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HETEROGENEITY IN THE CONFORMATION OF DIFFERENT PROTEIN FRACTIONS FROM THE HUMAN ERYTHROCYTE MEMBRANE

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We have isolated 5 families of proteins from human red blood cell membranes and characterized their secondary structure by ultraviolet circular dichroism measurements. The protein families were prepared by selective solubilization from ghosts under nondenaturing conditions. We find that the intact ghost has a mean α-helix fraction of 0.37, whereas a low-ionic-strength extract (bands 1, 2, 5, "spectrin") has a substantially higher helix fraction, 0.55. Further extraction of the ghosts with para-chloromercuribenzoate yields bands 2.1, 4.1, 4.2, and 6; their helix content is only 0.17. Finally, the major intrinsic protein, band 3, was solubilized by a non-ionic detergent. Its helix fraction is 0.38.

INTRODUCTION

The major polypeptides of isolated human red blood cell membranes (ghosts) have been enumerated by PAGE following their dissociation in SDS (1–3). Recent studies indicate that these polypeptides differ characteristically in their molecular weight, chemical composition, function, asymmetric localization in and mode of association with the membrane (4–6). However, a detailed understanding of the architecture of the diverse proteins in the membrane is still lacking.

One approach to the study of the red blood cell membrane proteins has been the estimation of their secondary structure by measurement of the ultraviolet optical activity of ghosts (cf. Ref. 7). Thus far, data have been limited to averaged values for unfractionated protein mixtures. In the present study, we resolved the ghost proteins into several families by

Abbreviations: 5P8, 5mM sodium phosphate buffer, pH 8; EDTA, ethylenediamine tetraacetic acid; PAGE, polyacrylamide gel electrophoresis; CD, circular dichroism; pCMB, para-chloromercuribenzoate; SDS, sodium dodecylsulfate.

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selectively solubilizing certain proteins from the ghosts under nondenaturing conditions. We observed that the elution procedures did not appear to alter the secondary structure of the membrane proteins. Furthermore, the various protein classes we obtained differed distinctively in their α-helical content.

METHODS

Materials

Reagents for polyacrylamide gel electrophoresis were essentially those of Fairbanks et al. (2). EDTA was obtained from Baker and Ammonyx LO from Onyx Chemical Co.; and pCMB from Sigma Chemical Co.

Polyacrylamide gel electrophoresis (PAGE)

The polypeptide profile of all samples was established by PAGE in 0.2% SDS, as described by Fairbanks et al. (2) and as modified by Steck and Yu (8). Bands stained by Coomassie blue are numbered according to previous convention (6).

Circular Dichroism

CD measurements were carried out at 27°C with a Cary 6003 instrument calibrated with d-10-camphorsulfonic acid by the Cassim-Yang ORD-CD method (9). Cylindrical fused silica optical cells of 1-mm and 5-mm path length were employed. In all cases the solution optical density was less than 1.5. Measured CD data θ (degrees) were reduced to molar ellipticity $[\theta]$ using the relation $[\theta] = \frac{100\,\theta\,\bar{M}}{lc}$ where l is the light path in cm, c is protein concentration in gram/liter, and $\bar{M}$ is the mean residue weight of the protein. We used $\bar{M} = 109$ in all cases; this is the average residue weight calculated from the overall amino acid composition of the human erythrocyte ghost (10).

Protein Concentration

The protein concentration in solutions for CD measurement was determined by a modification of the Lowry method described by Hartree (11); this method yields a linear photometric response. Bovine serum albumin (Sigma, Fraction V) was used as the standard.

Fractionation

Six different fractions enriched in various membrane polypeptides were prepared as follows.

A. Hemoglobin-free human erythrocyte membranes (ghosts). These were obtained by hypotonic hemolysis of fresh or outdated bank blood in 5P8, according to the procedure of Fairbanks et al (2).

B. Low ionic strength extracts. Extracts of bands 1, 2, and 5 were prepared following the procedure of Fairbanks et al. (2). Ghosts were incubated for 10 min at 37°C in 7 vol of 1×10^{-4} M EDTA, pH 8.0. The mixture was then centrifuged at 15,000 rpm for 60 min in a Sorvall SS-34 rotor. The supernatant was dialyzed exhaustively against 5P8.

C. Low ionic strength residue. The residue was prepared by resuspending the pellet obtained from step B in 5P8 followed by exhaustive dialysis against 5P8.

D. pCMB extract. A solution enriched in bands 2.1, 4.1, 4.2, and 6 was prepared by incubating residue C for 15 min at 0°C in 5P8 containing 3 mM pCMB (8). After

centrifugation for 1 hr at 30,000 rpm in a Spinco 40 rotor, the supernatant fraction was made 25 mM in 2-mercaptoethanol and then dialyzed exhaustively against 5P8.

E. pCMB residue. Membranes depleted of bands 1, 2, 2.1, 4.1, 4.2, 5, and 6 were prepared by resuspending the pellet from step D in 5P8 containing 25 mM 2-mercaptoethanol and by dialyzing it exhaustively against 5P8.

F. Band 3. The major membrane polypeptide was prepared by a modification of the method of Yu and Steck (12). Ghosts were first depleted of band 6 by incubation in Na phosphate, pH 8, $\mu = 0.15$ (Ref. 2). The ghosts were then washed with the same buffer at $\mu = 0.1$ and resuspended in 5 vol of this buffer containing 0.1% Ammonyx LO (13). (This detergent is favored over polyethoxyphenolic surfactants such as Triton X-100 because of its low optical absorbance in the ultraviolet.) After an incubation of 20 min on ice, the sample was centrifuged at 15,000 rpm for 30 min in a Sorvall SS-34 rotor. The supernatant fraction was collected and adjusted with 100 mM pCMB in 0.01 N NaOH to a final concentration of 3 mM pCMB. After 20 min of incubation on ice, a 5 ml sample was loaded onto an aminoethyl cellulose column (0.6 × 5 cm) equilibrated with 0.1% Ammonyx LO in Na phosphate, pH 8, $\mu = 0.1$. The column was washed with this buffered detergent at $\mu = 0.13$; band 3 was then eluted at $\mu = 0.24$. The band 3 eluate was made 10 mM in 2-mercaptoethanol and then dialyzed exhaustively against 0.075% Ammonyx LO in 5P8.

RESULTS

A series of six samples were prepared under nondenaturing conditions, each enriched in a different set of red cell membrane polypeptides. Their electrophoretic profiles in gels stained with Coomassie blue are shown in Fig. 1. Sample A is of unfractionated, hemoglobin-free ghosts, comparable to those previously reported (1–3, 8). Specimen B is rich in bands 1, 2, and 5, which are characteristically eluted from the cytoplasmic surface of ghosts under conditions of low ionic strength and alkaline pH, in the absence of divalent cations (2). Gel C shows the polypeptides retained by the sedimented membrane vesicles following the low ionic strength extraction; it is seen that the overall electrophoretic profile is conserved in the supernatant and pellet fractions following this treatment.

In sample D, the polypeptides remaining loosely bound to the cytoplasmic surface of the membranes, principally bands 2.1, 4.1, 4.2, and 6, were eluted by 3 mM pCMB. The pCMB residue (sample E) evinces bands 3 and 7, and a complex zone designated 4.5. It also contains the membrane lipids and glycoproteins which are not apparent here but are visualized by the periodic acid-Schiff stain for carbohydrates (8). As before, the pCMB extract and residue show good conservation of the stained profile; no polypeptides are lost or degraded. However, as has been noted elsewhere (12), the pCMB treatment generated a small amount of an irreversibly aggregated dimer of band 3 [designated as $(3)_2$ in Fig. 1]. Such discrete, SDS-resistant aggregates have also been encountered with other hydrophobic membrane proteins such as the red cell sialoglycoprotein, PAS-1 (or glycophorin) (14), and opsin (15, 16). Finally, gel F shows a specimen of band 3 purified in the nonionic (and presumably nondenaturing) detergent, Ammonyx LO; periodic acid-Schiff stain of a duplicate gel showed no glycoprotein contaminants.

The ultraviolet CD spectra of four of these samples are shown in Fig. 2; data for all six are summarized in Table I. The spectra, especially for $\lambda > 200$ nm, reflect primarily the average secondary structure of the proteins; the chromophores of lipids and carbohydrates are important only for $\lambda < 200$ nm (7). A considerable difference in secondary

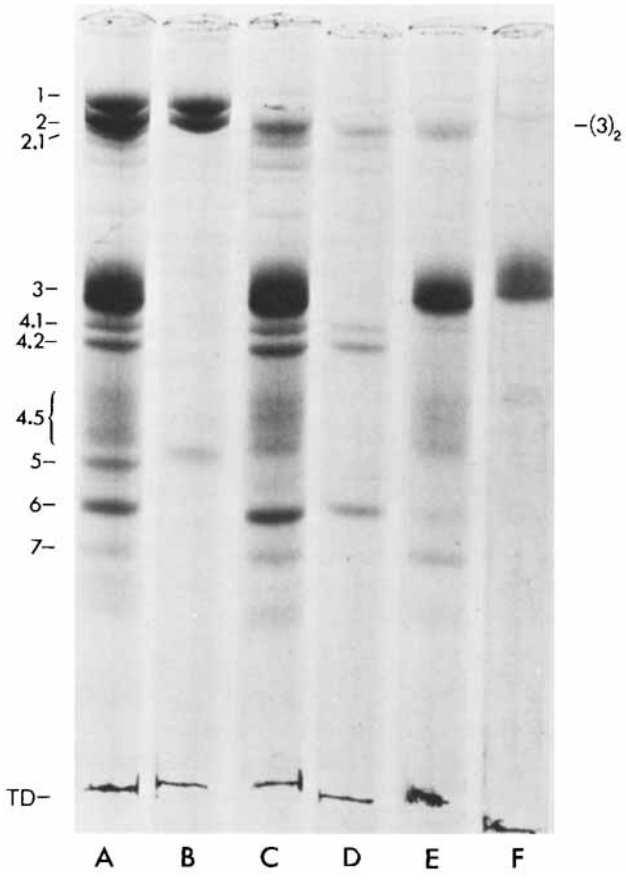

Fig. 1. Polypeptide profiles of erythrocyte membrane fractions. Preparations: A, unfractionated ghosts; B, low ionic strength extract; C, low ionic strength residue; D, pCMB extract; E, pCMB residue; F, purified band 3. Membrane fractionation, polyacrylamide gel electrophoresis in 0.2% SDS, and Coomassie-blue staining were performed as described in Methods; TD, tracking dye.

structure is apparent among the different proteins. These differences in CD curves can be interpreted in terms of the relative amount of α-helix, using either synthetic polypeptides or proteins of known structure as standards (17–19). We prefer the method of Chen et al. (17), which utilizes as standards the CD curves of 5 soluble proteins whose crystal structures are known from X-ray analysis. We have chosen to interpret our data using a simplified form of this approach which depends only upon $[\theta]$ at 222 nm, as proposed by Chen and Yang (18). We use the value of the CD at 222 nm as a measure of helix content because of concern for scattering contributions to the observed CD in particulate suspensions at other wavelengths. One expects the contributions of scattering to be small near 222 nm since the refractive indices of the proteins toward left and right circularly polarized light are nearly equal in the spectral region (20–23).

The CD spectrum of the intact ghost (Fig. 2) resembles that reported previously by us and by others (7, 20, 24–27). It is similar to that of a protein with α-helix fraction equal to 0.37 but with characteristic spectral shifts and distortions which arise from the

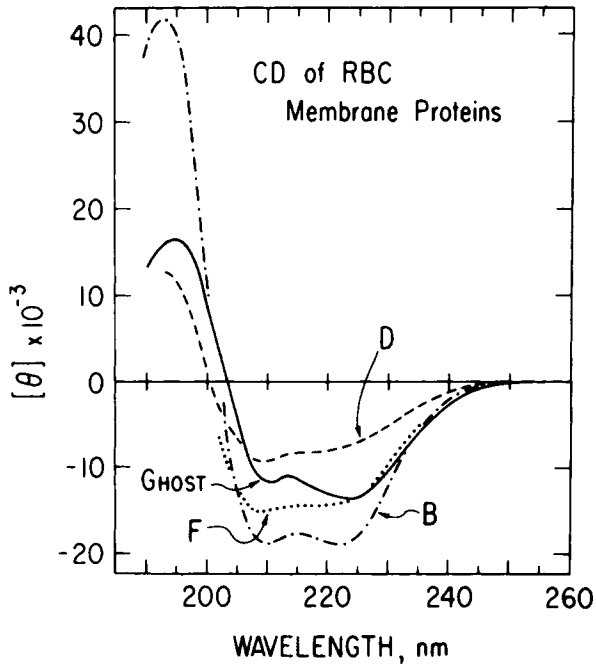

Fig. 2. Circular dichroism spectra of erythrocyte membrane protein fractions. (————): intact ghost, preparation A, unfractionated. (— · — · — · — ·): preparation B, low ionic strength extract containing bands 1, 2, and 5. (– – – – – – –): preparation D, pCMB extract containing bands 2.1, 4.1, 4.2, and 6. (· · · · · · · ·): preparation F, purified band 3.

particulate nature of the sample (20, 26, 27). The CD intensity at 222 nm is −13,500 deg · cm^2 · decimole^{-1} for the intact ghost. This value can be compared to earlier values: −14,000 (Ref. 24, using a mean residue weight of 130); −16,000 (Ref. 25); −13,200 (Ref. 26).

The CD curve for sample B, the low ionic strength extract (containing principally components 1, 2, and 5) shows much stronger CD than does the intact ghost. The curve shape resembles that of metmyoglobin ($[\theta]_{192}/[\theta]_{222}$ = 2.2 for sample B, 2.2 for myoglobin (17), but only 1.1 for intact ghosts). It is noteworthy that this fraction scatters light much less than do intact ghosts. The helix fraction for this extract is estimated as 0.55. This high value is consistent with an anisometric structure, which has been observed for similar proteins from beef erythrocyte membranes (28) and liver plasma membranes (29).

The CD curve for the membrane vesicles lacking components 1, 2, and 5 (preparation C) exhibits weaker CD than does the intact ghost ($[\theta]_{222}$ = −12,300, Table I), and particulate distortions are reduced. The direction of the CD change of this preparation, compared to intact ghosts, is also consistent with the high helix content of bands 1, 2, and 5.

The pCMB extract, preparation D, containing principally components 2.1, 4.1, 4.2, and 6, shows a CD curve typical of a much lower helix content than the intact ghost. From Fig. 2, $[\theta]_{222}$ = −7,500, suggestive of a helix fraction of 0.17. The fact that the CD amplitude at 210 nm exceeds that at 222 nm is consistent with a low α-helix fraction.

The CD of the pCMB residue (preparation E) exhibits $[\theta]_{222}$ = −14,800 (Table I). This value is not far from that of the original ghosts, to which the preparation E polypep-

TABLE I. Characteristics of Membrane Protein Classes

Preparation	Designation	Principal polypeptide bands present*	Approximate fraction of total protein weight*	$[\theta]_{222}$ deg · cm · decimole^{-1}	Mean fractional α-helix†
A	Intact ghost	All	1.00	−13,500	0.37
B	Low ionic strength extract	1 2	0.30	−19,000	0.55
		5	0.045		
C	Low ionic strength residue	2.1 3 4.1 4.2 4.5 6 7 sialoproteins	0.65	−12,300	0.33
D	pCMB extract	2.1 4.1 4.2	0.09	− 7,500	0.17
		6	0.055		
E	pCMB residue	3 4.5 7 sialoproteins	0.50	−14,800	0.41
F	Major intrinsic protein	3	0.24	−14,000	0.38

*Notation and data from Refs. 6 and 8.

†We use the relation $f_h = \frac{[\theta]_{222} + 2{,}340}{-30{,}300}$ from Ref. 17. The uncertainty in helix fraction is estimated as ± 0.05.

tides contribute about half the protein mass. The lack of major change in the CD of the pCMB residue can be attributed to a balance between the elution of polypeptides of high helical content (preparation B) and low helical content (preparation D) from the ghosts.

Component 3 in Ammonyx LO solution exhibits $[\theta]_{222} = -14{,}000$. This suggests a helix fraction of 0.38. These values are similar to those found for preparation E, to which component 3 contributes approximately 50% of the protein. From these data we can infer that the other proteins in the pCMB residue (preparation E) must also contain, on the average, a comparable helix content.

DISCUSSION

The major uncertainties in the interpretation of these data are three: (1) errors in protein concentration; (2) artifacts arising from particulate nature of some samples; (3) the usual reservations arising in the use of CD to estimate secondary structure. It is well known that different proteins may yield different calibration curves in the Lowry

method of protein estimation (30). The modification used here is less susceptible to underestimation of protein in particulate samples or glycoproteins, since it includes a 10 min digestion at 50°C in a strongly alkaline medium (11). Nevertheless, various proteins inevitably show significant differences in color yield. In the worst case examined, Hartree (11) found that the ratio of the absorbance of equal weights of bovine serum albumin and insulin was 0.57.

The magnitude of errors arising from light scattering is now at least partially understood (20, 23, 27). The error in helix fraction arising from this source is almost certainly small compared to the uncertainty in protein concentration.

The uncertainties in conformational analysis of proteins from their CD curves has been examined by others in detail. For example, Yang and co-workers have used the results of X-ray analysis of protein crystals to test their method (17). Absolute accuracies for the estimation of the fractional helix content are probably ± 0.05 and are worst at lower helix fractions.

We infer from this study that the CD of ghosts is the average of individual polypeptides whose helical contents vary widely. Preparations A–E should themselves be resolved into purified components; their structure could then be examined in further detail. It is important to note that these fractions appear not to have gained or lost helical content during their separation; that is, their weighted ellipiticities sum up to a value close to that found in the intact ghosts. We have evidence that the $[\theta]_{222}$ value given for purified band 3 (preparation F) is close to that which it bears in ghosts (12). It is significant that the readily elutable, water-soluble proteins (Fractions B and D) and the tenaciously bound, hydrophobically anchored proteins (8, 13) (Fractions E and F) both contain significant α-helix. Thus, high α-helix content does not correlate with any particular mode of binding to the membrane.

Future applications of CD to membrane structural analysis should include determination of the helix content of the individual major proteins, purified in a nondenatured state. Furthermore, it would be of interest to compare the optical activity of the polar and hydrophobic portions of membrane-penetrating proteins, such as band 3, perhaps by gentle proteolytic dissection. Finally, one could study the net orientation of helical segments in these proteins by measuring the CD of oriented membranes. Recent measurements of the components of the rotational strength tensor in the α-helix (31) would allow interpretation of such experiments.

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