Metabolic Dependence of Protein Arrangement in Human Erythrocyte Membranes. I. Analysis of Spectrin-rich Complexes in ATP-depleted Red Cells

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The discocyte-echinocyte transformation and the decrease in deformability associated with red cell ATP depletion have been attributed to changes in the physical properties of spectrin and actin, membrane proteins located at the membrane-cytosol interface. We investigated the spontaneous formation of spectrin-rich complexes in human erythrocyte membranes, employing two-dimensional SDS-polyacrylamide gel electrophoresis. Membranes of red cells depleted in ATP under aerobic conditions exhibited (1) an increase in components 4.5 and 8 and globin subunits, (2) a spontaneous formation of heterodimers of spectrin 1 + 2 and spectrin 2 + component 4.9, and (3) a large molecular weight (>10^6 daltons) protein complex with a high spectrin to band 3 ratio. These complexes were dissociated with dithiothreitol and were prevented by anaerobic incubation or the maintenance of red cell ATP and GSH levels with glucose, adenine, and inosine. The complexes 1 + 2 and 2 + 4.9 were also seen in acetylphenylhydrazine-treated, glucose-6-phosphate dehydrogenase-deficient fresh erythrocytes that showed marked GSH depletion but preserved >70% of the original ATP level. However, membranes of these cells did not contain the >10^4-dalton aggregate with a high spectrin to band 3 ratio. We concluded that the formation of the latter complex results from rearrangement of spectrin and other polypeptides in membranes of ATP-depleted red cells. Under aerobic conditions, the rearranged proteins undergo spontaneous intermolecular crosslinkings through disulfide couplings.

RED CELL GHOSTS, when examined in thin sections by transmission electron microscopy, are seen to contain a fibrillar material attached to the inner surface of the membrane. This material consists of three prominent polypeptides, components 1 and 2, termed spectrin, and polypeptide 5, recently identified as actin. These membrane proteins have the characteristics of being eluted with solutions of low ionic strength and reassociating into fibrillar structures when the ionic strength is raised with magnesium or calcium plus ATP. Physical, biochemical, and functional properties of these proteins have been recently reviewed.

Alterations in physical properties and content and an unconfirmed decrease in phosphorylation of these proteins in hereditary spherocytes and ATP-depleted normal erythrocytes has led to the postulate that these proteins are involved in the regulation of cellular shape and deformability. Furthermore, removal of such material or incorporation of antispectrin antibody into ghosts was found to be associated with modification of their shape response to magnesium, calcium, and ATP.

Recent applications of two-dimensional gel electrophoresis and various tech-
niques of crosslinking of nearby proteins of red cell membranes\textsuperscript{21-25} have permitted a study of the spatial arrangement of these proteins and their interaction in the membrane. This communication is the first of a series reporting on application of these techniques to investigation of membrane protein rearrangements of red cells undergoing ATP depletion and calcium accumulation. In this first report, we demonstrate that ATP depletion is associated with the formation of large molecular weight spectrin-rich complexes produced by intermolecular disulfide coupling. Part of the results were previously reported in preliminary form.\textsuperscript{26}

**MATERIALS AND METHODS**

**Separation of Red Cells**

Venous blood was collected into heparin (5 mg/dl blood) and was centrifuged at 2500 g for 15 min. The supernatant and buffy coat were discarded after aspiration and the red cells were washed three times in 5 volumes of isotonic sodium chloride solutions buffered with 10 mM glycyl glycine or Tris-HCl, pH 7.4. The layers of leukocytes and platelets remaining were carefully removed after each washing.

**Incubations**

Washed red cells free of white cells and platelets were prepared as 20\textsuperscript{o} suspensions with 50 mM glycyl glycine (pH 7.4 at 37\textdegree C) containing 5 mM KCl, 2 mM CaCl\textsubscript{2}, 2 mM MgCl\textsubscript{2}, and NaCl up to isotonicity. Fifty units penicillin and 20 \textmu g streptomycin were added per ml cell suspensions. The suspensions were transferred into plastic erlenmeyer flasks, flushed with air for 10 min, sealed with rubber stoppers, and incubated in a water bath at 37\textdegree C for 24 hr with shaking to deplete ATP. In some experiments, incubation under deoxygenated conditions was performed with a constant flow of prewashed humidified nitrogen gas through 18-gauge hypodermic needles perforating the stoppers. After 3 hr of incubation the pH of the suspensions dropped to 7.2 \pm 0.1 and was readjusted to 7.4 \pm 0.04. Thereafter the pH values of the suspensions remained between 7.3 and 7.4. In some samples intracellular ATP content was maintained with the addition of 0.5 mM adenine, 12.7 mM inosine, 2 g/liter glucose, and 2 mM phosphate; periodic pH adjustments were made throughout the incubation.

In the experiments with glucose-6-phosphate dehydrogenase (G6PD)-deficient erythrocytes (Worcester variant, as previously described\textsuperscript{27} red cell suspensions were incubated under aerobic conditions for 4 hr in the presence of acetylphenylhydrazine (APH), 4 mM.\textsuperscript{30}

**Ghost Preparation**

Erythrocyte ghosts were prepared by hypotonic lysis of washed fresh or incubated cells at 0-4\textdegree C in 30 volumes of 10 mM Tris, pH 7.4, followed by centrifugation (29,000 g for 10 min) and two washings.

**Sodium Dodecylsulfate (SDS)-Polyacrylamide Gel Electrophoresis**

Our detailed procedure of the two-dimensional gel electrophoresis was previously described.\textsuperscript{25} The buffer and catalyst formulations for the first dimension were similar to those used by Steck.\textsuperscript{21} The gels containing 2.5\textsuperscript{o}, acrylamide and 0.3\textsuperscript{o}, agarose were cast according to Peacock and Dingman.\textsuperscript{28} The discontinuous buffer system described by Laemmli\textsuperscript{29} was employed in the second-dimension slab. The stacking and separating slabs were formed of 2.67\textsuperscript{o}, and 8\textsuperscript{o}, acrylamide, respectively.

For two-dimensional fractionation, the membranes solubilized in SDS without a reducing agent were run first in cylindrical agarose/acrylamide composite gels. After the tracking dye had migrated 7 cm, the gel was removed from the glass tube, transferred to the top of the slab, and sealed in place with melted agarose solution containing 1\textsuperscript{o}, SDS, 40 mM dithiothreitol (DTT), 125 mM Tris pH 6.8, and pyronin Y. Electrophoresis was carried out with a current of 20
mA per slab for 18 hr. The slab was stained with Coomassie blue following the procedure of Fairbanks et al. Densitometric scans of gels were performed at 525 nm with the Quick Scan Jr. (Helena Laboratories). Apparent molecular weights of protein aggregates were estimated from their relative mobilities and the calibration curve of logarithmic molecular weight versus mobility plot of the known molecular weight of the major erythrocyte membrane proteins.

Measurements of ATP and GSH

Levels of ATP and reduced glutathione (GSH) were measured using methods detailed by Beutler.

RESULTS

Membrane Protein Associations During ATP Depletion

Figure 1A shows SDS–polyacrylamide gels of membrane proteins from red cells undergoing ATP depletion during aerobic incubation without glucose. The densitometric scans are depicted in Fig. 2. ATP depletion and a concomitant decrease in GSH content were associated with formation of slowly migrating complexes of apparent mol wts of 260,000 and 450,000 daltons. In addition, a large complex (> 10^6 daltons), which increased progressively with time of incubation, appeared at the origin. These complexes were first noted at 17–19 hr
of incubation, when $94^{\circ}\alpha \pm 4^{\circ}\alpha$ of the cells had been transformed into echinocytic shapes. The $>10^6$-dalton complex was decreased by $60^{\circ}\alpha$, when ATP content was increased and concave shape was restored by incubation with adenine and inosine (not shown). Furthermore, all the complexes were completely prevented from forming when ATP, shape, and GSH were maintained or when oxygen was excluded during the incubation (Fig. 1B). The latter conditions also delayed discocyte-echinocyte transformation. For example, at 15 hr of incubation the percentage of discocytes in anaerobically and aerobically incubated samples was $19^{\circ}\alpha$ and $52^{\circ}\alpha$, respectively, while after 24 hr both samples contained nearly $100^{\circ}\alpha$ spheroechinocytes. Treatment with a reducing agent (DTT) reversed the crosslinking reaction (Fig. 1B). These results suggest that the high molecular weight complexes resulted from the formation of intermolecular disulfide bonds upon oxidation.

Additional changes of membrane protein patterns of cells undergoing ATP depletion included an increase in band $4.5$ (mol wt 60,000), recently identified as monomeric catalase,\textsuperscript{31,33} band 6 (mol wt 35,000), globin subunits (mol wt 16,700), and a decrease of band $2.1$ (mol wt 195,000). The increase in 6 and the decrease in 2.1 in ATP-depleted cells were prevented by DTT reduction, which, however, produced an increase in band 8 (mol wt 18,000). Incubation under deoxygenated conditions produced a gel pattern similar to that of aerobically
incubated cells subjected to DTT treatment, with the exception of the increase in band 8.

These alterations in membrane protein composition of ATP-depleted cells were noted in both the presence and the absence of external calcium (2 mM), with the exception of the retention of 4.5 and globin subunits, which required the presence of external calcium (not shown).

Fig. 3. Two-dimensional gel electrophorograms of membrane proteins from fresh and ATP-depleted red cells. (A) Fresh ghosts (100 µg protein) were fractionated in the first dimension without DTT. Electrophoresis in the second dimension was performed in a slab, incorporating a DTT zone to cleave the possible disulfide bonds. The pattern of a membrane sample applied directly is shown at the left for reference. The pattern obtained by electrophoresis in the first dimension is shown in a stained replicate gel placed at the top of the slab. Off-diagonal elements indicated by dotted curves probably represent glycoproteins (see text). The individual polypeptides are numbered according to Fairbanks et al. * indicates the start of the gel. (B) Ghost protein (250 µg) from ATP-depleted cells incubated under air for 24 hr was applied on the gel. After electrophoresis in the second dimension all the material was dissociated and entered the second dimension. Arrow indicates the large aggregate (>10^6 daltons) in the first dimension and the cleaved components in the second dimension.
Identification of the Individual Polypeptides of the High Molecular Weight Protein Complexes by Two-dimensional Polyacrylamide Gel Electrophoresis

Since the high molecular weight protein complexes could be dissociated with DTT reduction, we treated the first-dimension gels containing the high molecular weight complexes with DTT, followed by electrophoresis on slab gels in the second dimension to identify the cleaved components. Figure 3A shows a two-dimensional gel of membrane polypeptides of fresh erythrocytes. The individual components on the slab appear as distinct spots falling in a diagonal skewed curve. The individual membrane proteins are denoted by numbers. The off-diagonal elements (indicated by dotted curves) appear to be glycoproteins, since they can be stained by periodic acid–Schiff reagent (not shown).

The resolution of the high molecular weight membrane protein complexes of ATP-depleted red cell membranes by two-dimensional gel electrophoresis is shown in Fig. 3B. All of the slowly migrating complexes have been completely dissociated during their migration through a DTT zone, and their individual constituents are depicted on the two-dimensional gel as off-diagonal spots positioned at the same vertical line as the original complex. The 260,000-dalton...
complex can now be resolved as a monomeric $2 + 4.9$ association, as judged from the apparent molecular weights of the complex and its dissociated components. Similarly, the 450,000-dalton complex is identified as a heterodimer of $I + 2$. These two polypeptides are more clearly separated from each other at a lower protein loading, as shown in Fig. 4. The identity of these bands with spectrin $I$ and $2$ is further indicated by a fine smearing line between these constituents and the spots of the nonpolymerized spectrin separated in the second dimension (Fig. 4). Finally, the $>10^6$-dalton complex that remains near the origin of the first dimension was found to consist of multiple components, including spectrin, actin, bands $3, 4.1$, and $6$, globin, and several unidentified cytoplasmic proteins. However, whether the large aggregate represents a homogeneous complex or a series of complexes with their molecular weights reaching beyond the fractional resolution in the first dimension is not known.

Two-dimensional gel analysis of changes in bands $2.1, 5$, and $6$ revealed that part of band $5$ and band $8$ comigrated with band $6$ in the first dimension and contributed to the increased intensity at the band $6$ region. Upon DTT reduction, they were separated from band $6$ in the second dimension. The abnormal mobility of band $5$ was probably due to its formation of intramolecular disulfide linkages, which may cause a change of Stoke’s radius. However, band $8$, which overlapped with band $6$ in the first dimension, arose from its dimer, as judged from the apparent molecular weights of bands $6$ and $8$ (18,000 and 36,000 respectively). The increased contents of bands $4.5$ and $8$ and hemoglobin during ATP depletion probably reflected retention of cytoplasmic proteins. Evidence has been recently provided that band $4.5$ is a subunit of catalase and that its partitioning in membranes is ATP and calcium dependent.

Quantification of the Protein Composition of the Large Molecular Weight Protein Complex

We have attempted to quantitate the constituents of the $>10^6$-dalton complex by its densitometric scanning on the Coomassie blue-stained second-dimension gel. The scan was compared to a reference of membrane proteins from fresh erythrocytes (Fig. 5). The principal characteristic of this aggregate was its high spectrin to band $3$ ratio (2:1), which was almost twice that found in intact membranes of normal erythrocytes (1:1).

Spontaneous Protein Associations in G6PD-deficient Erythrocytes Treated with Acetylphenylhydrazine

These experiments were conducted to investigate the spontaneous formation of membrane protein complexes in APH-treated G6PD-deficient red cells that maintained relatively normal ATP levels (>0.7 μmoles/ml red cells) while they exhibited a marked depletion in GSH (<10% of preincubation value). Ninety percent of the cells exhibited five or more Heinz bodies per cell when stained with methyl violet. As shown in Fig. 4, such cells exhibited the formation of spectrin dimers and a complex of $2 + 4.9$. They also contained a large molecular weight protein complex that, however, consisted almost exclusively of globin subunits. A nearly identical two-dimensional gel pattern was seen in G6PD-deficient cells incubated aerobically for 24 hr with adenine, inosine, glucose,
Fig. 5. Densitometric scan of Coomassie blue-stained proteins. (A) Reference membrane sample as shown at the left-hand side of Fig. 3B. (B) Protein constituents released from the $>10^5$-dalton aggregate after DTT reduction, as indicated by an arrow in Fig. 2B.

and APH (1 mM) that also exhibited marked GSH depletion while maintaining their ATP content (not shown in Fig. 4). Normal red cells exposed to APH formed both spectrin dimers and the $2 + 4.9$ complex but contained only minimal amounts of a globin-rich complex and, accordingly, formed only a small number of Heinz bodies (Fig. 4). It should be pointed out that no membrane protein complexes were seen in untreated fresh G6PD-deficient erythrocytes (not shown).

DISCUSSION

We found that membranes of red cells depleted of ATP under aerobic conditions contain several high molecular weight spectrin-rich complexes, namely, a heterodimer of spectrin $(1 + 2)$, a complex of spectrin $2$ and polypeptide $4.9$ $(2 + 4.9)$, and a $>10^5$-dalton complex with a high spectrin to band $3$ ratio. We conclude that these complexes result from intermolecular disulfide couplings between the nearest-neighbor membrane and cytoplasmic proteins due to spontaneous oxidation and concomitant GSH depletion. This process can be prevented by maintaining red cell GSH levels and/or by anaerobic incubation and can be reversed by DTT reduction.

In order to explore whether or not any of these oxidative crosslinks reflect membrane protein rearrangement directly related to red cell ATP depletion, we investigated membrane protein composition of fresh APH-treated G6PD-deficient erythrocytes and G6PD-deficient cells incubated aerobically with energy supplementation. These cells showed marked GSH depletion while they maintained $70\%$ of the preincubation ATP levels. In such cells, we found the formation of the complexes $1 + 2$ and $2 + 4.9$. In addition, these cells formed
a globin-rich aggregate (85%, globin) cleavable by dithiothreitol (DTT) reduction, probably representing denatured globin (Heinz bodies) attached to the membrane. The composition of this aggregate was in striking contrast to the protein aggregate of aerobically ATP-depleted erythrocytes, which had a high spectrin to band 3 ratio (2:1, normal 1:1) and only small amounts of globin. On the basis of these findings, we concluded that the formation of the complexes 1 + 2 and 2 + 4.9 was related to membrane protein oxidation and GSH depletion but not to a decrease in cellular ATP.

In contrast, the formation of the > 10^6-dalton complex with high spectrin to band 3 ratio appeared to require additional depletion of cellular ATP. This finding suggests that in ATP-depleted red cells spectrin and some other polypeptides are rearranged into more intimate contact with each other. After a concomitant spontaneous oxidation during aerobic incubation, the rearranged proteins are crosslinked by disulfide bridges with subsequent formation of a spectrin-rich heteropolymer.

Formation of this complex in ATP-depleted red cells was independent of the presence of external calcium. However, normal human erythrocytes contain a small fraction of exchangeable calcium, presumably in the form of a calcium ATP complex, that after depletion of ATP may become available to interact with the cell membrane. The > 10^6-dalton complex should be distinguished from a recently described calcium promoted aggregation of red cell membrane proteins. The latter aggregate required high calcium concentration, was DTT insoluble, and was recently shown to be mediated by an intrinsic transglutaminase catalyzing protein polymerization through γ glutamyl-e-lysine bridges.

It is of interest that the large molecular weight spectrin-rich complex contains other membrane and cytoplasmic constituents. The contamination by cytoplasmic proteins including hemoglobin may account for the observation that the complex is only partially reversible with ATP repletion. The presence of other membrane proteins in the aggregate is presumably related to the fact that in normal red cell membranes spectrin forms reversible interactions with several membrane constituents, as recently documented in studies employing crosslinking agents and experiments relating spectrin aggregation to clustering of intramembrane particles.

In addition to spectrin rearrangement and oxidation, we observed an increased retention of several cytoplasmic components in ATP-depleted red cell membranes, namely, retention of bands 4.5 (catalase) and 8 and globin subunits during ATP depletion. While the globin membrane attachment had been previously in part related to the effect of aerobic conditions, the retention of catalase could be related directly to ATP depletion rather than to concomitant oxidation, since this change was not altered by anaerobic incubation. It is noteworthy that these changes also required the presence of calcium in the external solution, as also recently observed by others. The mechanism of the attachment of these components is presently uncertain but may in part be related to the changes in the physical state of spectrin and other proteins at the membrane cytosol interface, which in turn may alter the solubility properties of other cytoplasmic constituents. It should also be noted that the increase in the
band 6 region in ATP-depleted cells was not totally due to increased retention of polypeptide 6 (glyceraldehyde-3-phosphate dehydrogenase) but was also partially due to an increase in band 8, which in the absence of DTT formed a dimer overlapping with band 6.

The ATP dependence of red cell membrane deformability has been well documented and had been originally attributed to a sol-gel transformation of proteins at the cell membrane–cytosol interface. Currently under investigation is the question of whether or not such alterations are in part related to the formation of spectrin-rich complexes described above. However, we have recently shown that such complexes are seen even in ATP-depleted cells in which discoidal shape and near normal viscosity are maintained by addition of a cationic anesthetic to the incubation medium. In addition, ATP depletion is associated with accumulation of 1,2-diacylglycerol in red cell membranes, which has been suggested as the cause of echinocyte transformation. Thus the relationships among spectrin aggregation, cell shape, and deformability are apparently more complex and require further clarification.

ACKNOWLEDGMENT

We appreciate the skilled technical help of Maureen Monroe and Adolphus Anewke, the stimulating discussions of Dr. Grant Fairbanks, Dr. Hans Lutz, and Dr. Normand L. Fortier, and the excellent secretarial assistance of Betty Stevenson.

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