In the second dimension, 6 M urea (Figure 5B, left) or 0.05% SDS (Figure 5B, right), the protein appears in three regions.

In the case of urea in the second dimension, the tetramer position is established by the tetramers from the first dimension

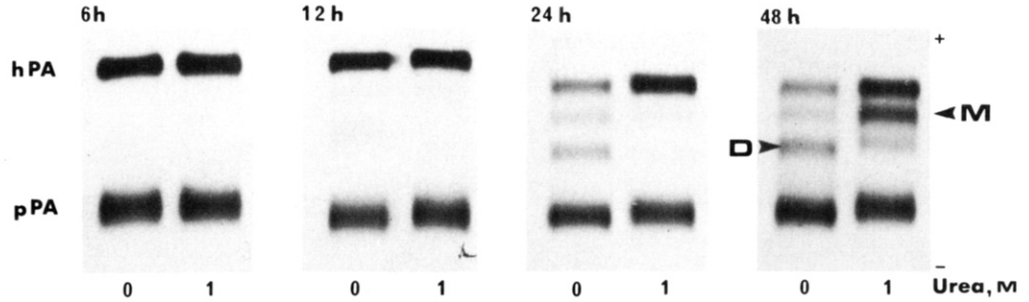

FIGURE 6: Prealbumin hybrids resolved by agarose electrophoresis. Hybridization between human prealbumin (hPA) and porcine prealbumin (pPA) was achieved by incubation for different times in 0.1 M Tris-HCl, pH 8.0, without urea or in the presence of 1 M urea. The hybrids were studied in agarose electrophoresis, pH 8.6, free from urea. In the absence of urea, dimers were preferentially exchanged as indicated by the increase of hybrid D. Introduction of urea into the incubation mixture resulted in a pattern of hybrids indicating a shift to monomer exchange. The tendency toward hybrids containing three human monomers as indicated by hybrid M reflects different resistance of the two parent prealbumins toward urea.

(as shown in Figure 5B, left, horizontal). The monomers M1–M4 are associated into tetramers in the second dimension. When the urea concentration (6, 5, 4, 3, and 1.5 M) of the gradient polyacrylamide gel is decreased, the reassociation can be brought to completion. In the conversion of monomers into tetramers, an intermediate form can be identified. This form is never a major form and has a lower mobility or, alternatively, a higher molecular weight than the major dissociated forms. This intermediate is concluded to represent the dimer. Of the different monomers, the forms M1, M2, and M4 predominate in positions corresponding to monomers and tetramers and M3 predominates preferentially in the position corresponding to dimers (Figure 5B).

In the case of SDS in the second dimension, monomers, dimers, and tetramers appear at positions as shown in Figure 5B, right. The tetramer position is again determined by the position of the nondenatured tetramers from the first dimension and by observation of the different extents of reassociation when the SDS concentration is decreased (0.1, 0.05, and 0.025%). The behavior as an intermediate form of the dimer is confirmed except for M3, which under these circumstances appears as dimer and does not reassociate into tetramers (Figure 5B, right).

Chromatography on Sephacryl S-200. The appearance of only two forms upon urea denaturation is further confirmed by exclusion chromatography of purified prealbumin in buffers at different pH (7.4, 6.5, 5.5, 4.8) in the presence of 3 M urea. Prealbumin appears always in only two peaks corresponding to molecular sizes between those of albumin (66 000) and retinol-binding protein (21 000), as shown for the two buffer systems at pH 7.4 and 4.8, in Figure 5C. The exact positions and the quantitative relation between the two peaks vary with pH. Acidic pH promotes dissociation as seen by the increase of the later eluting form.

Prealbumin Hybrids. Hybridization experiments using purified porcine and human prealbumins under nondenaturing conditions show a predominant exchange of dimers as evidenced by the position of the dominating hybrid D in Figure 6. When the two prealbumins are incubated in 1 M urea, a shift to monomer exchange can be seen as the reassociated hybrids are resolved by agarose electrophoresis containing no urea. This hybridization experiment confirms the previous finding that urea attacks predominately the monomer–monomer interactions of the dimer, thus resulting in complete dissociation into monomers.

Conclusions. Treatment of purified prealbumin with SDS or urea results in dissociation into monomers as shown in Figures 3A and 5A. The mobility of the tetramer is untraditional, and the behavior of the dimer is confusing upon electrophoresis but could be interpreted by two-dimensional resolutions in different denaturants (Figure 5B). Under both conditions of dissociative treatments of purified tetramers, dimers cannot be identified (Figure 3A and 5A). Serum itself dissociates the prealbumin tetramer without any sign of dimers under the nondenaturing conditions as shown in Figure 3B, and the serum forms M1a and M1b can reassociate into tetramers (Figure 3C). However, SDS treatment gives the serum monomeric forms only at a critical SDS concentration, beyond which the denaturation becomes irreversible (Figure 5A). In contrast, reassociation of urea-dissociated monomers is complete (Figure 5B, left; upon lowering the urea concentration) and gives back the original tetrameric pattern (data not shown). Only during reassociation of monomers to tetramers can an intermediate form be identified (Figure 5B), and this is concluded to represent the dimer. Thus, dissociation of prealbumin tetramers by SDS, urea, and serum goes directly to monomers. The reassociation, however, seems to pass via the dimer, as schematically shown in Figure 5D. Together, these observations indicate that an equilibrium between tetramers and monomers may exist in serum that is regulated by endogenous serum factor(s).

Studies of Thiol Groups in Prealbumin

Homogeneous Prealbumin Tetramers. Homogeneous tetramers consisting of M1 or M2 were prepared as described under Materials and Methods and were incubated in the presence of different thiol reagents. The effects were studied by denaturing isoelectric focusing as in Figure 2.

Apart from the effects of preincubations, there is a small spontaneous conversion of M1 to M2 (cf. Figure 3B). Both reduced (GSH) and oxidized (GSSG) glutathione shift some M1 into the position of M2; reduction with GSH, β-mercaptoethanol, or dithiothreitol shifts some of the M2 fraction into the position of M1; Ellman's reagent and iodoacetate react to a limited extent with both M1 and M2, giving rise to products at the M2 and M4 positions, respectively; oxidative treatment of M1 with hdyrogen peroxide is without effect, but that of M2 gives some M4; urea or $ZnCl_2$ do not affect the pattern. None of all these patterns are shown in detail, because the results do not allow final interpretations since Figure 3B indicates that both M1 and M2 are not necessarily homogeneous. Therefore, the same thiol reagents were instead incubated with serum and the effects on M1a, M1b and M2a–M2c were studied by crossed immunoelectrophoresis after nondenaturing isoelectric focusing, since these forms can be fully resolved.

Serum Prealbumin Monomeric Forms. Effects of incubation of serum prealbumin with different SH reagents are summarized in Figure 7. Addition of GSH causes an increase

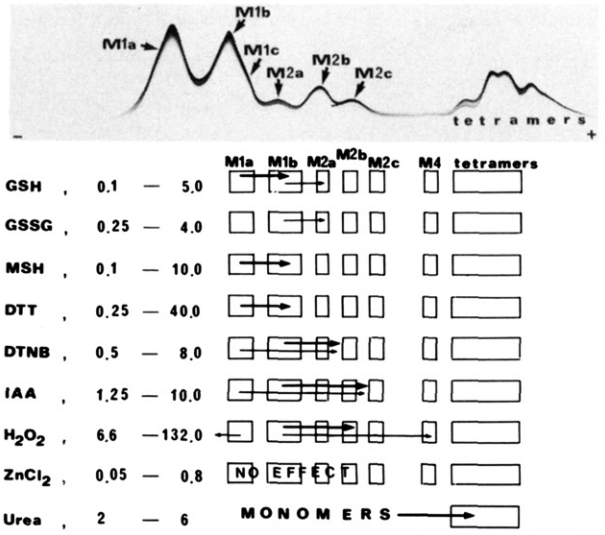

FIGURE 7: Relation between thiol groups of prealbumin and its microheterogeneity, studied by addition of thiol reagents. Schematic representation of conversions between serum prealbumin monomers upon addition of thiol reagents (concentration range indicated in millimolar, but in molar for urea; preincubation of samples was 2 h at 25 °C) to serum. Visualization is performed by nondenaturing isoelectric focusing followed by crossed immunoelectrophoresis. The conversions, indicated by arrows (thick for dominating conversions), show M1b to be the reduced form, M2b and M4 to be oxidized forms, and M2a probably to be an adduct with glutathione.

of the monomeric forms M1b and M2a, while M1a decreases. Addition of GSSG causes a decrease of M1b, while M2a slightly increases. Addition of dithiothreitol or β-mercaptoethanol causes a shift from M1a to M1b. Addition of dithiobis(nitrobenzoic acid) (DTNB) or iodoacetate causes a decrease of the M1b fraction and reveals an appearance of more acidic forms, not identical with M2a, M2b, or M2c. The decrease will finally also affect M1a. Addition of H_2O_2 gives a disapperance of M1b and an increase of M2b. Some material appears at a still more acidic position, identical with that of M4, and, at higher concentrations of H_2O_2, also at a more basic position than that of M1a.

Preincubation with urea gives larger shifts with patterns toward tetrameric forms, preventing judgment of monomer shifts. As shown above in Figure 5B, the monomers M1–M4 will reassociate into tetramers when separated from urea. In this case, the preincubation of serum with urea abolishes the dissociative effect that serum itself has on the prealbumin tetramer, which is compatible with the existence of a serum factor that normally promotes dissociation (cf. Discussion) but is sensitive to urea.

The effect of addition of retinol-binding protein, bovine albumin, and thyroxine to serum was studied. Neither retinol-binding protein nor bovine albumin affected the pattern. However, raising the thyroxine concentration above the prealbumin concentration resulted in a slight shift toward the tetrameric forms. Addition of trace amounts of ^{125}I-labeled thyroxine showed radioactivity associated only with the serum tetrameric forms.

Presence of More M1b in Serum Prealbumin Than in Purified Prealbumin: Relationship between M1b and the Retinol-Binding Protein. Isoelectric focusing under nondenaturing conditions of reduced purified prealbumin reveals a shift among the tetrameric fractions in the pH range 4.95–4.60. The tetramer pattern here is composed of two overlapping quintets, one of which can be shifted into the other by reduction (Figure 8A). Concomitantly, M1a is shifted to the position of M1b (Figure 8A). Without reduction, the purified prealbumin contains only small amounts of M1b and the tetramer quintet is of the type containing M1a and the oxidized form M2b.

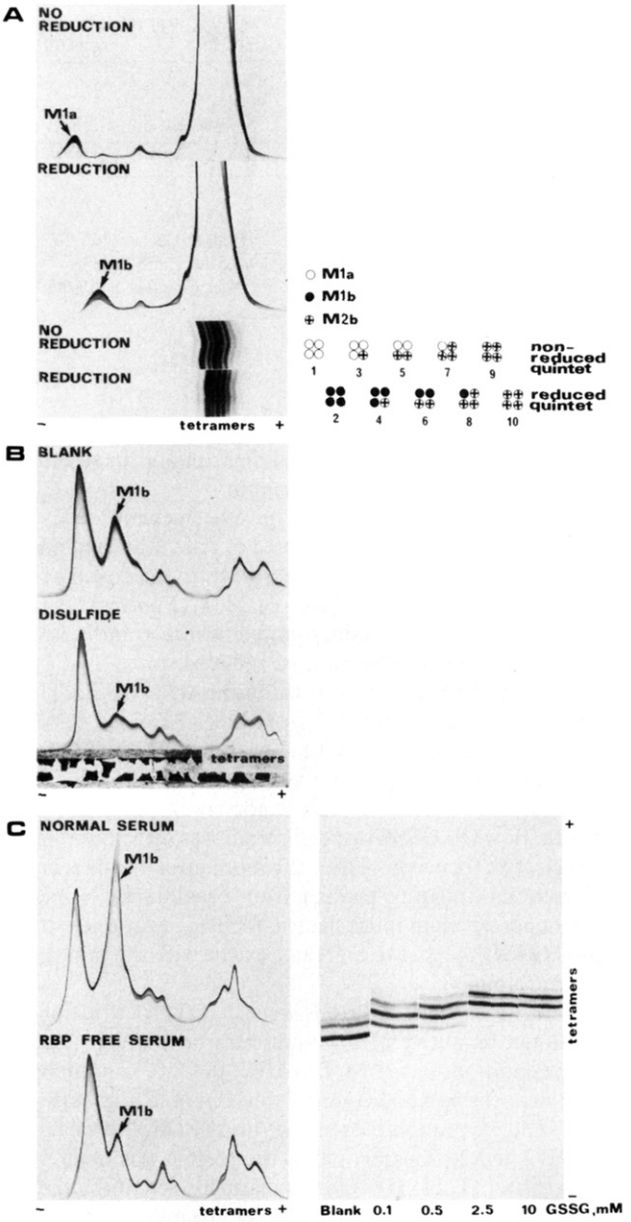

FIGURE 8: The presence of the reduced monomer M1b in purified prealbumin and serum and its relation to retinol-binding protein. (A) Reduction of purified prealbumin results in a shift between two quintets building up the tetrameric patterns and a conversion of M1a to M1b. The composition of the two interconvertible quintets is indicated to the right. (B) Electrophoretic transport of the serum monomeric forms, M1a, M1b, M2a, M2b, and M2c, through a gel strip of Sepharose-linked disulfide shows the form M1b alone to react with the fixed disulfide. (C) Left: Decreased M1b fraction in serum depleted in retinol-binding protein relative to fresh serum. Right: Acidic conversion in the tetramer heterogeneity pattern of prealbumin incubated (1 h) with GSSG (oxidized glutathione) in the concentrations indicated. Isolation from GSSG-treated serum by agarose electrophoresis before isoelectric focusing (cf. Figure 7).

The serum prealbumin forms were exposed to a fixed disulfide in crossed immunoelectrophoresis, where Sepharose-linked glutathione 2-pyridyl disulfide (Pharmacia, Uppsala, Sweden) was introduced in a gel strip between the isoelectric focusing strip and the antibody-containing gel. Only the M1b fraction decreased in size (Figure 8B), indicating a reaction with the disulfide when passed through the disulfide-containing gel strip. This finally establishes that M1b contains a reduced

Table II: SH Content of Native Prealbumin Tetramers and Homogeneous Prealbumin Tetramers Studied by Titration with Ellman's Reagent or Carboxymethylation under Different Conditions with or without Reduction

	Ellman's reagent			carboxymethylation		
	preparation	condition	SH/tetramer	preparation	iodoacetate, excess	SH/tetramer
no reduction	native tetramer	buffer	0.00	native tetramer	40	0.26
		8 M urea	0.06	M1 tetramer	40	0.62
		0.2% SDS; 95 °C; 5 min	0.06		120	2.08
		2.5 M 2-propanol	0.11	M2 tetramer	40	0.57
		3 M Gdn-HCl; 6.5 M 2-propanol	0.00		120	1.21
				M3 tetramer	40	0.46
reduction	native tetramer	buffer	2.5	native tetramer	40	2.8
		8 M urea	2.6	M1 tetramer	40	3.3
				M2 tetramer	40	2.34
				M3 tetramer	40	3.81

thiol and is present in a much higher amount in serum prealbumin than in purified prealbumin.

The serum prealbumin forms have been examined in fresh normal serum and in serum depleted of retinol-binding protein. In the absence of retinol-binding protein, the concentration of M1b has decreased in favor of M1a (Figure 8C). This shows that the retinol-binding protein is important in keeping a fraction of the prealbumin in a reduced state. This is also observed in the reaction of prealbumin with GSSG. If the prealbumin zone is prepared from GSSG-treated serum by agarose electrophoresis at pH 8.6 prior to isoelectric focusing, almost all of the prealbumin has reacted with GSSG (Figure 8C, right). This is in contrast to the result presented above (Figure 7), when GSSG-treated serum was directly submitted to isoelectric focusing. Thus, the more effective dissociation of the retinol-binding protein from prealbumin in agarose electrophoresis than in isoelectric focusing (data not shown) allows GSSG to react to a greater extent with the thiol groups of the protein.

Titration with Ellman's Reagent. DTNB titration was carried out on native prealbumin tetramers under nondenaturing conditions in 0.1 M Tris-HCl, pH 8.0, and under different denaturing conditions (Table II); no SH groups were found, not even in the presence of urea, SDS, 2-propanol, or Gdn-HCl and 2-propanol, unless the protein was treated with dithiothreitol (Table II). After treatment with dithiothreitol, the DTNB reaction detected 2.5 SH groups per prealbumin tetramer under nondenaturing conditions and 2.6 in the presence of 8 M urea (Table II).

Carboxymethylation. Results were similar to those with Ellman's reagent (above) although in all cases somewhat higher values were obtained with the homogeneous tetramers. Without pretreatment of prealbumin and with a 40-fold iodoacetate excess, 0.26–0.62 SH group per tetramer was carboxymethylated (Table II); increases of the iodoacetate excess resulted in a higher degree of carboxymethylation. After pretreatment with dithiothreitol, the same preparations showed from 2.2 to 3.8 SH groups per tetramer (Table II).

The relation between carboxymethylation and the pattern of microheterogeneity was also studied. Upon carboxymethylation of native prealbumin tetramers without initial reduction, radioactivity was found in all fractions of the microheterogeneity pattern and there was no change of the pattern from that obtained with noncarboxymethylated samples.

Sequence Analysis

The N-terminal region was analyzed in native prealbumin tetramers and in homogeneous M1, M2, and M3 tetramers. The native tetramers were studied untreated, and the M1, M2, and M3 tetramers were carboxymethylated with ^{14}C-labeled iodoacetate before and after pretreatment with dithiothreitol, respectively. In all carboxymethylated fractions, the carboxymethylation was found to be associated with Cys-10, as judged by the recovery of the corresponding phenylthiohydantoin during degradation. Most fractions contained protein chains starting at different positions. Thus, beside Gly-1, which is the ordinary prealbumin N-terminal residue, prealbumin chains were found to start at positions Pro-2 (5% in M3) and Thr-3 (10% in M3, 5% in M2, and only traces in M1) but not at further positions in any appreciable yield. The results establish an N-terminal heterogeneity of M1, M2, and M3, but the ragged N-terminus does not explain the multiple prealbumin forms observed by electrophoresis.

Discussion

Heterogeneity of Prealbumin

Native Conditions: Basic Patterns of Two Quintets from Three Monomers. At least 10 bands were seen upon isoelectric focusing in the pH gradient 6.5–4.0 (Figure 1A) corresponding to different tetramers. This microheterogeneity is obtained with all three isolation methods used, but the more time-consuming the purification procedure, the more pronounced the shift toward acidic forms. This is probably due to oxidation, as the shift is diminished at anaerobic conditions (Felding & Fex, 1984). Thus, preparative agarose gel electrophoresis was found to yield the most native pattern; in a commercial prealbumin preparation (Behringwerke), almost all material appears at a more acidic position upon isoelectric focusing. The 10 fractions of the microheterogeneity pattern comprise two quintets, each corresponding to every second subfraction. The quintet with the more basic forms can be shifted into the other quintet by reduction. Such a pattern could be explained by the presence of three different monomers pairwise combined into tetramers (Figure 8A).

Upon expansion of the pH gradient, each of the 10 tetrameric subfractions is found to be further microheterogeneous (Figure 1A), indicating more than three different monomers. Up to 32 fractions can be distinguished, suggesting at least 4 different monomeric forms.

Dissociation into Monomers. Prealbumin zones appear at different isoelectric points when serum is directly submitted to isoelectric focusing under nondenaturing conditions. Examination of these forms by isoelectric focusing under denaturing conditions suggests that they are due to dissociation into monomers. Moreover, the conversion of the serum pattern into the urea-denatured pattern shows that the serum forms M1a and M1b give rise to the urea-denatured fraction M1. Similarly, M2a, M2b, and M2c give rise to M2. This excludes the possibilities that M1a, M1b and M2a–M2c are aggregates, heterodimers, or heterotetramers. If instead these forms

represent serum-specific homotetramers or homodimers, the serum prealbumin tetramers would be either M1 or M2 homogeneous. This would be in contrast with purified prealbumin, where only the most basic tetrameric form is M1 homogeneous; the rest are M1 and M2 heterogeneous (Figure 3A). Furthermore, purified prealbumin added to serum is also split into the M1 and M2 homogeneous forms M1a, M1b and M2a–M2c. Finally, SDS treatment with or without heating results in dissociation of purified prealbumin into monomers as shown in Figure 5A. These monomers are of a type different from the urea-produced monomers, as purified prealbumin heated in the presence of SDS with or without reduction also gives rise to the forms M1a, M1b and M2a–M2c upon nondenaturing isoelectric focusing (Figure 4). Consequently, all results strongly suggest that the serum prealbumin forms are monomers, where M1a and M1b on the one hand and M2a, M2b, and M2c on the other hand are converted to M1 and M2, respectively, in the presence of urea. This interpretation of dissociation into monomers is further supported by the fact that the serum monomeric forms can be interconverted by chemical means (Figure 7).

It is unclear why prealbumin of untreated serum samples, but not of purified preparations, dissociates during isoelectric focusing. However, there is (are) apparently some component(s) in serum enhancing the dissociation, as dilution of serum significantly shifts prealbumin toward tetramers and addition of purified prealbumin to serum results in dissociation (cf. below).

It may be concluded that the three dominating monomeric forms in the prealbumin tetramers are M1a, M1b, and M2b, which explains why the purified prealbumin tetramers exist as 10 main subfractions corresponding to tetramers formed by M1a, M2b and M1b, M2b, respectively. Less common monomers are M1c, M2a, M2c, M3, and M4, explaining some of the further complexity of the microheterogeneity of prealbumin.

Upon isoelectric focusing of purified prealbumin or of serum in the presence of urea, prealbumin is dissociated mainly into four different subfractions, M1, M2, M3, and M4, representing another type of monomers (Figure 3A), which are apparently only seemingly unique, as M1a, M1b and M2a, M2b, and M2c are all converted to M1 and M2, respectively, upon urea treatment. The urea dissociation has also been shown by Altland et al. (1981).

Importance of the Single SH Group in the Monomer. There is a single SH group (Cys-10) of the prealbumin monomer. On addition of thiol reagents to isolated M1 or M2 tetramers, conversions take place, but the changes in the patterns are not easily interpreted. The relation between M1 and M2 has also been studied by Felding and Fex (1984), but the further complexity of M1 and M2 was then not noticed.

However, clear conclusions can be drawn from studies of the serum forms M1a, M1b, M2a, M2b, and M2c after incubation with thiol reagents (Figure 7) and from the fixed disulfide electrophoresis (Figure 8B). Thus, the thiol group of the form M1b only is free and reacts with GSSG, DTNB, iodoacetate, H_2O_2 (Figure 7), and the fixed disulfide (Figure 8B); M2a is apparently the GSH adduct and M2b an oxidized form (Figure 7); M4 could represent a still higher oxidition state (Figure 7); M3 is one further adduct with an SH-reactive component, probably a GSH metabolite (see below); the existence of M2c lacks final explanation, but it could be due to spontaneous endogenous carbamylation, as uremic patients seem to have an elevated amount of this monomer (data not shown). In M1a, the condition of the SH group is obscure. This form reacts to a slight extent with iodoacetate and completely with DTNB at high reagent excess, contrasting the result with purified preparations. It reacts poorly with H_2O_2 but is easily reduced to give M1b. Possible explanations could be either a conformational change of M1a rendering the thiol group less accessible for reaction or a so far unidentified adduct.

In purified prealbumin the monomeric forms M1a and M2b dominate. Only small amounts of the SH-reactive monomer M1b is present, and without pretreatment with dithiothreitol, the protein SH content is low. This finding is not changed by separate pretreatments in 8 M urea, 2% SDS (90 °C; 5 min), 2.5 M 2-propanol, or 3 M Gdn-HCl and 6.5 M 2-propanol, respectively. After reductive pretreatment, thiol contents from 2.2 to 3.8 SH groups per prealbumin tetramer were found (Table II).

Earlier reports on prealbumin thiol content are conflicting and have not penetrated the problem in detail. Raz et al. (1970) reported only one disulfide bond of prealbumin to be readily accessible to alkylation after mild reduction. However, present results show that there is no disulfide bond in prealbumin under any circumstance now studied. Rask et al. (1971) reported all four cysteinyl residues to be accessible to alkylation or reactive with DTNB in 6.5 M 2-propanol under denaturing conditions with or without reduction. We could not repeat the effect of 2-propanol. Felding and Fex (1984) showed, in accordance with our results, reduction to be a prerequisite for the subsequent alkylation of all cysteinyl residues of purified prealbumin. The present identification scheme reveals an interpretable relation between thiol group status and the prealbumin heterogeneity that can explain previous inconsistencies, and for the first time, the different monomeric forms of prealbumin here discussed can now also be identified.

N-Terminal Processing. Sequence analysis revealed some differencies in starting position of the constituent protein chains, with lack of the first one or two amino acid residues, Gly-1 – Pro-2, to a limited extent (5–10%). Such a loss is not infrequent in other biologically active peptides (Jörnvall et al., 1986). However, the N-terminal heterogeneity has no significance in relation to the electrophoretically defined microheterogeneity of prealbumin.

Presence of both Prealbumin Tetramers and Subunits. The prealbumin tetramer has long been considered to represent an extremely stable configuration (Branch et al., 1972). This is now shown to be true only under certain conditions. Isoelectric focusing of serum prealbumin under nondenaturing conditions also results in dissociation into forms concluded to be monomers, but of a different type than those obtained by isoelectric focusing in 7.0 M urea, which gives complete dissociation of prealbumin tetramers into monomers. The serum forms can also be seen upon SDS treatment of purified prealbumin (Figure 4). However, purified prealbumin under nondenaturing conditions appears more stable and has little free monomers (Figure 1B). The hybridization experiment with purified prealbumin from pig and man showed a predominant dimer exchange under nondenaturing conditions with a shift to monomer exchange when urea was introduced (Figure 6). Thus, urea, SDS, and under certain circumstances serum itself dissociate the prealbumin tetramer into monomers. Monomers dissociated with serum or urea, however, can reassociate into tetramers (Figures 3C and 5B).

These observations indicate the existence in serum of an equilibrium between monomers and tetramers with intermediate dimers. This dissociation–reassociation of the prealbumin tetramer in serum is probably due to dissociation-enhancing

Table III: Amino Acid Sequence around Reactive Cysteine Residues in Prealbumin and Other Glutathione-Binding Proteins[a]

	30	31	32	33	34	35	36	37	38	39	40	41	42	
ALBUMIN	- Tyr	Leu	Gln	Glu	Cys	Pro	Phe	Asp	Glu	His	Val	Lys	Leu	-
	6	7	8	9	10	11	12	13	14	15	16	17	18	
PREALBUMIN	- Gly	Glu	Ser	Lys	Cys	Pro	Leu	Met	Val	Lys	Val	Leu	Asp	-
	18	19	20	21	22	23	24	25	26	27	28	29	30	
GLUTAREDOXIN	- Ile	Lys	Pro	Thr	Cys	Pro	Tyr	Cys	Arg	Lys	Thr	Gln	Glu	-
	- Gly	Arg	Ser	Gly					Val	Arg	Ala	Lys	Asp	-

[a] Human albumin, human prealbumin, and glutaredoxin (from calf thymus, top in glutaredoxin line; *Escherichia coli* below with residues listed only at positions where they differ from the calf protein) contain a Cys-Pro configuration at the positions given by the numbers. Significantly, Cys-Pro is absent in the glutaredoxin-related (Höög et al., 1983; Klintrot et al., 1984) thioredoxin structures. No significant similarities were observed among the proteins compared outside the regions shown.

molecule(s) in serum. In fact, a so far unidentified substance has been isolated from serum that enhances the dissociation of purified prealbumin into monomers when studied in the presence of urea (unpublished results). Interestingly, the dissociation of the prealbumin tetramer by either urea, SDS, or serum passes directly from tetramers to monomers. Dimers can be identified only upon reassembly of tetramers from monomers (Figure 5B) or, indirectly, in hybrid molecules (Figure 6).

Ligand Interactions with Prealbumin

Thyroxine. Binding of thyroxine to prealbumin stabilizes the tetramer configuration (Nilsson et al., 1975) and restricts binding of another thyroxine molecule to the second binding site (Ferguson et al., 1975). This negative cooperativity could possibly now be explained by the observed balanced dissociation–reassociation of prealbumin tetramers and subunits. Thus, thyroxine molecules associated to prealbumin monomers or dimers are immediately trapped in the prealbumin tetramer configuration. On addition of [^{125}I]thyroxine to purified prealbumin or serum, activity appears preferentially in the less acidic tetramers (unpublished results), which are those tetramers that are most prone to dissociation (Figure 3B,C).

These interpretations can be correlated with the results of Blake and Oatley (1982), who studied thyroxine binding and conformational stability of prealbumin. X-ray studies of prealbumin crystals showed large displacements in the outer part of the thyroxine binding site, and the displacements are apparently obligate to give the ligand access to the inner part of the binding site. The possibility that a serum factor could influence the monomer/tetramer status and hence regulate the binding and release of thyroxine in vivo could explain the frequent underestimation of the importance of prealbumin as thyroxine carrier in serum.

Retinol-Binding Protein. The retinol-binding protein circulates complexed with prealbumin in a 1:1 molar ratio (Van Jaarsveld et al., 1973; Kopelman et al., 1976; Trägårdh et al., 1980). Two (Kopelman et al., 1976; Trägårdh et al., 1980) or four (Van Jaarsveld et al., 1973) identical binding sites on prealbumin, with negative cooperativity, have been proposed. In serum freed from retinol-binding protein by affinity chromatography on prealbumin–Sepharose, the conversion of reduced M1b to M1a during storage is accelerated (Figure 8). Thus, the presence of retinol-binding protein seems to keep some of the prealbumin monomers in a reduced state. This might indicate one further functional relation between the two proteins and retinol, in addition to the well-known complexing of the two proteins.

Zinc. Recent (unpublished) results suggest that prealbumin binds one Zn^{2+} per monomer. However, the present results show that Zn binding is not associated with the prealbumin microheterogeneity noticeable upon isoelectric focusing.

Glutathione. One of the observed monomeric forms, M2a, is apparently an adduct with glutathione (Figure 7). A fraction of small molecules including GSH metabolites have been found to be coisolated with prealbumin when the affinity chromatography purification system is used. Incubation of native prealbumin with this fraction increases the amount of the M3 form, which may possibly be an adduct with a small molecule.

In this context, it is of interest to note that serum albumin is considered to be a transport protein for glutathione (Peters, 1975). The binding of glutathione is thought to involve the single free cysteinyl residue of albumin. Another protein known to interact with glutathione is glutaredoxin, in which two closely positioned cysteinyl residues are found in known structures of the N-terminal part (Höög et al., 1983; Klintrot et al., 1984). The amino acid sequences of the glutathione-binding region of albumin, the active site of glutaredoxin, and the Cys-10 region of prealbumin are given in Table III. A Cys-Pro configuration is present in all four proteins. This should not be taken to indicate ancestral connections but could well suggest convergent similarities from common functional roles.

Still another carrier function has been proposed for prealbumin, regarding the small peptide thymuline (identical with the serum thymic factor/zinc complex) (Dardenne et al., 1980). However, the only peptide possibly associated with prealbumin that we so far have recognized is glutathione.

Multifunctional Relationships. The heterogeneity of prealbumin in the different electrophoretic systems is explained by the status of the single SH group of the monomer and the equilibrium between tetramers and monomers. A relation appears to exist between the thiol group, the binding of retinol-binding protein and retinol, the tetramer–monomer equilibrium, and thyroxine binding. These facts, together with the following characteristics of prealbumin—an association with glutathione, a zinc binding, a DNA complementary structure (Blake, 1981), a presence in several organs including endocrine tissues (Jacobsson et al., 1979; Felding & Fex, 1982; Aleshire et al., 1983; Soprano et al., 1985), a thymic hormone-like activity (Burton et al., 1978), a highly conserved protein (Larsson et al., 1985), and distant homologies with other proteins (Jörnvall et al., 1981)—make prealbumin highly multifunctional, which is of great physiological interest. Possible further relationships between prealbumin, the 5′-deiodinase (Köhrle & Hesch, 1984), and the triiodothyronine nuclear receptor (Eberhardt et al., 1979; Azimova et al., 1985) have been pointed out. Prealbumin could thus exhibit several important physiological functions. Against this background, the present finding of different types of prealbumin complexity is not surprising, and the distinction of the various forms and their interrelations, now characterized, would appear to be important in future physiological studies.

ACKNOWLEDGMENTS

We are grateful to Asta Stendahl for assistance in the typing of the manuscript and to Rolf Andersson for preparation of the figures.

Registry No. Thyroxine, 51-48-9.

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